Identification of α6ß1 Integrin Positive Cells in Synovial Lining Layer as Type B Synoviocytes

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ABSTRACT. ***Objective.*** In rheumatoid arthritis (RA) the synovial lining is responsible for cartilage destruction. Laminin is one of the major matrix molecules surrounding the lining cells. We investigated the laminin adhesion mechanism of synovial lining cells by analyzing the presence of its receptor, α6ß1 integrin, on type A and type B synoviocytes.

Methods. The α6 integrin subunit and a macrophage marker were simultaneously localized by immunohistochemistry in 29 RA derived, 6 osteoarthritis derived, and 2 healthy synovial samples by light and electron microscopy. We also used enzyme treatments to release cells from synovial tissue samples and localized the same antigens on adherent cells.

Results. The α6ß1 integrin positive cells were localized in basal areas of the lining layer and many of them were negative for the macrophage markers. By immunolabeling electron microscopy the α6 integrin positive cells were confirmed to represent the fibroblast-like type B cells. Further, in freshly isolated synoviocyte cultures the type B cells were positive for α6 integrin, whereas all other cell types were negative for this laminin receptor.

Conclusion. Integrin α6ß1 is known to be a laminin receptor of endothelial cells, adipocytes, and macrophages, not usually expressed on fibroblasts. However, in synovial lining layer it is expressed on fibroblastic type B cells, but the macrophage population is negative. The unique characteristics of synovial lining cells distinguish them from other connective tissue cells and must be taken into account in all considerations of the pathogenic mechanisms of rheumatoid disease. (J Rheumatol 2001;28:478–84)

Key Indexing Terms:
RHEUMATOID ARTHRITIS INTEGRINS SYNOVIOCYTE SYNOVIAL LINING

In normal synovium the lining cells form a thin layer against the joint cavity. Two different lining cell types have been identified: Type A cells originate from the bone marrow and belong to the monocyte-macrophage cell lineage[1,2]. Type B cells resemble fibroblastic cells with a well formed endoplasmic reticulum and Golgi apparatus[3]. They arise from the mesenchymal tissue, but may still express HLA-DR antigens[3]. The interaction between synovial fibroblasts and their cellular environment is thought to be one of the key phenomena in the tissue destruction taking place in the rheumatoid joint[4].

The extracellular matrix surrounding the lining cells contains laminin and type IV collagen[5,6]. Tenascin[7] and fibronectin[7-9] are present as well. Specific cell surface receptors, the integrins, mediate the interaction between cells and the matrix[10,11]. Integrins are heterodimeric α/ß-type complexes. Twenty-four different integrins are known at present, and at least 19 of them are matrix receptors.

Integrins can play an important role in rheumatoid inflammation. They mediate the attachment of lining cells to their surroundings and may participate in the interaction between lining cells and cartilage. Integrins also regulate cellular functions, including the release of connective tissue degrading matrix metalloproteinases[12-14]. We and others have described the expression pattern of multiple putative integrin-type laminin, collagen, vitronectin, and fibronectin receptors in synovium[5,15-18]. The main result in our studies has been that the α6 integrin is the major α subunit associated to ß1 integrin in lining cells. In addition, the number of cells positive for α6 integrin is much higher in the lining cell layer of a healthy joint than in the proliferating lining cell layer of a joint affected by rheumatoid arthritis (RA)[16]. Further, αV is an abundant integrin subunit on the lining cells of healthy joints, and its partner seems to be ß5 rather that ß3[5]. The expression

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of these integrins is remarkably reduced in the rheumatoid lining cell layer[5].

We recently showed that cytokines also regulate the expression of integrins on synovial cells and suggested that this may be one of the mechanisms behind the downregulation of integrin expression in RA[19]. We have analyzed the expression of α6ß1 integrin specifically on both type A and type B synovial lining cells. Our results indicate a unique pattern of integrin localization in rheumatoid synovial lining cells compared to any other tissue.

MATERIALS AND METHODS

Patients. Synovial tissues in this study were obtained from patients undergoing orthopedic surgery. Six patients had osteoarthritis (OA) (mean age 63 yrs) and 29 had RA (mean age 54 yrs). In the latter group all fulfilled the American Rheumatism Association revised criteria for classic or definite RA[20]. Disease duration in patients with RA ranged from one to 36 years. The healthy synovial tissue used in this study was removed from 2 cadavers donating bone transplants.

Primary synovial cell cultures. During surgery the pieces of synovial tissue removed from the patients were collected to cell culture medium. Within a few hours the samples were cut to small pieces and incubated for 2 h at 37°C with 0.5% trypsin and bacterial collagenase (type II, Sigma Chemical, St. Louis, MO, USA) in Hanks' buffer containing 1 mM $CaCl_2$ as described[21]. Cells released from tissue were collected, washed, and allowed to attach to cell culture wells in the presence of medium containing 10% fetal calf serum. After 24 h cells were fixed with 2% paraformaldehyde.

Immunohistochemistry. Tissue specimens were either snap frozen or fixed in 10% buffered formalin for routine paraffin embedding. All sections corresponding to those used for immunolocalization studies were stained with hematoxylin and eosin for histopathological analysis. Frozen sections (6 µm) of the synovial tissue were cut in a cryostat and fixed with –20°C acetone for 5 min and stored at –70°C until used. Monoclonal antibody G0H3 was used to analyze the expression of α6 integrin subunit[22]. Macrophages were detected by human alveolar macrophage marker (HAM56) antibody (Enzo Diagnostics, New York, NY, USA) and by antibody against CD68 (PG-M1; Dakopatts, Glostrup, Denmark) in paraffin sections, and with antibodies against CD14 (HB-246; ATCC, Rockville, MD, USA) and CD18 (HB-10164; ATCC) in frozen sections. HLA-DR was recognized by antibody from Dakopatts. Immunolocalization of the antigens was performed as described using an avidin-biotin technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA)[23]. The immunoperoxidase reaction for HAM56 and CD68 were performed by Techmate 500 automate (Dako A/S, Glostrup, Denmark). Double staining was performed using the Vectastain ABC kit with Vector Blue or Vector Red as chromogens. Alternatively, 3,3′-diaminobenzidine tetrahydrochloride (DAB; Polysciences Inc., Warrington, PA, USA) giving a brownish color was used as the chromogen[24]. The specimens were mounted and examined using a Leitz Aristoplan epifluorescence microscope and photographed using photoautomat Wild MPS (Wild Leitz 46/53 Photoautomat).

Analysis of immunohistochemistry. Synovial samples in which the lining layer was not clearly evident were excluded from study. All sections were examined under 400× magnification. In the lining cell layer, the number of positive cells was expressed as a percentage of the total number of lining cells counted, up to a maximum of 300 cells.

Electron microscopy. The pre-embedding method was used[25]. Briefly, the specimens were unfixed but stabilized with 0.1% sodium azide before incubation with anti-α6 integrin (GOH3) antibody overnight at 4°C followed by Vectastain ABC immunoperoxidase with prolonged incubations and washes. DAB was used as the chromogen prior to fixation with 5% glutaraldehyde. The specimens were then postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812 for conventional electron microscopy.

RESULTS

Typing of cells in the synovial lining layer. In all rheumatoid synovial tissue samples there were infiltrates of inflammatory cells, and proliferation of synovial stromal fibroblasts was evident. The synovial lining cell layer was hypertrophic, forming villous projections into the synovial space. In OA derived synovial tissue some signs of inflammation were present although to a lesser extent than in those derived from patients with RA. In OA derived synovial samples there were fewer CD14 and CD18 expressing synovial macrophages compared with RA samples. The 2 samples representing healthy synovium had a thin lining cell layer mainly consisting of one cell layer. Few synovial cells expressing CD14 or CD18 were present and there were no signs of angiogenesis or inflammation in the synovial stroma. In healthy synovial lining layer 17.5% of cells were positive for CD68 and 18.5% of cells expressed HAM56. In RA lining layer 53.0% (SD ± 3.3) of cells were CD68 positive and 27.4% (SD ± 3.8) were HAM56 positive. In the lining of OA synovium 52.3% (SD ± 2.4) and 25.4% (SD ± 6.6) of cells were positive for CD68 and HAM56, respectively. Thus, in inflamed synovium there were significantly more CD68 positive than HAM56 positive cells. The CD68 positive cells were often found on the superficial synovial lining layer, whereas the HAM56 positive cells were located mainly in the basal cell layer of synovial intima (Figure 1). The immunoreaction of both macrophage markers was weaker in healthy synovial samples than in the inflamed synovium.

Integrin α6 is located on type B cells both in vitro and in vivo. In accord with our previous observations[16], synovial lining derived from noninflamed synovium showed strong reaction with α6 integrin antibody, whereas in inflamed synovium there were fewer α6 integrin positive cells in the lining cell layer. In the double immunoreactions the macrophage markers (CD14 or CD18) and α6 integrin were located in different cells (Figure 2, panel A). In inflamed and noninflamed synovium very few cells were positive for both markers. In inflamed synovium the amount of macrophages was increased and α6 integrin expressing cells were often located in the basal lining layers (Figure 2, panel B).

In electron microscopy, the cells identified as macrophage-like (type A) had micropinocytotic vesicles and filopodia, whereas the fibroblast-like (type B) cells had a very prominent Golgi apparatus. The type A cells were smaller than the type B cells. The nuclei of type A cells were dense compared to type B cells. In immunolabeling microscopy the α6 integrin subunit was localized on the surface of type B cells, and cells representing type A were negative for α6 integrin subunit (Figure 3).

In primary synovial cell cultures 4 different types of cells were present, as described[21]. Spindle shaped cells, representing type A cells, expressed macrophage markers (CD14 or CD18) (Figure 4, panel A), but were negative for α6 integrin. In different 24 h primary cultures, about 10–30% of cells

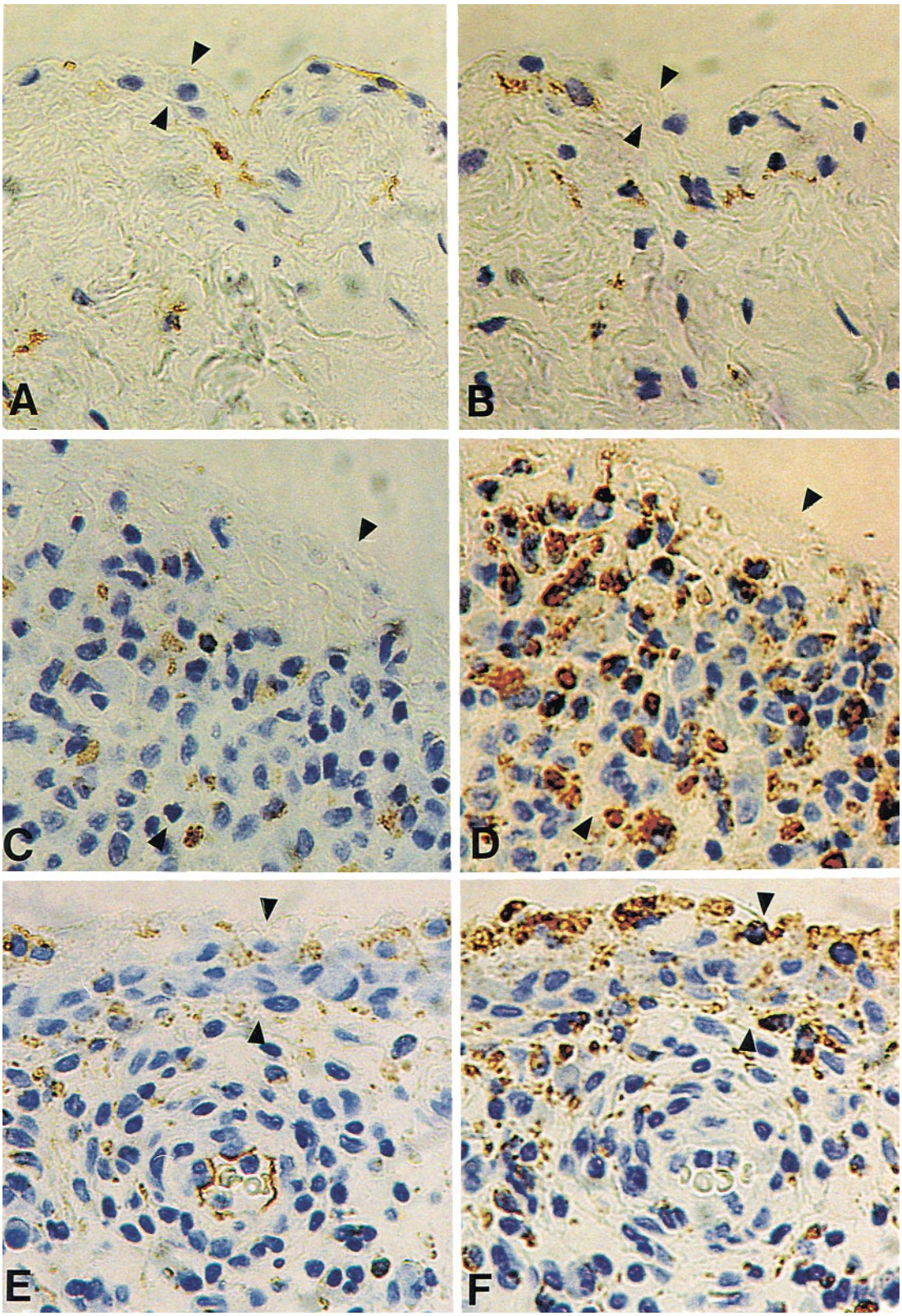

Figure 1. Immunohistochemical localization of the HAM56 and CD68 macrophage markers by brown peroxidase reactions in adjacent paraffin embedded sections. Panels A and B represent healthy synovium, panels C and D rheumatoid synovium, panels E and F osteoarthritic synovium. Panels A, C, and E show the expression of HAM56, and panels B, D, and F show CD68. Arrowheads show the thickness of the lining cell layer. The nuclei are counterstained blue by hematoxylin. Magnification 420×.

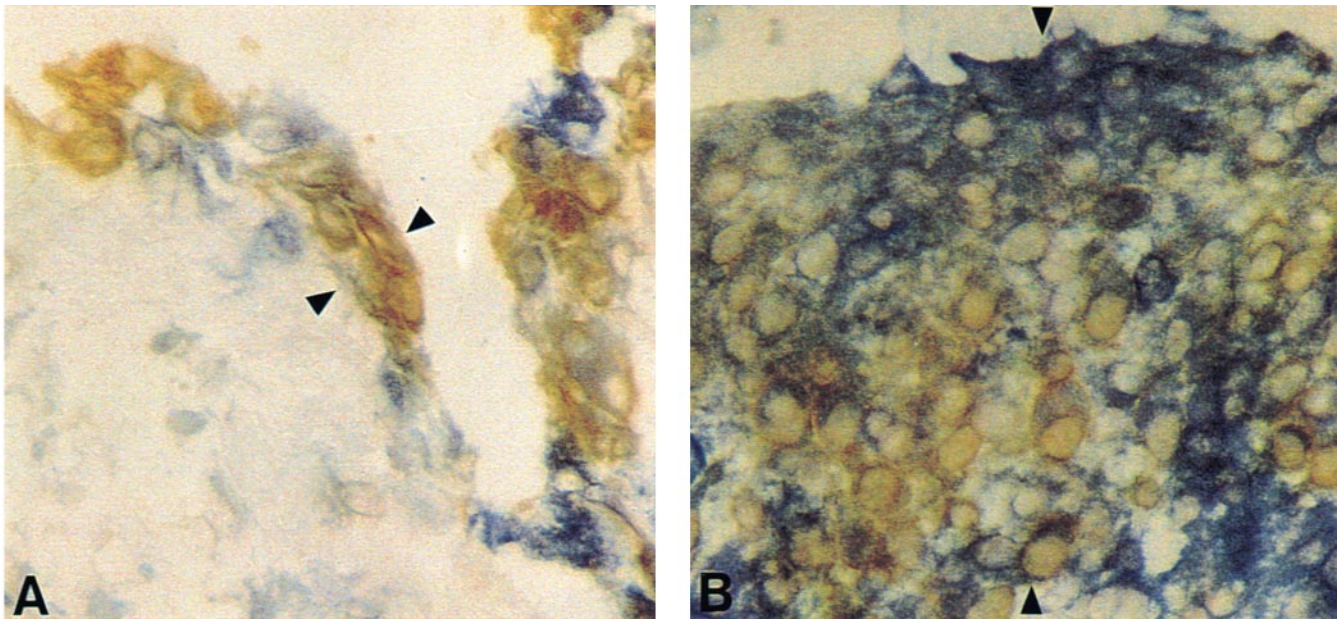

Figure 2. Immunohistochemical localization of the α6 integrin subunit (brown) and CD18 macrophage marker (blue) in noninflamed (A) and rheumatoid (B) synovium. The lining cell layer is thicker in rheumatoid synovium, the portion of macrophages has increased, and the portion of α6ß1 integrin positive cells decreased. The synovial lining surface is delineated by arrowheads. Magnification 610×.

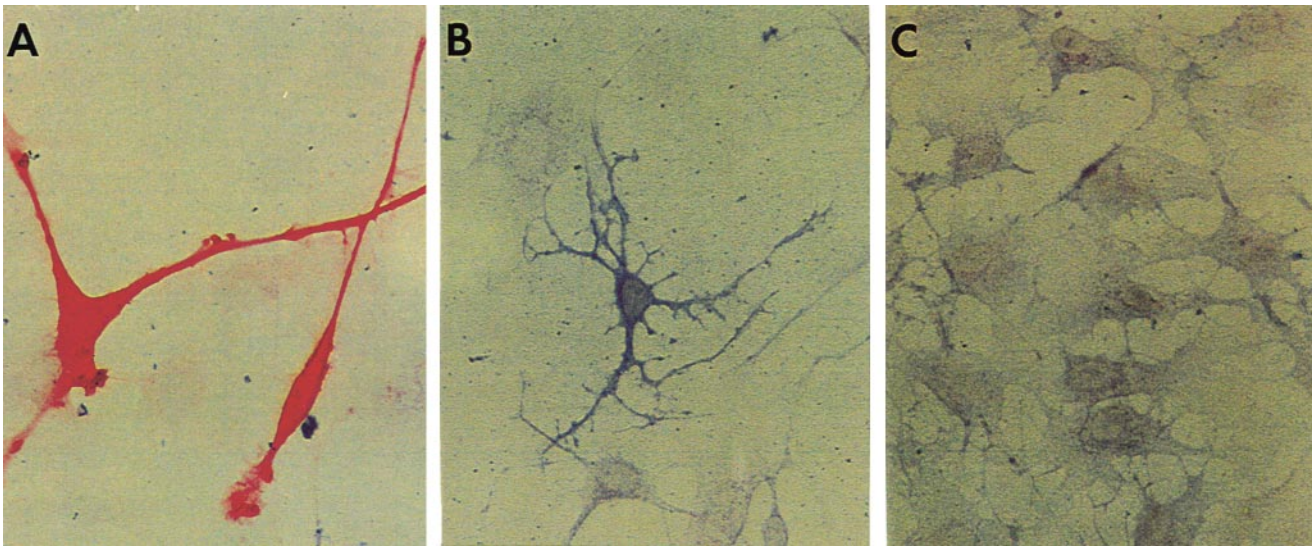

Figure 4. Expression of α6 integrin (blue) and the macrophage marker CD18 (red) in adherent cells in culture derived from rheumatoid synovium. Panel A shows a macrophage-like cell (synovial type A cell). Panel B shows a stellate shaped cell (synovial type B cell). Panel C shows fibroblasts. Magnification 420×.

belonged to this category. They also expressed HLA-DR antigen (not shown). The cells with numerous long branching processes, representing type B cells, contained α6 integrin (Figure 4, panel B) and HLA-DR (data not shown). About 20% of cells belonged to this category. Other cells, namely typical fibroblastic cells and smaller round cells resembling peripheral blood monocytes, were negative for α6 integrin (Figure 4, panel C).

DISCUSSION

The role of synovial lining cells in the pathogenesis of RA has been emphasized recently. These cells are considered as potential therapeutic targets[26]. The basic cell biology of synovial lining cells is incompletely known. A growing body of evidence indicates that the phenotype of synovial lining cells as well as the structure of the extracellular matrix surrounding them is significantly different compared to any

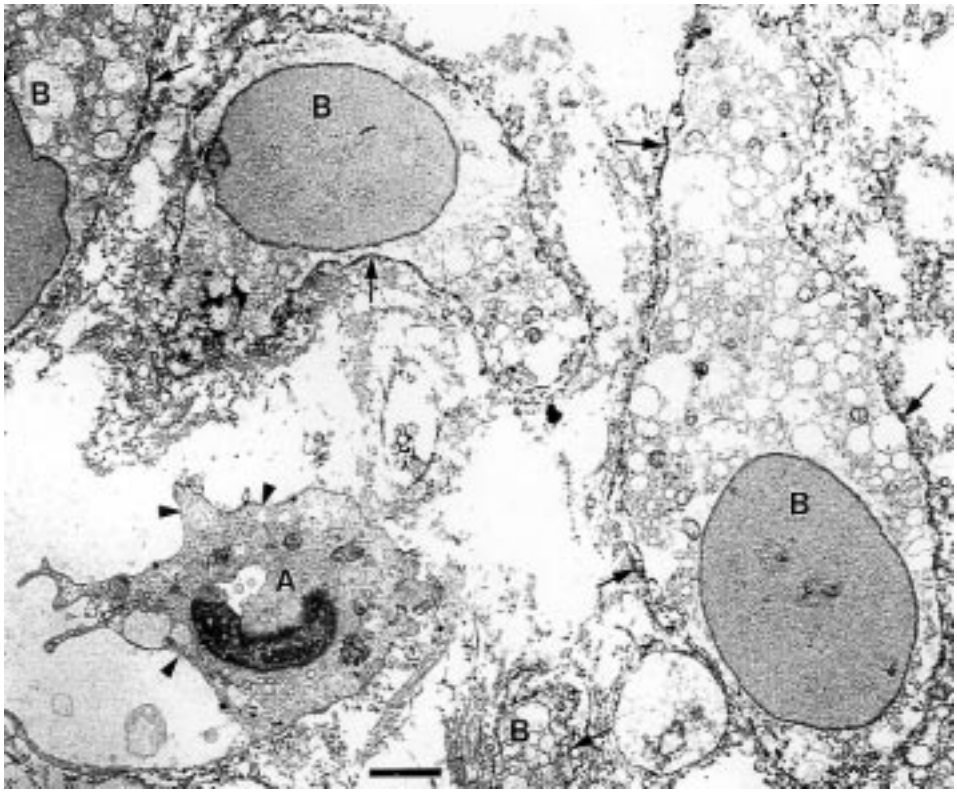

Figure 3. Subcellular localization of α6 integrin by immunolabeling electron microscopy on the plasma membrane (arrows) of fibroblast-like cells (B) (type B cells). The adjacent type A cell (A) is negative (arrowheads). The nucleus and cytoplasm were counterstained lightly with uranyl acetate to confirm the localization of the black reaction product (arrows) on the plasma membrane. The endoplasmic reticulum has degenerated into vesicular structures, because the antigenicity did not allow proper fixation. Scale bar 2 µm.

other tissue[27,28]. In particular, the integrin-type cell adhesion receptors may regulate cellular functions, such as release of matrix metalloproteinases[12-14], that are important for the progression of rheumatoid disease. Studies indicate marked changes in integrin expression in rheumatoid synovial lining cells[5,15-18,29].

The lining layer in RA is thick and invasive, and the relative number of α6ß1 integrin positive cells is decreased[15-17] concomitantly with the increase in the number of type A cells. With light microscopy it is not possible to distinguish between type A and type B cells by their morphology, and the localization of integrins in the 2 specific cell types has been undetermined. Integrin α6ß1 is typically a laminin receptor of endothelial cells, adipocytes, and macrophages and is not expressed on fibroblasts[30,31]. Thus the original hypothesis was that the type A cells contained α6 integrin. Our results, however, unveiled the unique localization of adhesion receptors on synovial lining cells.

The synovial lining seems to have at least 2 distinct populations of macrophages[32]. Here, macrophage-type cells were studied more closely by using 2 macrophage markers. Human alveolar macrophage marker, HAM56, can recognize "mature" macrophages. Another widely used marker of monocytes and macrophages is CD68. We show here that 2 macrophage markers, HAM56 and CD68, are differentially located in inflamed synovium, whereas there is no difference in their appearance in healthy synovium. The CD68 positive lining cells are more numerous than HAM56 positive cells in inflamed synovium and they are often located on the superficial part of lining cell layer. The cellular functions of HAM56 antigen and CD68 are unknown. Surprisingly, the cells expressing macrophage markers did not have α6 integrin on their surfaces. The α6 integrin positive cells were mainly located in the basal areas of the lining cell layer and they were negative for the macrophage markers. Immunolabeling electron microscopy showed that in rheumatoid synovium the cells considered as macrophages, or type A cells, were negative and the fibroblast-like type B cells were positive for α6 integrin. The results suggest that in RA the disappearance of α6ß1 integrin on lining cells is mainly due to the reduced proportion of the type B cells.

The expression of α6ß1 integrin on synovial lining cells was further tested in experiments in which we used enzyme treatments to release cells from rheumatoid synovial tissue. The adherent synovial cells could be divided into 4 different populations, as we have shown[21]. The elongated cells were positive for CD18 and CD14 and they were supposed to represent type A cells. They did not express α6ß1 integrin. The

cells with a stellate body and numerous branching filopodia-like processes were supposed to represent type B cells. They are HLA-DR positive and negative for the macrophage markers[21]. Here, they were the only cells expressing α6 integrin. When the synovial cells are cultured *in vitro* for few passages the type A cells disappear[21] and the fibroblastic cells no longer contain α6 integrin[19]. Studies have revealed the gradual dedifferentiation of the stellate cells to fibroblasts[33]. These cells are also the main producers of collagenase-1[34,35]. The *in vitro* dedifferentiation may reflect the importance of type A cells for α6 integrin expression in type B cells. It is tempting to speculate that the type A cell derived factors, such as cytokines, may regulate the phenotype of type B cells also *in vivo*.

The fibroblast-like cells in the synovial lining are needed to maintain the cellular structure of synovial intima[36]. Pannocytes are thought to be a special entity of fibroblasts that most likely are responsible for the final cartilage destruction. It has been suggested that pannocytes are transformed type B cells[26,37]. In spite of their important role in the pathogenesis of rheumatoid disease, type B cells are not well characterized. Our results indicate that synovial type B cells differ from other fibroblastic cells since in both inflamed and noninflamed synovium they contain α6 integrin. Synovial lining cells are surrounded by laminin, the ligand for α6ß1 integrin[5,6]. Studies have shown the importance of integrin mediated cell adhesion for cell proliferation, phenotype, and survival, emphasizing the importance of type B cell–laminin interaction. Further, the integrin–ligand interaction may trigger matrix metalloproteinase expression and initiate matrix destruction or remodeling. That type A cells did not express α6ß1 integrin laminin receptor may contribute to the mobility of macrophages and to their ability to interact with other matrix components. The unique features of the synovial lining cell types must be taken into account when the pathological mechanisms of rheumatoid disease are studied.

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