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Basement Membrane Proteins in Synovial Membrane: Distribution in Rheumatoid Arthritis and Synthesis by Fibroblast-like Cells

M. SCHNEIDER, B. VOSS*, J. RAUTERBERG**, M. MENKE, TH. PAULY***, R.K. MIEHLKE+, J. FRIEMANN++, U. GERLACH**

Summary **Rheumatoid arthritis is a complex disease of unknown origin. In consequence of some immunological reactions, proliferative invading synovial tissue leads to destruction of normal joint architecture. The aim of this study was to investigate qualitative changes in extracellular matrix distribution of proliferating rheumatoid synovium and their cellular origin. Synovial tissues from 57 clinically indicated arthrotomies were investigated with immunofluorescence, using specific antibodies against extracellular matrix proteins in tissue slides and cultured cells, which were also studied for collagen biosynthesis. Results indicated that synovial fibroblast-like cells synthesize and secrete basement membrane proteins laminin and collagen type IV as e.g. endothelial cells or organogenic fibroblasts. Laminin and collagen type IV were specifically demonstrated pericellularly in the hyperplastic lining layer of active rheumatoid synovitis. These findings are discussed with respect to the possible implication of altered cell-matrix interactions in rheumatoid synovial proliferation.**

Key words **Rheumatoid Arthritis, Synovium, Extracellular Matrix, Basement Membrane Proteins, Immunohistochemistry.**

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic connective tissue disease leading to joint destruction. Initiated by an unknown antigen in genetically predisposed persons, activation of various cell types, e.g., macrophages, T- and B-lymphocytes, endothelial cells and fibroblasts, may eventually induce the characteristic changes in the synovial membrane: mononuclear cell infiltration, angiogenesis and lining cell hyperplasia (1,2). The degradation of articular cartilage, bone and ligaments is an outcome of final invasion by an activated synovial proliferating connective tissue (3).

The cellular origin of proliferating synovial tissue is still under discussion, with data from experimental arthritis indicating bone marrow as a source of primarily infiltrating cells (4). Local proliferation in response to activated mononuclear cells may be the cause in RA (2,5-7), and tumour-like proliferation is also considered feasible (3).

From cell culture studies, proliferating synovial cells are generally characterized as fibroblast-like with collagen synthesis comparable to normal skin fibroblasts (8). In RA, proliferation in conjunction with increased extracellular matrix protein synthesis of synovial fibroblast-like cells may be vital to pannus formation. Cytokines, e.g. TNFα, Il-1, IFN-gamma and growth factors, are capable of inducing proliferation of cultured synovial cells, enhancing synthesis of glycosaminoglycans and prostaglandines, activating collagenase and inducing expression of Ia antigen, resembling most of the alterations found in rheumatoid synovium (5,9-13). In part, even activated synovial fibroblast-like cells may contribute in an autocrine or paracrine way to chronic inflammation and proliferation by synthesizing and secreting proinflammatory mediators such as Il-1, IL-6, bFGF, TGFβ1 and GM-CSF (14-16).

Enhanced synthesis of collagens is known to be attended by quantitative changes in relative amounts of

Medical Clinic and Policlinic B, University of Münster; *Professional Associations' Research Institute for Occupational Medicine, Bochum; **Institute for Arteriosclerosis Research, Münster; ***Rhine Rheumatology Center Meerbusch; +Northwest German Center of Rheumatology Sendenhorst; ++Department of Environmental Pathology of the Institute for Environmental Hygiene at the University of Düsseldorf.

different collagen types (17-21). The aim of the present study was to evaluate possible qualitative changes in extracellular matrix distribution in rheumatoid synovium and related cells.

MATERIAL AND METHODS

Synovial cell cultures

Specimens of human synovial tissue were obtained at clinically indicated arthrotomies from 18 patients with rheumatoid arthritis, 31 with meniscal lesions and 8 with osteoarthrosis requiring joint replacement. After removing adherent adipose tissue and capsula, specimens were minced, transferred to sterile plastic tubes, and washed three times in PBS. Half of each sample was directly explanted in 75-cm^2 plastic culture flasks (Nunc, Roskilde, Denmark) using DMEM (Merck, Darmstadt, Germany) containing 2mM L-glutamine and 12.8% fetal calf serum (bibco Karlsruhe, Germany). The other material was dispersed with collagenase (Seromed Biochrom, Berlin, Germany; diluted to 60 U/ml in PBS) for 4 hrs. at 37° C. After centrifugation (10 min., 1,800 rpm) cells were cultured as described.

Immunofluorescence studies

Collagen types I and III were prepared from human skin as described by Miller (22), and types IV and V and laminin P1 from human placenta (23,24). Fibronectin was isolated from human plasma (25). Antisera were raised in goats (collagen types I and II and laminin) and rabbits (collagen type IV, 7S-collagen and fibronectin) and specific antibodies were isolated according to Voss (26). FITC-conjugated antibodies against goat and rabbit IgG were purchased from Behringwerke (Marburg, Germany). Monoclonal antibodies against vimentin and desmin were obtained from Boehringer (Mannheim, Germany), against factor VIII related antigen came from Immuno (Heidelberg, Germany). Immunofluorescence analyses were performed in 3rd passage unless otherwise indicated. Antigens were demonstrated intra- and extracellularly and in tissue specimens as described by Voss (27). Thereby cell surface and extracellular matrix are stained using moist cells; after 24 hrs. drying at 37°C cell membranes are permeable for antibodies. Immunofluorescence was observed with a Leitz Orthoplan epiiluminated fluorescence microscope (Leitz, Wetzlar, Germany).

Collagen-biosynthesis

Collagen polypeptides were demonstrated in cell layer and medium after incubation with ^{14}C-glycine for 24 hrs. as previously described (28). The cell layer was incubated with 1M sodium chloride overnight at 7°C to facilitate removing from the flask. 100µg human collagen type I was added to the medium and suspended cell layer. Following dialysis against 0.1% acetic acid the mixture was incubated with 100µg pepsin and collagen precipitated by sodium chloride (12% w/v). Non-dialysable radioactivity in final solutions is a parameter for collagen biosynthesis, whereas the amount of secreted protein was estimated from the radioactivity of the medium after dialysis against 0.1% ammonium bicarbonate (Compu-gamma, LKB, Freiburg, Germany). Both medium and cell layer samples of 3,000 cpm marked collagen were separated in 5% acrylamide/5% SDS gels (Serva, Heidelberg, Germany; SE 600, Hoefer Scientific Instruments, USA). After 3hrs. in 2.5-diphenyloxazole (22% in dimethylsulfoxide) and 1hr. in 7% acetic acid (with 1% glycerine), dried gels were incubated at - 70°C for 3 to 21 days with an X-ray film. Processed films were evaluated by densitometric scanning of gels performed under nonreducing conditions to allow separation of collagen type I and III peptides (Ultroscan II, LKB, Freiburg, Germany).

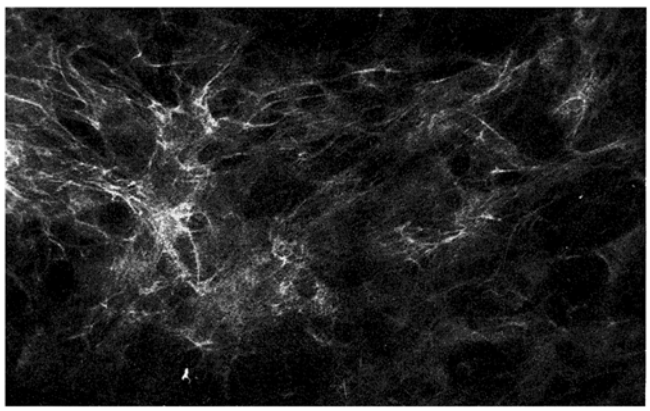
Stimulation with cytokines

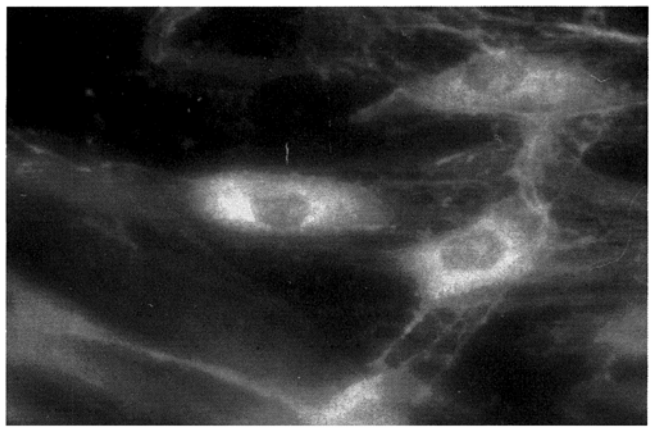
Stimulation studies were performed with Il-1β (Genzyme, Boston, USA; 10U Il-1β/ml FCS-free medium with 20 ml medium/75 mm^2 flasks and 10^6 cells) and rIFN-gamma (100U/ml FCS-free medium; Bioferon, Laupheim, FRG). Cells were incubated for 72hrs. for immunofluorescence, cell proliferation counting and determination of collagen synthesis.

RESULTS

Immunofluorescence in cultured cells

Explanted directly from tissue specimens, about 90% of growing cells were spindle-like with parallel orientation in culture. Collagenase treatment of synovial tissues led to 70-80% stellate or dendritic cells; 15 to 20% could be morphologically classified as lymphocytes. Only a small number of cells exhibited large vacuoles within cytoplasma, like macrophages. Up to 15% cells showed a positive reaction using antibodies against factor VIII-related antigen indicating these cells as endothelial cells. There were some variations in relative cell amounts derived from different synovial tissues, a strong relation to the underlying disease could not be determined. In active synovitis, up to 20% lymphocytes and 15% macrophages were observed.



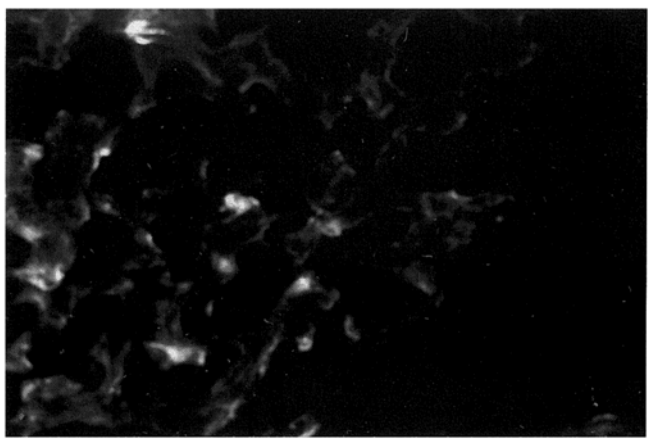


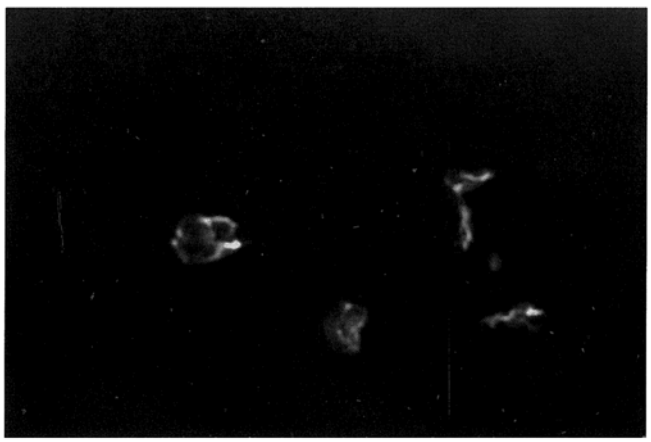
Figs. 1a and 1b: Immunofluorescence microscopic detection of collagen type IV in cultured synovial fibroblast-like cells (a) after collagenase dispersion of synovial tissue extracellular and cell-surface associated (×510) and (b) derived after collagenase treatment after 72 hrs. incubation with IL-1β (10 U/ml): intra- and extracellular, cell-surface associated reaction on cells (×850).

From the 3rd passage onwards, differences between collagenase-treated und untreated tissues disappeared. With the 2nd passage of collagenase-treated tissue, lymphocytes and macrophages are absent and only 15-20% cells are dendritic, decreasing with any medium exchange. Both the spindle-like and the dendritic cells showed intracellularly a positive reaction with antibodies against vimentin, whereas no fluorescence could be detected using anti-desmin antibodies.

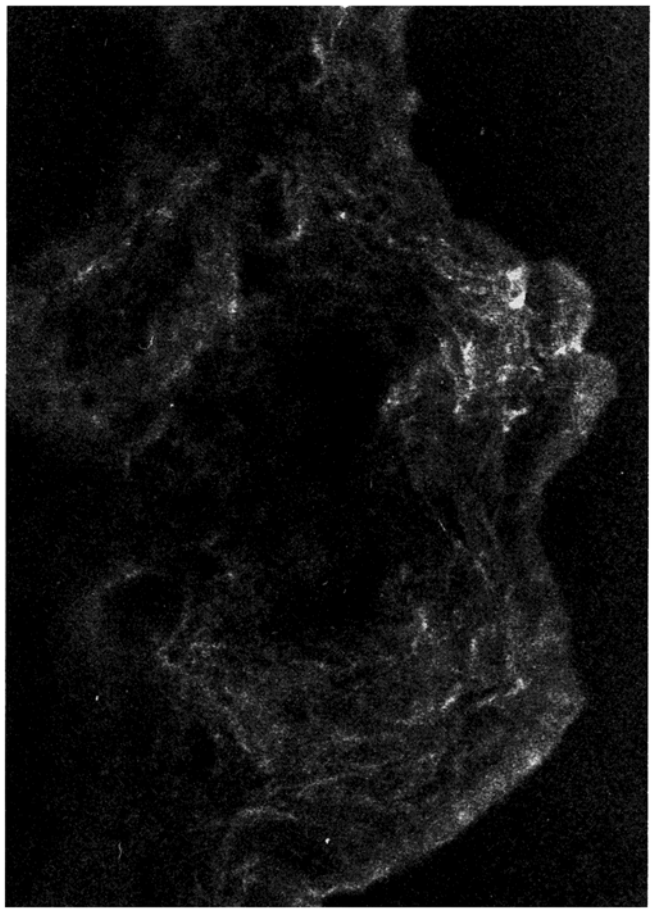
Immunofluorescence studies with antibodies against extracellular matrix proteins exhibited no significant qualitative differences between cultured cells from various sources. Collagen types I and III were found extracellularly and in nearly all cells exhibiting a granular pattern. Intensity of collagen type I specific immunofluorescence was generally higher in comparison with type III, both intra- and extracellularly. Collagenase digestion of synovial tissue was attended by enhanced staining for collagen type III in early passages. Antibodies against fibronectin and collagen type V gave weaker intracellular staining than collagen type I. Extracellularly, thin fibers were seen predominantly at cell surface with an intensity comparable to collagen type III.

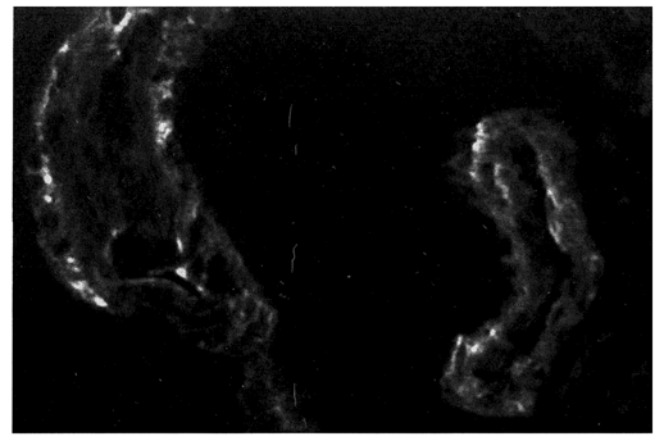
Immunofluorescence reactions with antibodies specific to basement membrane proteins showed significantly different results depending on cell culture procedure. Only a few cells grown from explants exhibited minimal intracellular staining for type IV collagen; extracellularly, a minimal cell surface distribution was detected. The same pattern was seen using anti-laminin antibodies. After collagenase digestion of synovial spec-





Figs. 2a and 2b: Extracellular matrix distribution in a morphologically normal synovial tissue, detected by indirect immunofluorescence (×850): (a) Collagen type I is found in synovial stroma in regular thin fibers, (b) Collagen type IV occurs in vessel wall basement membranes only.





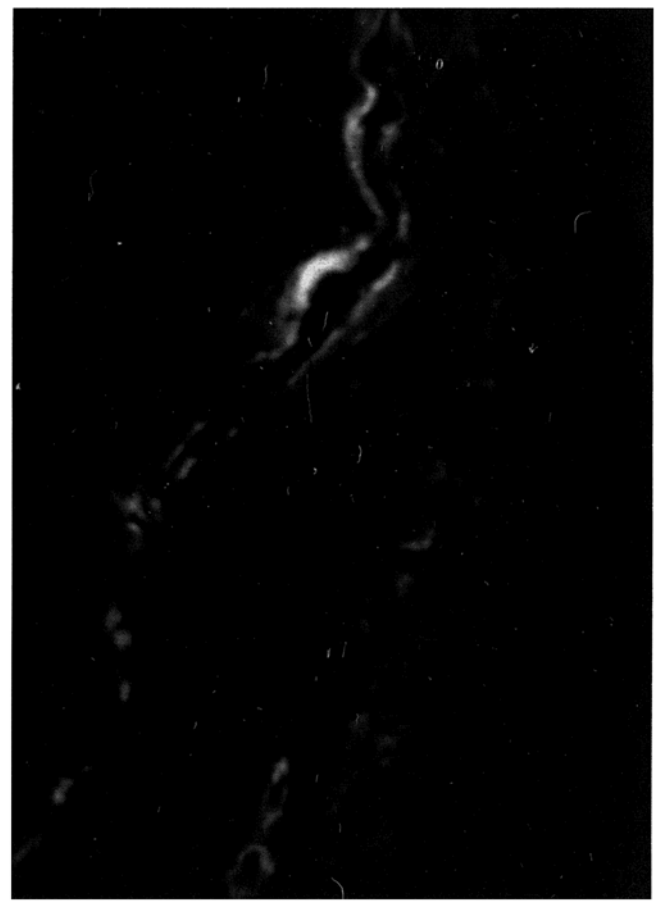


Fig. 3: Immunofluorescence detection of extracellular matrix proteins in active synovitis during RA (×850):

↑ a) Besides intensified staining, immunofluorescence for collagen type I shows a more uniform reaction with no clear fibers;

↗ b) With antibodies against laminin, broadened basement membranes of synovial vessels are detected;

→ c) Collagen type IV clearly stained in sublining layer and pericellularly in hyperplastic lining layer (right side).

imens, intensity of fluorescence was slightly expressed on nearly all cell surface (Fig. 1a). Independent of cell source, intracellular occurrence of both basement membrane components was enhanced by incubation with interleukin-1β, as shown in Fig. 1b for collagen type IV. Extracellular distribution was also increased, but less than intracellularly. γIFN-gamma incubation had no effect on immunofluorescence staining for laminin and collagen type IV.

^{14}C-Polypeptide chains of collagen

In synovial cell culture, proliferation rates (2-4 - 7.6 × 10^5 after 96 hrs. starting with 2 × 10^5) and total protein synthesis (0.26 - 4.29 × 10^6 cpm) varied in

marked degrees, irrespective of underlying disease. After 24hrs. incubation, about 90% of ^{14}C-collagen was found in the culture medium, 10% in the cell layer. Separation by PAGE of polypeptide chains of labelled collagens showed the typical bands of collagen types I and III. Unstimulated and stimulated cells were lacking the bands for collagen types IV and V shown in umbilical vein endothelial cells and human dermal fibroblast cultures respectively. There was no change in this pattern with a longer incubation time up to 96hrs.

Immunofluorescence studies in synovial tissues

In morphologically normal synovial tissues from meniscopexy, collagen types I, III and V and fibronectin were distributed regularly in thin fibers in synovial stroma; the lining cell layer was not marked (Fig. 2a). Collagen type V and fibronectin were additionally found in synovial vessel walls. Type IV collagen and laminin were only seen in vascular basement membranes (Fig. 2b). Synovial fibrosis state of RA was characterized by larger, defined threads of interstitial collagens, which were also found between the cells of the hyperplastic lining layer. Basement membrane proteins were distributed comparably to normal synovium. Synovial specimens of osteoarthrotic joints exhibited an increased deposition of collagen type I, III and V and fibronectin in stroma; lining layer was not changed in comparison to normal tissue.

Active rheumatoid synovitis showed a pronounced immunofluorescence of type I and especially type III collagen with a uniform pattern (Fig. 3a). Vessels of rheumatoid synovium showed broadened basement membranes marked by laminin antibodies (Fig. 3b). A qualitative alteration was the staining with type IV collagen and laminin antibodies in the sublining area of the rheumatoid synovium, a region known for vascular proliferation in RA (Fig. 3c). In addition, basement membrane proteins were found pericellularly in the hyperplastic lining layer of active inflammation (Fig. 3c). This distribution was unique in active synovitis.

DISCUSSION

Initiated by arthrotropic pathogens in predisposed subjects, RA develops into a chronic damaging joint disease. Expansion of synovial membrane eventually leads to nonphysiological proliferation over the articular cartilage, with damage in contact areas. The present study, directed to the proliferating actived synovial membrane in RA, analyzes changes in extracellular matrix distribution. In normal synovial stroma, extracellular matrix is built by collagen types I, III, V, VI and fibronectin as described by others (18,21,29,30) and also found here. In rheumatoid synovium and in synovial fibrosis, some quantitative changes in this distribution are described, as confirmed by our studies.

In addition to these alterations of interstitial matrix proteins, our immunofluorescence studies corroborate distinct changes in extracellular deposition of basement membrane components in rheumatoid synovium. In humans, synovial membrane is unique as a marginal layer to a cavity normally formed without a limiting basement membrane. This structural feature is confirmed by our immunofluorescence analysis of normal synovial tissues: positive reactions with anti-type IV collagen or anti-laminin were found in vessel walls only. In RA, laminin-specific immunofluorescence marks the extensive vascular proliferation in sublining areas and labels the thickened basement membranes of capillaries as also found by Matsubara (31) and Scott (32). This basement membrane protein deposition is attributed to endothelial cells and pericytes of synovial capillaries (31).

Besides localisation in areas of vascular proliferation, our investigations demonstrate both laminin and collagen type IV pericellularly in the hypertrophic lining layer of active RA. In our immunofluorescence investigations, staining was limited to the lining cell area of active synovitis and most prominently in deeper parts. In contrast to the basement membrane deposition described by Pollock (33), our results indicate that this distribution is characteristic of the proliferating synovial lining layer in active synovitis and differs qualitatively from normal, osteoarthritic and fibrotic synovium of RA. Pollock's (33) result of type IV collagen distribution in a normal synovial lining layer is derived from one sample only showing a diffuse marginal staining with no regard to cell surfaces; extravascular staining in uninflamed osteoarthritic synovium is demonstrated by a few marked cells in an altered lining layer. In line with Pollock's studies we found that basement membrane distribution is most prominent in inflammed synovial tissues distributed especially pericellularly on the lining cells and the deep area of this structure.

In general, basement membrane proteins, laminin and type IV collagen are synthesized by basement membrane associated cells e.g. endothelial and epithelial cells (34). Fibroblasts from human skin may also contribute to dermal-epidermal basement membrane formation in organogenesis as indicated by in vitro type IV and laminin synthesis (35,36). In culture, our studies by immunofluorescence demonstrate that laminin and collagen type IV are also produced by proliferating synoviocytes, with the latter confirmed by Okada for rheumatoid synoviocytes (37). Collagen polypeptide differenti-

ation after pepsin digestion failed to confirm type IV collagen synthesis by synociocytes, which may be due to reduced sensitivity to collagen type IV in PAGE, resulting from the low turnover of collagen type IV (38). High amounts of synthesized interstitial collagens in synoviocytes may further reduce a probable collagen type IV peptide staining.

The here found production of type IV collagen and laminin by synoviocytes of different sources in vitro and the deposition in the inflamed hypertrophic lining layer of RA may be a sign of proliferating and transformed fibroblast-like cells; transformation is discussed as a possible cause of chronic synovitis (2,18,39). Enhanced staining by IL-1 stimulation may indicate that the induction of basement membrane protein synthesis is related to inflammatory reactions in RA. In vitro synthesis of collagen type IV and laminin by cells derived from uninflamed and inflamed tissues may show that the proliferating cells in active rheumatoid synovitis derive from cell types normally distributed in synovium, e.g. endothelial cells or type B synoviocytes.

Basement membrane protein finding in the active rheumatoid synovial lining layer and proliferating synoviocytes may be important for chronic synovitis because of its biological activities in cell-matrix interactions. The function of basement membrane components from the liver (40) and from peripheral nerves (41) is confirmed in organ regeneration. In chronic damage, type IV collagen may support the development of fibrosis (42). These activities would imply basement membrane deposition in the rheumatoid synovial lining layer as a repair mechanism after injury, with synovial fibrosis as a final stage of rheumatoid synovitis.

Laminin and collagen type IV may also be important in cell-matrix interactions for controlling capillary morphogenesis in concert with cytokines (43-45). Deposition of basement membrane proteins in the lining cell layer may support endothelial cell proliferation and angiogenesis, one early important step in pannus formation (1,46-50). In this process, growth factor activities (51-53), regulation of cell adhesion (54) and differentiation of various cell types (51,55-58) by basement membrane proteins may contribute to proliferation and destructive activity of rheumatoid synovium, and stimulation of procollagenase synthesis, found in skin fibroblasts cultured on a basement membrane matrix (59), may provide joint damage.

In addition to effects on mesenchymal cells, laminin is also known to stimulate T cell response and interacts with neutrophils via β1 and β2 integrins (60). Basement membrane protein functions in invasion and metastasis (61-63) are reminiscent of the tumour-like proliferation of synovium described by Fassbender (3).

In RA, persistence of an arthropathic antigen or autoimmunity are favoured as possible sources of chronic joint damage. Fibroblast-like cells are generally discussed as subsequently stimulated and important producers of proteases such as collagenase. Our findings of basement membrane proteins deposited in the rheumatoid lining layer and their synthesis by cultured synoviocytes may give some support to a more active function of mesenchymal cells and extracellular matrices in the development of R.A. e.g. differentiation and activation of mononuclear and mesenchymal cells. Additional studies are needed to corroborate the importance of cell-matrix interaction in the pathogenesis of RA.

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Correspondence to: M. SCHNEIDER, M.D.,
Center for Rheumatology, Medical Clinic and Policlinic B, Westfalian Wilhelms-University, Albert-Schweitzer-Str. 33, D-48129 Münster, Germany.