Abstract

Intercellular communication mediated by cell surface antigens is important in the maintenance of synovial tissue (ST) integrity. Chronic inflammation is a common feature of osteoarthritis (OA). Cellular attachment to and migration into ST is one of the critical aspects of chronic inflammation. This study was undertaken to examine the tissue distribution of a broad spectrum of monoclonal antibodies (mAbs) containing tetraspan antigens (CD9, CD63, CD151), endothelial cell antigens (CD31, CD36, CD105, CD106, CD146), integrins (CD49a-f, CD29, CD41, CD51, CD61), CD39, CD98, CD99, CD143 and, CD147 supplied from fifth and sixth international workshops and conferences on human leucocyte differentiation antigens in a comparative manner in human OA and normal synovium. Ten primary OA patients and six normal individuals were included in this study. The average age of the patients was 65.0 ± 8.3 years and the average age of the controls was 31.8 ± 5.3 years. Sections were screened using an indirect immunoperoxidase method. Tetraspan antigens and CD98 presented rather unique staining pattern in OA synovium suggesting special roles for each antigen on the synovial lining layer (SLL). Endothelial cells and type A synoviocytes expressed CD31 and CD36 in OA, but only endothelium in normal subjects. Integrins presented a uniform staining pattern in both groups. There was a positive reaction in some of the ST stromal elements for CD143 in all specimens. In conclusion, human normal and OA synovium were comparatively reviewed by a broad spectrum of mAbs with special attention being given to their functional aspects. This data suggests a significant difference in antigenic phenotype of SLL cells in OA and ST not to be considered at a normal-like state in OA. The fact that their activation was independent of the degree of lymphocyte infiltration further emphasizes the possible central importance of SLL.

Osteoarthritis (OA) is the most widespread acquired connective tissue disorder that affects the synovial joints. This disease involves all the tissues of the joint including the synovium. Clinically, the disease is characterized by joint pain, tenderness, limitation of movement, and functional disability. Chronic inflammation and vasculogenesis is present to some extent in the natural course of OA. Studies on OA point out multiple biological and biochemical synovial tissue factors that are involved in the pathogenesis of the disease. The biology of the SLL in detail is not completely identified until now. It is assumed that in OA, this layer may have potential importance in the development and progression of the disease. Exceptional composition of connective tissue lying beneath the SLL and the absence of a basal membrane between them elaborated the essence of research to clarify cell extracellular matrix (ECM) interactions in inflammatory joint diseases including OA. Cell surface antigens are supposed to be mediator molecules for cell-cell and/or cell-ECM interactions in the pathogenesis of OA. It is hypothesized that comparative staining of normal and OA human ST with a wide panel of mAbs may give new
insights into the pathogenesis of the disease.

In this study, cell surface antigens expressed by different cell groups with special reference to their synovial compartments were used to collect an adequate amount of reliable data on immunophenotypic characterization of normal and OA synovial tissues. These antigens, which were covered by Cluster of Differentiation (CD) codes, were selected from the tetraspan, endothelial cell, integrin, and activation antigen families to establish a reference database for future immunological OA studies. The expression of certain antigens on SLL and vascular endothelium has been investigated in detail. Some of our data included the antigens that had been previously reported for several comparable investigations; while some others represented previously unpublished data.

Materials and Methods

Subjects
Specimens were obtained from ten primary osteoarthritic patients and six normal individuals. The average age of the patients and controls were 65.0 ± 8.3 and 31.8 ± 5.3 years, respectively. Demographic data on the sex, age, diagnosis, and surgery is provided in Table 1. All patients in the study group were undergoing total joint replacement due to OA, and presented severe joint pain and range of motion limitation with radiological features of Kellgren and Lawrance Grade 4. Information on the study was given to all subjects before the operation and a written consent was obtained. All procedures were in full compliance with the Helsinki Declaration of Human Rights.

Light Microscopical Study on Semi-Thin Sections
Tissue specimens were fixed in 2.5% glutaraldehyde in Sorensen's phosphate buffer. After washing with PBS, they were postfixed in 1% osmium tetroxide at 4°C for 1 hour. Specimens were then dehydrated in graded series of ethanol to absolute ethanol in preparation for embedding in araldite Cy 212 (Agar, Germany). Semithin sections were prepared using a microtome and stained with methylene blue-AzurII; then they were examined under the light microscope and documented.

Immunohistological Study
All specimens were immediately frozen in liquid nitrogen and stored at -30°C. Staining of sections was complete within two or three days following surgery. Cryostat sections (6 to 8 µm thick) were obtained on gelatin-coated slides and kept in humidity-free containers at room temperature until evaluation.

Monoclonal Antibodies
The monoclonal primary antibodies used in this study were supplied from the “Leukocyte Typing V and VI” International Workshop and Conferences on Human Leukocyte Differentiation Antigens. Staining intensity was scored in a scale from 0 to 3; “0” meaning “no staining” and “3” meaning “intense staining.” Scores were expressed as minimum, maximum and median standard deviations. Data was analyzed using Mann Whitney U test to assess statistical significance; p < 0.05 was considered significant. Cell surface antigens were grouped according to their general reactivity, molecular family and/or function; and their antibodies were listed according to their clones and originators (Table 2).

Staining Procedure
The indirect immunoperoxidase procedure used in this study was described in detail previously. In brief, sections were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. Then, they were incubated with the primary antibodies for 60 minutes. After washing with 0.01 M PBS at pH 7.4, the sections were covered with rabbit anti-mouse IgG peroxidase (Sigma, USA). The dilution of IgG in PBS was 1:200 and contained 0.2% bovine serum albumin (BSA) and 1% normal human serum. The slides were then stained for peroxidase activity with 3,3’-diaminobenzidine-tetrahydrochloride (DAB, Sigma, USA) (0.5 mg/ml Tris-HCl buffer, pH:7.6, containing 0.01% H2O2). A counterstaining with hematoxylin was performed respectively. Control staining were performed by omitting the initial primary antibody staining step and using a control mouse IgG.

Acid Phosphatase Staining Procedure
Following the secondary antibody and the DAB staining steps, a group of tissues were placed into the humidified chamber and incubated at 37°C for 45 minutes with acid phosphatase solution. The specimens were then washed with PBS and counter-stained with hematoxylin.

Results

Light Microscopical Study on Semi-Thin Sections
Synoviocyte hyperplasia was observed in some areas of the sections taken from the OA specimens. In some other areas, however, the SLL appeared as a single cellular layer. In the hyperplastic and hypertrophic areas, both type A and B synoviocytes were present and an increase in size and number was observed (Fig. 1A and B). Detachment of some particles of debris material was present in the synovial space near the SLL. The subsynovial tissue (SST) was hypervascular and hypercellular beneath the hyperplastic SLL. The synovium of the control group had a normal appearance with its SLL consisting of one or two layers of synoviocytes. The synovial

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Immunohistological Study
Descriptive statistics of immunohistological data (Figs. 1 and 2) are presented in Table 3.

Tetraspan Antigens
Reactivity for CD9, CD63, and CD151 of variable intensity was detected on the SLL and vascular endothelium of both normal and OA samples. In the OA group, CD9 immunoreactivity was prominent in the round shaped macrophage-like cells (type A synoviocytes) located at the outermost layer compared to that of the fibroblast-like spindle shaped cells (type B synoviocytes) present at the inner layer (Fig. 1C and D). CD9 expression decreased significantly in type B cells in the OA group when compared to the control group (p < 0.05; Table 3). A positive reaction was also noticed in stromal fibroblasts and macrophages that were located near the vessels. Generally, CD9 and CD63 expression was more intense than that of CD151 in all samples. CD63 expression significantly increased in vascular smooth muscle, and fibroblasts in SST of the OA group (p < 0.05, Table 3).

Endothelial Cell Antigens
Arterial, venous, and capillary endothelia were strongly reactive with CD31, CD36, CD105, CD106, and CD146 in both normal and OA samples (Figs. 1E and 2A-D). In the OA group, CD31 and CD36 presented a moderate reaction with macrophage-like cells of the SLL but not with the stromal macrophages. This reaction was statistically significant compared to the control group (p < 0.05 and p < 0.05, respectively, Table 3). CD106 exhibited a moderate to strong immunoreactivity with the SLL; the reaction shifted significantly onto fibroblast-like type B synoviocytes located at the upper layers in the OA group (p < 0.05). CD106 additionally strongly stained stromal fibroblastic and dendritic cells in the OA group (Fig. 2B-D).

Integrins
SLL and vascular smooth muscle moderately, and vascular endothelium and especially capillaries strongly expressed CD49a, b, c, f, and CD29 in both the normal and the OA groups (Fig. 1G-I). CD51 and CD61 were mild to moderately expressed on SLL, vascular endothelium and smooth muscle. CD51 expression was significantly more obvious in vascular smooth muscle of the OA group comparing to that of the control group (p < 0.05, Table 3, Fig. 1F). CD51 presented a prominent expression on the outermost layer of the SLL reacting with both type A and type B synoviocytes with no statistically significant difference between normal and OA groups (Table 3). CD41 presented a rather restrictive expression pattern limited to the vascular endothelium.

Miscellaneous
An intense reaction on the SLL and vascular endothelium was noted for CD98 in both normal and OA groups. Immunoreactivity was prominent at the outermost lining layer type A macrophage-like cells and stromal macrophages in the OA group. Type B cells presented significantly lower CD98 expression in the OA group when compared to that of the control group (p < 0.05, Table 3, Fig. 2E-G). CD39, villi also appeared normal.

### Table 2 Cell Surface Molecules and Their Monoclonal Antibodies

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<tr>
<th>CD family</th>
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CD99, CD143, and CD147 presented a restricted expression pattern basically limited to the vascular endothelium with no statistically significant difference among groups (Table 3).

**Discussion**

Synovial tissue is known to have important immunological features and is the target tissue in the articular joint during inflammation. Synovium is considered to be at a normal-like state in OA and its immunohistochemical aspects are compared to that of rheumatoid arthritis (RA) in many studies since the inflammation is episodic and mainly absent in this disease.\(^\text{10,11}\) In fact, there is still some controversy concerning the pathological criteria of the synovial involvement in OA.\(^3\) Oechler and colleagues\(^5\) reported ST alterations in all specimens that they examined deriving from patients with diagnosed OA. A large spectrum of alterations was found in different stages of OA and four different basic patterns of synovial reactions could be identified: (1) hyperplastic, (2)
inflammatory, (3) fibrotic, and (4) detritus-rich synoviapathy. They also documented that in OA synovium significant inflammation can occur. In this study we observed a hyperplasia and hypertrophy of the SLL. In some samples, hypercellularity and hypervascularity and in some others fibrotic changes were evident in the SST. This data was consistent to those of Oehler and colleagues. This suggests a distinct immunogenic role of the synovium in OA pathogenesis.

CD9, CD63, and CD151 are members of the tetraspan or transmembrane 4 (TM4) superfamily presenting a high degree of homology in their membrane-spanning domains. CD9 immunoreactivity was prominent on type A cells located at the outermost layer and significantly decreased in type B cells of the OA group. CD63 expression significantly increased in the vascular endothelium and smooth muscles of the OA group. SLL macrophage-like cells and SST mast cells but not the SST macrophages expressed CD9 in OA and RA patients. Upregulation of CD9 and CD63 expression in OA synovium may indicate the possible act of these molecules as facilitators that increase the formation and stability of functional signalling complexes in the SLL and vascular endothelium in the pathogenesis of OA. These molecules are reported to be involved in cell activation in different organs. SLL isolated from end-stage OA patients contains some cells that expressed typical combinations of mesenchymal progenitor cell surface markers, including CD9, and have the potency of osteogenic and chondrogenic differentiation. CD9 may be involved in one of the cellular transdifferentiation steps. The staining pattern of CD9 and CD63, even though double staining was not accomplished in this study, may reflect a co-localization with some integrins as reported by Demaziere and associates. Relatively weak and restricted expression of CD151 (PETA-3, platelet endothelial tetraspan antigen-3) on vascular endothelium that was
detected in the present study was consistent with its splenic expression pattern.\(^8\)

In this study, CD31 and CD36 presented a moderate reaction with macrophage-like cells of the SLL but not with stromal macrophages in the OA group. According to Vallejo and coworkers,\(^16\) consistent with the idea that lesions in rheumatoid synovitis are sites of antigenic recognition, the characteristic focal expression of thrombospondin and its receptor CD36 on antigen presenting cells such as macrophage and fibroblast-like synoviocytes suggest a central role of this molecular interaction in the expansion of tissue infiltrating T cells. CD31 and CD36 may be involved in an interaction of the OA pathogenesis. On the other hand, detection of endothelial precursor cells in the ST of RA and OA patients by using a combination of CD34+ and CD31-reaction provided evidence for vasculogenesis induced by precursor cells that arise in situ or from circulating progenitors.\(^19\) In other studies CD31 was reported to be expressed by normal, OA, and RA patients’ SST vascular endothelium equally; but it was present on most RA and OA SLL cells and macrophages and on significantly less myeloid cells in normal ST.\(^10,11\) Our immunohistological data is consistent with the findings of Szekanecz\(^10\) and Johnson.\(^11\) CD36 mAbs were previously suggested to be valuable tools for studying macrophages and endothelial cells and their shared antigenic determinants.\(^18\) As a conclusion we suggest the use of CD31 and CD36 as possible candidate markers for visualization of myeloid activated cells in ST inflammation.

Our findings on CD105 and CD106 immunoreactivity with SLL, and stromal fibroblastic and dendritic cells, are consistent with previous studies.\(^19,20\) In this study, CD146 was expressed almost exclusively by vascular endothelium in both the normal and the OA groups. This finding was relevant with Neidhart and colleagues,\(^21\) who suggested that high levels of soluble CD146 found in RA synovial fluid, particularly in patients with early disease, could reflect increased activity of endothelial cells and angiogenesis. In vivo expression of CD146 on synovial fluid T cells of RA patients may also suggest the possible involvement of this adhesion molecule in the extravasation and/or homing of activated T cells.\(^2\)

Our findings on the tissue distribution of integrins were in agreement with other studies.\(^5,11,24\) Similar to the report by Nikkari and associates,\(^6\) in spite of the proliferation and activation of the SLL in inflammation, the ECM remained as if it is normal in this study.

CD98 presented an intense reaction on the SLL and vascular endothelium in both groups; its immunoreactivity was prominent at the outermost lining type A macrophage-like cell layer and stromal macrophages in the OA group. This activation antigen’s functional importance in ST needs to be clarified.

CD39, CD99, CD143, and CD147 presented rather restricted expression patterns that were mainly limited to the vascular endothelium in both groups. CD39, CD99, and CD147 expression in human ST have not been reported to date. CD143 is known to be identical with angiotensin converting enzyme (ACE).\(^25\) Walsh and coworkers reported that locally generated angiotensin II may act via its synovial receptors to modulate synovial perfusion and growth\(^26\) and that ACE was upregulated in the SST in RA.\(^27\) According to that study, increased tissue ACE may result in increased local generation of the vasoconstrictor and mitogenic peptide angiotensin II and thereby potentiate synovial hypoxia.

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**Figure 2** A presents CD105 immunoreactivity on synovial lining layer and vascular endothelium. B, C, and D present CD106 immunoreactivity on synoviocytes and a lymphoid nodule. E, F, and G present CD98 immunoreactivity on synovial lining layer. OA, osteoarthritis; N, normal; Arrow, blood vessel; S, synovial lining layer; ML, macrophage-like type A synoviocyte; FL, fibroblast-like type B synoviocyte; Large arrow, lymphoid nodule; *immune positive cells.
and proliferation in RA. In our study, vascular CD143 expression did not show any significant difference between normal and OA samples. It can be speculated that ACE was not a part of the pathogenesis of OA at least in this study.

**Conclusion**

In conclusion, human normal and OA synovium were comparatively evaluated by detailed immunohistological staining using a broad spectrum of mAbs in this study. Tetraspan antigens and CD98 presented rather unique staining patterns in OA synovium suggesting special roles for each antigen on the SLL. Both endothelial cells and type A synoviocytes expressed CD31 and CD36 in OA synovium. On the other hand, a marked variation in the immunohistological reaction of mAbs encountered with the SLL macrophage-like cells and the SST macrophages was presented. ST may have important immunological features as a target tissue in the immunopathology of OA. The potential role of chronic SLL activation deserves further attention.

**Acknowledgment**

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**References**