Loss of laminin and of the laminin receptor integrin subunit α6 in situ correlates with cytokine induced down regulation of α6 on fibroblast-like synoviocytes from rheumatoid arthritis

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Abstract

Objective—To investigate in situ the expression of the integrin receptor subunits α6 and β1 and the distribution of the ligand laminin in the synovia from osteoarthritis (OA) and rheumatoid arthritis (RA) patients and to study the effect of cytokines and antirheumatic drugs on the expression of the α6 and β1 integrin subunits on long term cultures of fibroblast-like synoviocytes (FBS) derived from OA and RA.

Methods—The expression of the α6 and β1 integrin subunits and the distribution of laminin were examined immunohistochemically in normal synovia and in synovia from patients with OA and RA. The effect of proinflammatory cytokines (IL-1β and TNFα), and of antirheumatic drugs (salicylic acid, dexamethasone, and methotrexate) on the α6 and β1 expression of cultured normal FBS and FBS from patients with OA and RA was determined by flow cytometry.

Results—In normal synovia and in OA synovia samples with a low grade of inflammation, synovial lining cells (SLC) showed a parallel expression and distribution of α6 and laminin. In synovia samples of OA with a higher grade of inflammation and in the majority of RA synovia samples laminin was pericellularly distributed in a low number of SLC, whereas α6 was expressed on the surface of a high number of SLC. In RA synovia samples with severe inflammatory changes the gradual loss of laminin generally corresponded to a decrease of the α6 integrin subunit. β1 was always strongly expressed in all synovia samples detected. Proinflammatory cytokines up regulated the expression of α6 and β1 on OA-FBS, whereas these effectors decreased α6 and β1 on RA-FBS. In contrast, antirheumatic drugs, in particular methotrexate and dexamethasone, reduced the expression of α6 and β1 on OA-FBS, whereas the same treatment on RA-FBS stimulated the expression of these integrin subunits.

Conclusion—The gradual loss of laminin in chronic synovitis may contribute to the altered expression of α6 in SLC. IL-1β and TNFα down regulated the expression of the α6 and β1 integrin subunits on long term cultures of FBS derived from RA. Therefore, these cytokines may be among the effectors regulating the expression of the α6 integrin subunit in SLC in vivo. As antirheumatic drugs increase the expression of α6 on RA-FBS, the presence of the laminin receptor may confer a protective effect on the synovia in vivo.

Normal synovia is organised into an intimal layer of synovial lining cells and a subintima composed of fat and connective tissue that contains blood vessels and scattered leucocytes. Although, ultrastructurally no distinct basement membrane separates synovial lining cells (SLC) from the subintima, it has been shown immunohistochemically that different extracellular matrix proteins like laminin, entactin, and collagen type IV present a defined distribution in the synovium. This network of extracellular matrix components might provide an anchoring basement membrane for the synovia. The structural organisation of the normal synovia is stabilised through cell-cell and cell-matrix interactions mediated, in part, via a large family of cell surface adhesion molecules known as integrins. Integrins consist of an α/β heterodimer. Through the extracellular receptor domain, which is built up by both subunits, and the cytoplasmic portion of the β subunit, which interacts with talin and α actin, integrins connect the extracellular milieu with the cytoskeleton. In chronic synovitis inflammatory changes contribute to the remodelling process of the extracellular matrix. These changes in the composition of the extracellular matrix might lead to an alteration of the cell-matrix interaction and to an altered regulation of the integrin expression of the cells.

The aim of this study was to determine immunohistoologically the distribution of the basement membrane component laminin and of the corresponding adhesion receptor α6β1 in chronic synovitis in situ. We observed changes associated with the intensity and the aetiology of inflammation. As cytokines have been recognised as important factors involved in the regulation of integrin expression, we investigated the effect of various cytokines and of antirheumatic drugs on the expression of α6 and β1 on fibroblast-like synoviocytes in vitro by flow cytometry. The differential effect of cytokines on the expression of α6 and β1 was related to the aetiology of the arthritic process.
Methods

SYNOVIAL TISSUE

Synovial tissues were obtained at arthroplasty from different joints (table 1) of 23 patients with rheumatoid arthritis (RA) and 18 patients with osteoarthritis (OA) (according to the criteria of the American College of Rheumatology, formerly, the American Association of Rheumatology). Laboratory tests, erythrocyte sedimentation rate, rheumatoid factor; NSAID, non-steroidal anti-inflammatory drug; ND, not determined.

FBS ISOLATION

In vitro cultures of FBS were obtained by standard methods, as previously described. The tissue was minced into small pieces and digested with collagenase type Ia (Sigma Chemical Company, St Louis, MO, USA) in serum free basal Iscove medium (Seromed-Biochrom, Berlin, Germany). The samples were washed and suspended in basal Iscove medium, supplemented with 10% fetal calf serum (FCS, Bio Pro, Karlsruhe, Germany) and with penicillin-streptomycin-amphotericin B (10 units/ml, 10 mg/ml and 0.25 mg/ml). Finally, isolated cells were cultured in a humidified 5% carbon dioxide atmosphere. After overnight culture, non-adherent cells were removed. Fresh medium was added and the incubation continued. At confluence, cells were trypsinised, split at 1:3 ratio, and recultured. The medium was changed twice each week. FBS from passages 3 to 9 were used in these experiments. Each of the three cultures of OA-FBS and the four cultures of RA-FBS were detached with EDTA (0.02%) and centrifuged at 1000 rpm for five minutes. The cultured cells comprised a homogeneous population of FBS with respect to morphological and immunocytochemical criteria (< 1% CD11b+, < 1% CD11a+, < 1% CD11c+, < 1% CD53+, < 1% CD3+, < 1% Factor VIII+).

STIMULATION OF FBS

FBS cultures from three patients with OA and from four patients with RA were cultured for 72 hours in the presence of cytokines and/or of antirheumatic drugs. One FBS culture (OA-FBS 1) was stimulated simultaneously with cytokines and drugs. Pilot studies were performed in vitro cultures of FBS were obtained by standard methods, as previously described. The tissue was minced into small pieces and digested with collagenase type Ia (Sigma Chemical Company, St Louis, MO, USA) in serum free basal Iscove medium (Seromed-Biochrom, Berlin, Germany). The samples were washed and suspended in basal Iscove medium, supplemented with 10% fetal calf serum (FCS, Bio Pro, Karlsruhe, Germany) and with penicillin-streptomycin-amphotericin B (10 units/ml, 10 mg/ml and 0.25 mg/ml). Finally, isolated cells were cultured in a humidified 5% carbon dioxide atmosphere. After overnight culture, non-adherent cells were removed. Fresh medium was added and the incubation continued. At confluence, cells were trypsinised, split at 1:3 ratio, and recultured. The medium was changed twice each week. FBS from passages 3 to 9 were used in these experiments. Each of the three cultures of OA-FBS and the four cultures of RA-FBS were detached with EDTA (0.02%) and centrifuged at 1000 rpm for five minutes. The cultured cells comprised a homogeneous population of FBS with respect to morphological and immunocytochemical criteria (< 1% CD11b+, < 1% CD11a+, < 1% CD11c+, < 1% CD53+, < 1% CD3+, < 1% Factor VIII+).

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performed to test the concentration of cytokines and drugs showing the most relevant effect on the expression of α6 and β1. On the basis of these data we used the following concentrations: IL1β (Genzyme; Cambridge, MA): 40 U/ml; TNFα (Knoll; Ludwigshafen, Germany): 10 ng/ml; salicylic acid (Sigma): 200 µg/ml; dexamethasone (Sigma): 500 nM; methotrexate (Sigma): 100 µM. In four experiments FBS cultures were cultured for 72 hours in the presence of basol Iscove’s medium, supplemented with 10% supernatant of synovial fluid from a patient with RA. At the end of the incubation, cells were washed with PBS, and detached with EDTA. Cell viability was measured by the trypan blue exclusion test that was > 95%. Before analysis, cells were washed twice in PBS and resuspended in RPMI 1640 (Gibco, Paisley, Scotland, UK).

REAGENTS

The monoclonal primary antibody (mAb) GOH3 (anti-α6; IgG2a) and the anti-laminin mAb 4C12 (IgG1) were purchased from Dianova-Immunotech (Hamburg, Germany). The mAb P4C10 (anti-β1; IgG1) was obtained from Telios (San Diego, USA). A polyclonal biotinylated sheep antibody to mouse Ig (reactive with all mouse and rat isotypes), a polyclonal biotinylated sheep antibody to rat Ig for detection of rat derived mAb GOH3 and 4C12 and a streptavidin-biotinylated peroxidase complex were purchased from Amersham (High Wycombe, UK). 3-amino-9-ethylcarbazole (AEC) and N’N-dimethylanilide (DMF) were obtained from Sigma. An anti-cytomegalovirus antibody (clone CCH2; IgG1) was used as a negative control and was obtained from Dako (Glostrup, Denmark).

IMMUNOSTAINING PROCEDURE

Serial frozen sections of about 1 cm² in area and 4–6 µm in thickness were fixed in acetone for 100% at room temperature, and immunostained immediately or after storage at −20°C for 1–3 weeks. Immunostaining procedures were carried out according to standard methods as previously described.17 Briefly, for immunohistochemistry after rehydration with phosphate buffered saline solution (PBS; pH 7.5), the frozen sections were incubated for one hour with primary mAbs. The primary mAbs were used in a protein concentration of about 5 µg/ml PBS. The sections were then incubated with biotinylated antimouse or antirat antibody (20 µg/ml protein) and streptavidin/biotin-peroxidase complex (10 µg/ml protein) for 30 minutes, respectively. All incubation steps were carried out in a humid chamber at room temperature. Between each incubation step the sections were rinsed twice with PBS and resuspended in RPMI 1640 (Gibco, Paisley, Scotland, UK). The monoclonal primary antibody (mAb) is heterogeneous, a simple semi-quantitative statement was made: 4+, meaning 75–100% cells positive; 3+, meaning 50–75% cells positive; 2+, meaning 25–50% cells positive; 1+, meaning 1–25% cells positive.

DEGREE OF INFLAMMATION

Haematoxylin and eosin stained sections from each synovial specimen were scored for the degree of inflammation by two independent observers, according to Rooney et al.12 Synovial lining cell depth was determined, and the results were recorded as 1 (1–3 cell layers), 2 (4–5 cell layers), 3 (6–8 cell layers), and 4 (≥ 9 cell layers). Proliferating blood vessels were determined by immunostaining using anti-von Willebrand Factor (Dako, Glostrup, Denmark) and recorded as 1+, (0–5 vessels/high power field (HPF)) or 3–19). The size of lymphoid aggregates on stained sections was recorded as 0+ (no aggregates), 1+ (1–20 cells/HPF), and 2+ (>20 cells/HPF). The number of vessels showing perivascular infiltrates was determined for the final score and regarded as 1+ (1–25% of the vessels involved), 2+ (25–50% of the vessels involved), 3+ (50–75% of the vessels involved), and 4+ (75–100% of the vessels involved). The degree of fibrosis and the presence of necrosis were assessed. Sections containing < 10% fibrous tissue in the membrane sublining layers were considered normal and graded 1. Sections containing ≥10–50% fibrous tissue were scored as 2+, whereas sections with ≥50% fibrous tissue were graded as 3+. The absence of necrosis was scored 0. The presence of necrosis was scored 2+. The scores of the individual items were added, and the sum was used as an estimate of the overall intensity of inflammation using histological criteria (range of possible values 3–19).

FLOW CYTOMETRIC ANALYSIS

FBS (1 x 10⁶) were suspended in RPMI 1640, 10% FCS, 0.1% NaH3 (Merck, Darmstadt, Germany), and 2% HEPES buffer (Seromed-Biochrom). The expression of α6 and β1 was assayed by flow cytometry, performed on a FACScan (Becton Dickinson) using the Lysis
II Software. W6/32, a monoclonal antibody that reacts with the HLA-A,B,C/β2m-complex, served as a positive control. The mAb CD21 (clone BU-36), recognising an epitope on mature B lymphocytes, was used as a negative control. The cells were incubated with the primary mAbs GOH3 and P4C10 at a concentration of 20–100 µg/ml for one hour at 4°C. Subsequently, after washing, FBS were counterstained with the polyclonal fluorescein isothiocyanate (FITC) coupled goat-antimouse antibody (Dianova-Immunotech) diluted 1:50, and placed for 45 minutes on ice. For the rat derived mAb GOH3, a goat derived antirat mAb was used. After extensive washing cells were resuspended in 300 ml of FACS medium containing 1 mg/ml propidium iodide (Sigma). Cells that had taken up propidium iodide were regarded as damaged or dying and were excluded from further analysis by gating on propidium iodide negative cells. The number of positive cells was assessed by gating on a precise upper limit of control antibody fluorescence. The interface channel for positivity was set at the point where 1–5% of the control fluorescence was positive. The mean fluorescence of each experiment was calculated by subtracting mean control fluorescence values from mean fluorescence with target antibodies.

STATISTICAL ANALYSIS
Correlations between α6/laminin staining and inflammation score/treatment were calculated using the rank correlation Spearman test.

Results
INFLAMMATION SCORE, CLINICAL AND IMMUNOSTAINING DATA
Table 1 gives the histological scores of inflammation, immunohistochemical results, and patient data. Normal synovial membranes were histologically devoid of inflammatory changes, the synovial lining layer was generally two cells in thickness. All SLC strongly expressed the α6 (fig 1A) and β1 integrin subunits. In contrast with a faint pericellular laminin positivity in the synovial layer (fig 1B), subintimal arterial and venular vessels were strongly laminin positive.
Laminin loss correlates with cytokine induced down regulation of α6 on fibroblast-like synoviocytes from RA

Table 2  Effect of cytokines and a synovial fluid (SF) derived from a patient with RA on the expression of the α6 and β1 integrin subunits

<table>
<thead>
<tr>
<th>Medium</th>
<th>IL1β</th>
<th>TNFα</th>
<th>SF from RA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Positive</td>
<td>MF</td>
<td>% Positive</td>
</tr>
<tr>
<td>OA-FBS 1</td>
<td>α6</td>
<td>12.3</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>39.3</td>
<td>13.1</td>
</tr>
<tr>
<td>OA-FBS 2</td>
<td>α6</td>
<td>17.2</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>81.7</td>
<td>19</td>
</tr>
<tr>
<td>RA-FBS 1</td>
<td>α6</td>
<td>65.7</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>99.1</td>
<td>63</td>
</tr>
<tr>
<td>RA-FBS 2</td>
<td>α6</td>
<td>27.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>46.7</td>
<td>25</td>
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</tbody>
</table>

MF, mean fluorescence. SF from RA, medium with synovial fluid derived from a RA patient.

Table 3  Effect of anti-inflammatory drugs on the expression of the α6 and β1 integrin subunits

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dexamethasone</th>
<th>Methotrexate</th>
<th>Salicylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Positive</td>
<td>MF</td>
<td>% Positive</td>
</tr>
<tr>
<td>OA-FBS 1</td>
<td>α6</td>
<td>12.3</td>
<td>3.1</td>
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<tr>
<td></td>
<td>β1</td>
<td>39.3</td>
<td>13.1</td>
</tr>
<tr>
<td>OA-FBS 3</td>
<td>α6</td>
<td>12.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>86</td>
<td>21</td>
</tr>
<tr>
<td>RA-FBS 3</td>
<td>α6</td>
<td>2.8</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>26.8</td>
<td>33</td>
</tr>
<tr>
<td>RA-FBS 4</td>
<td>α6</td>
<td>17.4</td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>β1</td>
<td>55.3</td>
<td>11.8</td>
</tr>
</tbody>
</table>

With regard to the expression of the β1 integrin subunit, all or the majority of SLC in 12 of 15 OA samples and in 18 of 19 RA samples were strongly positive. By contrast, α6 and laminin staining in SLC of OA and RA samples was rather heterogeneous.

OA samples presented a higher histological score of inflammation (mean (SD), 6.2 (1.9)). The histological score correlated with the thickness of the synovial intima (p = 0.019).

In 7 of 15 samples (samples 3, 5, 6, 8, 9, 14, and 16) the majority of SLC showed α6 and laminin staining. However, as with normal synovia, laminin staining was weaker in comparison with α6. In these OA synovia samples SLC were usually flat and distributed in a low number of cell layers. In 4 of 15 OA samples (samples 7, 10, 11, and 15) a faint laminin positivity was found in a low number of SLC, whereas α6 was strongly expressed and distributed in a high number of SLC (fig 1C, D). In 4 of 15 OA samples (samples 4, 12, 13, and 17) only a subset of SLC (1–25%) was α6 positive and showed laminin staining. Three of these four OA samples presented a higher degree of inflammation (> 7) in comparison with the other OA samples. No correlation was found between the degree of inflammation, α6 and laminin staining, and the joint site of the corresponding synovia samples. Inflammation scores of the detected OA synovia samples did not correlate with the laboratory parameter erythrocyte sedimentation rate (ESR).

RA samples presented a high grade of inflammation (mean (SD), 10.4 (3.2)). The degree of inflammation correlated statistically (p = 0.010) with the synovial lining thickness, but no correlation was found between the degree of inflammation and laboratory tests (ESR, rheumatoid factor (RF)).

In 3 of 19 RA synovia samples α6 and laminin were distributed in a large number of SLC (samples 21, 22, and 36). In 12 of 19 RA synovia samples laminin staining was restricted to a small number of SLC (samples 18, 19, 23, 26, 27, 29, 30, 31, and 32), or was negative (samples 20, 33, and 34) in comparison with the number of the α6 positive. SLC had a plump cell shape with strong α6 positivity confined to the surface cell membrane. In 3 of 19 RA samples (samples 25, 28, and 35) α6 and laminin were distributed only in one subset of SLC, whereas in 1 of 19 RA samples (sample 24) SLC lacked these antigens (fig 1E, F). α6 and laminin staining inversely correlated with the inflammation score (p = 0.004, and 0.006, respectively) and with the synoviocyte hyperplasia (p = 0.034, and 0.008, respectively). α6 and laminin staining decreased in RA samples with a high grade of inflammation and with hypertrophic lining layers. However, the decrease in laminin staining was stronger than in α6 staining. No correlations were found between α6, laminin staining and treatment or joint site of origin of the synovia samples.

Expression of the A6 and B1 Subunits on Cytokine and Drug Treated FBS

To investigate whether cytokines and drugs influence the expression of α6 and β1, long term cultures derived from OA and RA synovia were kept in the presence of cytokines (IL1β and TNFα) and of drugs (dexamethasone, methotrexate, and salicylic acid), and were analysed for the expression of α6 and β1 by FACS analysis (tables 2 and 3). Having treated OA-FBS with IL1β and TNFα we observed an increase in α6 and β1 expression on the two detected FBS cultures of OA. The most prominent effect on the expression of α6 was...
induced by IL1β in OA-FBS 1 and by TNFα in OA-FBS 2. The most relevant up-regulating effect on the expression of α6 was obtained by IL1β in OA-FBS 1, whereas in OA-FBS 2, IL1β and TNFα had a similar effect.

The effect of cytokines was different in RA-FBS. The expression of α6 was markedly reduced by treatment with IL1β and TNFα in RA-FBS 1 and RA-FBS 2. In RA-FBS 1 the maximal reduction on β1 expression was obtained by treatment with TNFα.

To reproduce in our model in vitro the effect of factors that play a part in the inflammatory process in vivo, OA-FBS and RA-FBS were cultured in the presence of the supernatant of a synovial fluid obtained from an arthritic joint of a patient with RA. Treatment of OA-FBS with this synovial fluid up-regulated the α6 and β1 expression in both FBS cultures, whereas the addition of this synovial fluid to RA-FBS decreased the expression of α6 and only minimally decreased β1 in one of the RA-FBS cultures.

Drug treatment of OA-FBS resulted in a down-regulating effect on the expression of α6. This effect was observed in particular using methotrexate and salicylic acid in the two studied OA-FBS cultures and using dexamethasone in OA-FBS 3 (table 3). Treatment of OA-FBS with dexamethasone and methotrexate resulted in a down-regulating effect on the expression of the β1 on OA-FBS 1 and 3. This effect was also observed upon treating OA-FBS 3 with salicylic acid.

In contrast with these findings treatment of RA-FBS with the same drugs up-regulated the expression of the α6 and β1 integrin subunits (table 3). The most prominent up-regulating effect on α6 and β1 of RA-FBS was observed with dexamethasone and salicylic acid and, more intensely, with methotrexate (fig 2).
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α6 molecules. We and others have shown that α6 staining inversely correlates with the synovial lining thickness. It is still open to question whether changes in α6 and in laminin staining are typical for RA.

To substantiate further our in situ findings, in vitro studies on FBS cultures derived from OA and RA synovia samples were carried out. In particular, we studied the effect of cytokines and of drugs, on the expression of the α6 and β1 integrin subunits. Even if there were differences in the baseline expression of α6 and β1 on the FBS cultures studied, the effect of cytokines and drugs on the expression of these integrins was specific for RA-FBS and OA-FBS and regardless of the original level of the integrin subunits. In particular IL1β and TNFα increased the expression of α6 and β1 on OA-FBS, whereas these cytokines decreased α6 and β1 expression on RA-FBS. An analogous effect was seen upon treatment of the FBS with a synovial fluid obtained from an arthritic joint of a patient with RA. IL1β and TNFα are involved in inflammation induced tissue damage and joint destruction. Our findings suggest that these cytokines regulate the α6 expression in vivo. Our data in vitro on the cytokine stimulated RA-FBS can explain the decrease of α6 in SLC of RA synovia with a high grade of inflammation. In contrast with these findings we showed that antirheumatic drugs such as salicylic acid and dexamethasone and in particular methotrexate had an up regulating effect on the expression of α6 and β1 on RA-FBS raising the possibility that an increase in α6 and β1 expression on RA-FBS, suggesting that these cytokines may modulate the α6 expression on SLC in the rheumatoid process in vivo.

We would like to thank U Fabian and E Michalek for technical assistance.