Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins

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Abstract

***Objective*—To compare in vitro expression of β1, β3, and β4 integrins in normal fibroblast-like synoviocytes (FBS) and in FBS from rheumatoid arthritis (RA) synovium and to investigate the adhesion of normal FBS and RA-FBS to the integrin binding extracellular matrix (ECM) proteins: collagen type IV, fibronectin, laminin, and tenascin.**

***Methods*—Expression of integrin receptors of cultured FBS was detected by flow cytometry. Attachment of FBS to ECM proteins was quantified by adhesion assays. Inhibition studies were performed using monoclonal antibodies to the integrin subunits.**

***Results*—Compared with normal FBS, RA-FBS showed increased expression of α1 to α6, β1, and β4 integrin subunits and enhanced binding of ECM proteins. Binding to ECM proteins was partly or completely blocked by an anti-β1 integrin antibody and antibodies to α3, α5, and α6 integrin subunits. The blocking efficiency was significantly ($P < 0.05$) higher in RA-FBS than in normal FBS.**

***Conclusions*—The enhanced expression of the β1 integrin receptors on cultured RA-FBS correlated with increased attachment to ECM proteins. Adhesion of normal and RA-FBS to ECM proteins is mediated through β1 integrin receptors. Therefore, the tight binding of rheumatoid FBS to the matrix via β1 integrins might play a role in ECM remodelling in the rheumatoid process in vivo.**

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Integrins are transmembrane heterodimers consisting of non-covalently associated α and β subunits. At least eight distinct β chains and 15 α chains can complex to give rise to more than 20 different integrin heterodimers.[1 2] Based on different β subunits, integrins are subdivided into subfamilies. Receptors of the β1, β3, and β4 integrins have been shown to bind to a variety of ECM proteins and to some cell adhesion molecules (table 1). Integrins play an important role in different biological events such as inflammation, wound healing, and development.[1]

In situ, expression of various integrin receptors is increased in chronic synovitis.[3–5] Since integrins are adhesion molecules mediating interactions between cells and ECM components, their increased expression may contribute to the recruitment and retention of inflammatory cells into the inflamed joint.[6] Integrin receptors not only connect the cytoskeleton with the external milieu but also confer outside-in signals to cells, for example, α5β1 has been shown to mediate mitogenic signals.[7 8] Since α5β1 has been shown to be increased expressed in endothelial and synovial cells of inflammatory synovial tissues, this integrin receptor may be involved in synovial lining hyperplasia and in the increase of vascularity of chronic synovitis in the rheumatoid process in vivo. In vitro, receptors of β1 integrins were found decisive for cell adherence to ECM proteins and for migration on matrix proteins.[9 10]

In order to test whether the increase in integrin expression in synovial lining cells of rheumatoid synovium reflects a major adhesion capacity of cells to the ECM proteins, in vitro studies were carried out. Long term cultures of FBS from normal synovial membrane and FBS from rheumatoid arthritis synovium were performed. Expression of integrins on normal FBS and RA-FBS was analysed by flow cytometry. The functional relevance was studied in adhesion and adhesion-inhibition assays of normal FBS and RA-FBS to collagen type IV, fibronectin, laminin, and tenascin.

Methods

Synovial tissues were obtained from seven patients with rheumatoid arthritis (according to the criteria of the American College of Rheumatology[11]) undergoing joint replacement surgery or synovectomy. All patients with rheumatoid arthritis except one were known to be rheumatoid factor positive. Erythrocyte sedimentation rate was increased in six out of seven patients with rheumatoid arthritis. All patients had a destructive course, as demonstrated by radiologic findings. Seven normal synovial specimens were obtained at necropsy within six hours of death. The synovial membrane samples were dissected under sterile conditions, kept in phosphate buffered saline (PBS; pH 7.5), and immediately prepared for cultures of FBS.

Table 1 Integrin subunits detected and monoclonal antibodies used in this study

Antigen	*CD number**	*Function/ receptor for*	*Clone*	*Isotype*	*Reference for blocking effect on mAbs on integrin mediated adhesion*	*Source*
β1	CD29	*Common β chain of α1 to α6 and αv*	K20	IgG2a		Dianova, Hamburg, Germany Telios, San Diego, USA
			P4C10	IgG1	[14]	
β3	CD61	*Alternative β chain of αv*	SZ.21	IgG1	[15]	Dianova, Hamburg, Germany
β4	CD104	*Alternative β chain of α6*	3E1	IgG1	not known	Telios, San Diego, USA
α1	CD49a	Collagen IV/laminin (fragment E1)	TS2/7	IgG1	not known	T-Cell Sciences, MA, USA
α2	CD49b	Collagen/laminin/tenascin	Gi9	IgG1	[16]	Dianova, Hamburg, Germany
α3	CD49c	Collagen I and IV/laminin/ fibronectin/epiligrin/entactin	P1B5	IgG1	[17]	Telios, San Diego, USA
α4	CD49d	Fibronectin (IIICS region)/ VCAM-1 (CD106)	HP2/1	IgG1	[18]	Dianova, Hamburg, Germany
α5	CD49e	Fibronectin (RGD site)	SAM-1	IgG2b	[19]	Dianova, Hamburg, Germany
α6	CD49f	Laminin (fragment E8)	GOH3	IgG2a	[20]	Dianova, Hamburg, Germany

*According to the nomenclature committee of the 5th International Workshop and Conference on Leukocyte Differentiation Antigens, Boston, November 1993 (21).

CULTURES OF FBS

In vitro cultures of FBS were obtained by standard methods.[12] Briefly, the tissue was minced into small pieces and digested with collagenase type Ia (Sigma Chemical Co, St Louis, MO, USA) in serum-free basal Iscove medium (Seromed-Biochrom, Berlin, Germany). The samples were then filtered through a nylon mesh, extensively washed, and suspended in basal Iscove medium, supplemented with 10% fetal calf serum (FCS, Bio Pro, Karlsruhe, Germany) and with penicillin-streptomycin-fungizone (10 units ml^{-1}, 10 mg ml^{-1}, and 0.25 mg ml^{-1}). Finally, isolated cells were seeded in 25 cm^2 culture flasks (Falcon, Lincoln Park, NJ, USA) and 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. Pilot studies were performed in order to evaluate the influence of the culture time on the integrin expression of normal and RA-FBS. We found that the integrin expression were only slightly modified in four to six weeks old cultured normal FBS and RA-FBS from the third to fifth passages. On the basis of these data we used four to six week old cultured FBS from the third to fifth passages. In order to further minimise the effects of culture conditions on the integrin pattern and on the adhesive properties of the cells, we used standard conditions: FBS were analysed at confluence (5×10^5 cells per 25 cm^2 culture flask); the culture medium was changed 24 hours before the examination. Cultured FBS comprised a homogeneous population of cells with respect to morphologic and immunocytochemical criteria (< 1%

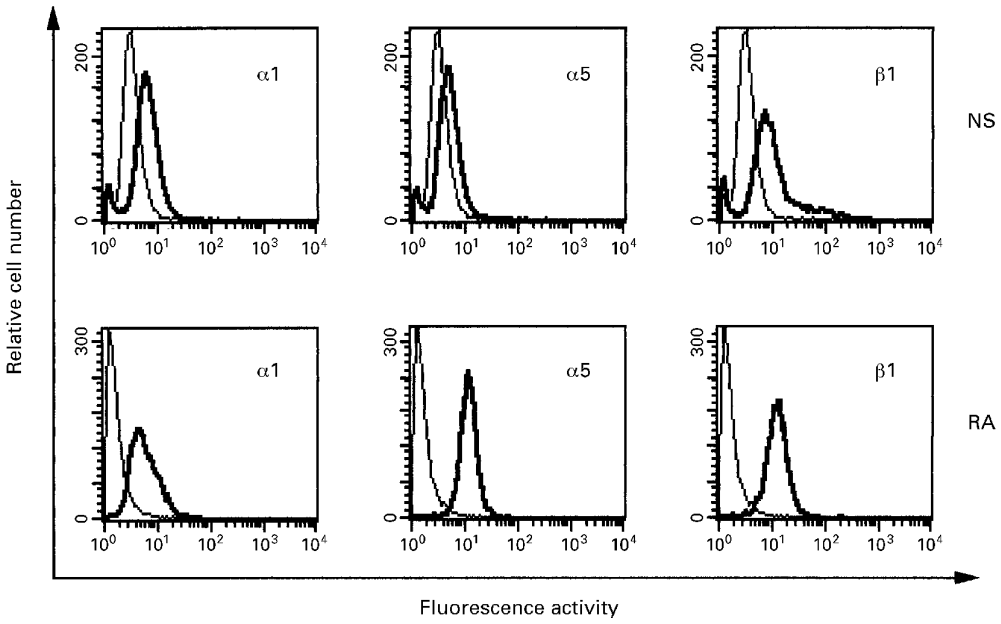

Figure 1 Histograms of flow cytometric analysis for the α1, α5, and β1 integrin subunits on fibroblast-like synoviocytes (FBS) from normal synovium (NS, upper panels) and from rheumatoid arthritis (RA, lower panels). The fluorescence obtained with isotypic negative control antibodies is plotted as a plain line, the results with anti-integrin antibodies as a bold line.

Table 2 Flow cytometric analysis of surface expression on FBS from normal synovium (NS) and from synovium derived from patients with rheumatoid arthritis (RA)

Antigen	NS (n=7)		RA (n=7)	
	% pos (SD)	mF (SD)	% pos (SD)	mF (SD)
α1	9.8 (4.3)	5.8 (4.9)	21.5 (4.6)*	6.7 (1.3)
α2	2.7 (1.6)	2.8 (2.3)	7.3 (5.3)*	5.01 (2.5)
α3	8.2 (6.7)	3.03 (1.5)	23.3 (16.2)*	5.8 (1.4)
α4	1.5 (0.4)	2.6 (1.4)	5.4 (1.3)*	4 (2.4)
α5	8.2 (8.4)	5.7 (4.6)	54.2 (10.4)*	8.7 (4)
α6	7.3 (4.3)	5.6 (2.1)	15.6 (5.3)	6.3 (4.8)
β1	37.6 (6.6)	14.8 (2.4)	71.8 (11.7)*	17.5 (1.3)
β3	12.4 (9)	4.2 (1.2)	10.4 (4.5)	2.8 (0.7)
β4	2.3 (1.2)	1.84 (0.2)	8.3 (2.3)*	4.7 (0.6)

mF, mean fluorescence
*P <0.05 *v* normal FBS.

CD11b+, < 1% CD11a+, < 1% CD11c+, < 1% CD53+, < 1% CD3+, < 1% Factor VIII+). Each of the seven cultures of normal FBS and each of the seven cultures of RA-FBS were detached with EDTA (Seromed-Biochrom) and centrifuged at 1000 rpm for five minutes, and used for FACS analysis and adhesion assays.

REAGENTS

Human placental collagen type IV was obtained from Becton Dickinson Labware (Mountain View, CA, USA). Human plasma fibronectin, human placental laminin, human tenascin (>98% pure by SDS-PAGE, <1% laminin and <1% fibronectin by enzyme linked immunosorbent assay [ELISA]) were purchased from Biomol (Hamburg, Germany). The primary monoclonal antibodies (mAb) against the integrin subunits used in this study are listed in table 1.

FLOW CYTOMETRIC ANALYSIS

We used 5×10^5 FBS per sample for flow cytometry. FBS, cultured in medium as described above, were suspended in FACS medium containing RPMI 1640 (Gibco, Paisley, Scotland, UK) 10% fetal calf serum (FCS, Bio Pro, Karlsruhe, Germany), 0.1% NaN_3 (Merck, Darmstadt, Germany), and 2% HEPES buffer (Seromed-Biochrom). Flow cytometric analysis were performed on a FACScan (Becton Dickinson) using Lysis II software. FBS were incubated with the purified antibodies at a concentration of 20-100 μg ml^{-1}. W6/32, a monoclonal antibody which reacts with the HLA-A,B,C/β_2m complex, served as a positive control. The mAb CD21 (clone BU-36) was used as negative control. The cells were incubated with the primary mAb for one hour at 4°C. Subsequently, FBS were washed extensively and incubated with the polyclonal fluorescein isothiocyanate (FITC) coupled goat anti-mouse antibody (Dianova-Immunotech, Hamburg, Germany) diluted 1:50 and placed 45 minutes on ice. For the rat derived mAb GOH3 (anti-α6), a goat derived anti-rat mAb was used. After washing, cells were resuspended in 300 ml of FACS medium containing 1 mg ml^{-1} 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. Mean fluorescence values were reported as arbitrary units from a 256 channel scale.

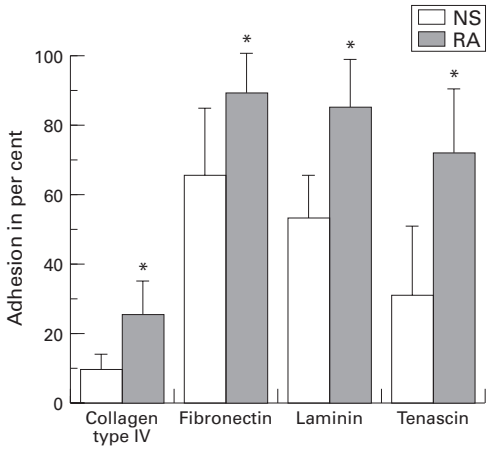

*Figure 2 Adhesion of fibroblast-like synoviocytes (FBS) from normal synovium (NS) and from synovium derived from patients with rheumatoid arthritis (RA). The assays were performed as described in Methods. The diagram shows percent adhesion of the total number of cells plated. Data are reported as the mean of assays of seven normal FBS cultures and seven rheumatoid arthritis FBS cultures. Bars = SD. *P < 0.05 v normal FBS.*

ADHESION ASSAYS

Flat bottomed 96-well plates (Titertek, Amstelstad, The Netherlands) were coated with collagen type IV (5 μg ml^{-1}), fibronectin (10 μg ml^{-1}), laminin (10 μg ml^{-1}), and tenascin (10 μg ml^{-1}) and incubated at 4°C overnight. Control wells were treated only with PBS. Non-specific binding was blocked by further incubation for two hours at 37°C with 100 μl of a 1% BSA/RPMI 1640 solution (pH 7.4). FBS were harvested with EDTA, pelleted, and resuspended in 1% BSA/RPMI 1640 at a concentration of 5×10^5 cells ml^{-1}. For blocking assays three cell cultures of normal FBS and three cell cultures of RA-FBS were used. For inhibition of cell attachment, the blocking efficiency of the different mAb was tested at various dilutions. Maximum blocking was achieved by preincubation of cells for one hour at 4°C using the following antibodies: P1B5 was used at a 1:300 dilution, P4C10 at a 1:200 dilution, Gi9, HP2/1, SAM-1, GOH3, and SZ.21 at a 1:20 dilution, which corresponded to an mAb concentration of 10-20 μg ml^{-1}. The maximum inhibiting effect of the antibodies was seen using the following concentration of ECM proteins: collagen type IV at 5 μg ml^{-1}, fibronectin at 1 μg ml^{-1}, laminin at 0.1 μg ml^{-1}, and tenascin at 10 μg ml^{-1}. Cells were added to microtitre wells (100 μl per well) and incubated for 20 minutes at 37°C. After five minutes of upside-down incubation, plates were gently flicked off and washed once with PBS. Adherence was quantified by measurement of hexosaminidase activity according to Landegren.[13] Briefly, 60 μl of a *p*-nitrophenol-*N*-acetyl-β-*D*-glucosaminide solution (Sigma) was added

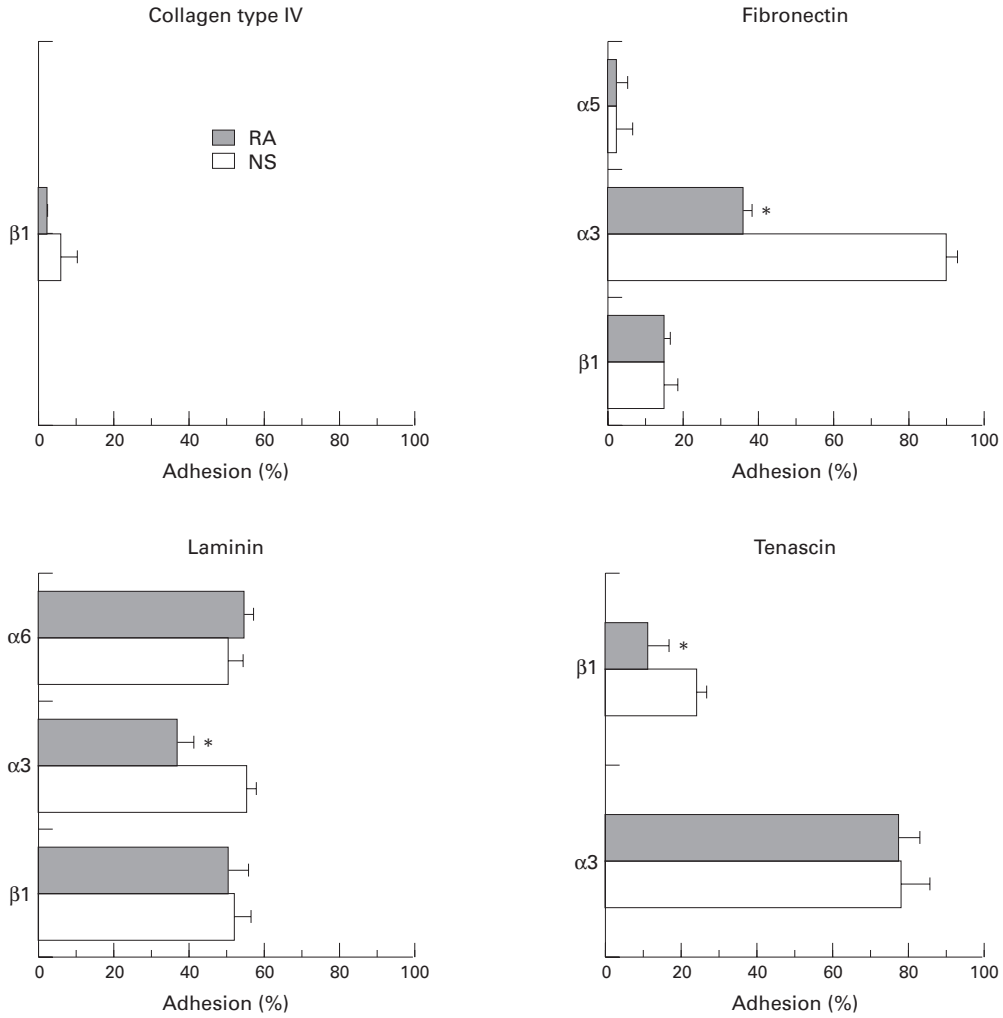

*Figure 3 Inhibition of adhesion of fibroblast-like synoviocytes (FBS) from normal synovium (NS) and from rheumatoid arthritis (RA) to the ECM proteins. Monoclonal antibodies (mAb) against α2, α3, α4, α5, α6, β1, and β3 were used; however, only mAb which blocked FBS binding are shown in the figure. Inhibition through the mAb is given as relative percent adhesion compared to adhesion without antibodies (100%). Data represent the means of assays of three normal FBS cultures and of three rheumatoid arthritis FBS cultures. Bars = SD. *P < 0.05 v normal FBS.*

to each well and incubated at 37°C. After three hours the reaction was stopped with 100 µl of glycine buffer (pH 10.4); 100% references were obtained by spinning equal volumes of cell suspension in Eppendorf tubes, performing the hexosaminidase reaction and transferring the developed colour solution to the plate. Absorbance was measured using a Titertek multiscan at 405 nm. Background adhesion was always less than 2% of the cells plated. For blocking studies, 100% reference values were obtained using the adherence to the ECM proteins of cell suspensions without mAb pretreatment. All of the experiments were performed in three independent series and the mean calculated on the basis of these values.

STATISTICAL ANALYSIS

Flow cytometric analysis and adhesion assays were carried out on cultured FBS derived from seven different control specimens and from seven rheumatoid arthritis patients. Data concerning integrin expression and ECM protein binding are reported as mean (SD) of the different experiments for both normal FBS and RA-FBS. The results for RA-FBS and normal FBS are compared by the Mann-Whitney test with $P < 0.05$ taken as minimum level of significance.

Results

Flow cytometry was performed to compare the expression of β1, β3, and β4 integrins of normal FBS and RA-FBS. Table 2 summarises the results showing the mean values of mean fluorescence and percentage of positive cells for the integrin expression of the different cultures of normal FBS (n = 7) and RA-FBS (n = 7). Significant differences in number of positive cells between normal and RA-FBS are marked by an asterisk. A strong difference in percentage of positive cells between RA-FBS and normal FBS concerned α5β1. A significantly ($P = 0.014$) higher percentage of RA-FBS was positive for α5 (54.2%) and β1 (71.8%) in comparison with normal FBS (8.2 and 37.6, respectively). Regarding the

expression of the other integrin subunits, we found that a major fraction of RA-FBS was positive for α1 (21.5%), α3 (23.3%), and α6 (15.6%) in comparison with normal FBS (9.8%, 8.2%, and 37.7%, respectively). For α1, α3, and α6 expression, only the increase in α1 on RA-FBS was significant (P = 0.014). A minor fraction of cells was positive for α2, α4, and β4 in normal FBS (2.7%, 1.5%, and 2.3% respectively). In comparison, a higher percentage of RA-FBS (P = 0.009) were positive (7.3%, 5.4%, and 8.3%, respectively). RA-FBS showed no significant differences in the staining of the β3 integrin (10.4%) in comparison with normal FBS (12.4%). Flow cytometric analysis showing a significant increase in expression of α1, α5, and β1 integrin subunits on RA-FBS when compared to normal FBS are given in fig 1.

To examine the interaction between ECM proteins and FBS, adhesion assays were carried out on collagen type IV, fibronectin, laminin, and tenascin coated microtitre wells. Data from the assays of the seven normal FBS cultures and of the seven rheumatoid FBS cultures are summarised in fig 2. RA-FBS bound significantly more strongly to collagen type IV (25.3%, P = 0.037), fibronectin (88.5%, P = 0.037), and laminin (85.6%, P = 0.037), and twice as strongly to tenascin (72%, P = 0.014), than normal FBS (64.8%, 9.3%, 51.2%, and 32.4%, respectively).

To investigate the role of integrins in FBS binding to extracellular matrix proteins, inhibition assays were performed (fig 3). For blocking studies we used mAb against α2, α3, α4, α5, α6, β1, and β3 integrin subunits. Of all tested antibodies, only the anti-β1 antibody (clone P4C10) inhibited, by approximately 95%, the adhesion of normal FBS and RA-FBS to collagen type IV at the concentration used for the assay. Adhesion to fibronectin was almost completely blocked by anti-β1 (85%) and anti-α5 (clone SAM-1) (98%) antibodies. A good blocking effect (about 65%) on the adhesion of RA-FBS to fibronectin was observed using anti-α3 (clone P1B5) antibody, whereas this antibody had minimal effect on the fibronectin-binding of normal FBS (P = 0.009). Adhesion to laminin was partly (approximately 50%) blocked by anti-α6 (clone GOH3) and anti-β1 antibodies in normal FBS and RA-FBS. Anti-α3 blocked the adhesion of RA-FBS to laminin by approximately 65%, whereas in normal FBS this was around 55%. This difference was statistically significant (P = 0.037). Adherence of FBS to tenascin was almost completely blocked by the anti-β1 antibody. However, this blocking effect was greater in RA-FBS (90%, P = 0.037)) than in normal FBS (75%). The anti-α3 antibody P1B5 blocked adhesion of normal FBS and RA-FBS to tenascin by about 20%.

Discussion

In vitro, functional characteristics of RA-FBS are different from those of normal FBS. RA-FBS in vitro differ in growth characteristics and in metabolic and ultrastructural properties, which persist for several passages in a long term culture.[22–26] This might be related to autocrine stimulation of the FBS in culture,[27] as supported by the finding that only RA-FBS are able to synthesise interleukin-1β spontaneously in long term cultures.[28] Our study now shows that long term cultured RA-FBS have considerably higher expression of β1 integrins and different subclasses of the α integrin receptor chains than normal FBS (table 2). Since the quantitative expression has been found to be not the only predictor for the integrin adhesiveness,[29] the functional behaviour was studied in adhesion and adhesion inhibition assays. We found that the integrin mediated adhesive interaction of RA-FBS with the corresponding ECM ligands, collagen type IV, fibronectin, laminin, and tenascin, was also significantly increased (fig 3). Considerable changes have been shown in the in situ expression of β1 integrins in synovial lining cells in the integrin α chain repertoire in chronic synovitis.[3–5] Our data suggest that differences in integrin expression of RA-FBS observed in situ persist under in vitro conditions.

Integrin receptors recognising collagen type IV as one of their ligands are α1β1, α2β1, and α3β1.[1] The expression of the α1, α2, α3, and β1 subunits was increased on RA-FBS in comparison with normal FBS. Analogous to the in vitro situation in chronic synovitis, in situ an increased expression of α1, α3, and β1 in the synovial lining cells has been described. The enhanced integrin expression correlates with an increased binding to collagen type IV. The attachment of FBS to collagen type IV is almost completely inhibited by the anti-β1 antibody P4C10.

We found that the enhanced expression of the fibronectin receptors α3β1, α4β1, and α5β1 on RA-FBS correlated significantly with an increased adherence of RA-FBS to fibronectin in comparison with normal FBS. In our in vitro system the capacity to adhere to fibronectin was partly blocked by anti-α5, anti-α3, and anti-β1 antibodies, whereas anti-α4 antibody had no blocking effects on fibronectin binding (fig 3). This suggests that α5β1 and α3β1 are involved in the binding of FBS to fibronectin. The blocking with anti-α3 antibody was greater in the case of RA-FBS than with normal FBS, which reflects the functionally active state of these integrins on RA-FBS. The available data on integrin expression of synovia in situ show an abundant expression of the integrin subunits α3, α5, and β1 in rheumatoid synovial lining cells,[4 5] whereas normal synovial lining are devoid of α3 and α5, and weakly express the β1 integrin subunit.[3] Thus the in vitro expression of the fibronectin receptors on rheumatoid FBS substantially reflects the in situ situation. This correspondence suggests an enhanced adhesiveness of synoviocytes to fibronectin in the rheumatoid process in vivo. Since the attachment to fibronectin confers mitotic signals,[7 8] this may play a role in the rheumatoid process in vivo.

The attachment to laminin can be mediated through both the multifunctional receptors α1β1, α2β1, and α3β1 and the monfunctional receptors α6β1 and α6β4.[1] We have found that

the subunits α2, α3, α6, and more clearly α1 and β1, are enhanced on RA-FBS. In accordance with these findings, we have shown that RA-FBS attach at a higher rate than normal FBS to laminin. In our in vitro system the adherence to laminin was only partly blocked by addition of anti α3 and β1 antibodies. However, the blocking effect on laminin attachment by anti α3 antibody was found to be markedly increased in RA-FBS in comparison with that found in normal FBS. This might be to due to the increased α3 expression found in RA-FBS and suggests that α3β1 plays a role in our cellular system as a laminin receptor.

At least three receptors of the integrin family, α2β1 and αvβ3 and recently α9β1, have been shown to mediate cell attachment to tenascin.[30 31] We show that there is a twofold increase in expression of α2 and β1 on RA-FBS compared to normal FBS (table 2). In accordance with these data, attachment of RA-FBS to tenascin is twice as strong as that of normal FBS (fig 2). Tenascin binding of FBS was clearly inhibited by an anti-β1 antibody (fig 3), whereas anti-α2 and anti-β3 antibodies showed no blocking effect on tenascin. Adhesion of RA-FBS to tenascin was partly inhibited, surprisingly, through anti-α3 antibodies. Since α3 is not a part of a known integrin receptor for tenascin, further studies are required to elucidate the role of α3β1 in recognising tenascin as one of its ligands on FBS. Since α9β1 was found to be a tenascin receptor,[31] β1 might heterodimerise with α9 to serve as tenascin receptor, explaining the only moderate blocking efficiency of antibody to α3. Concerning the expression of the α9 integrin subunit, no data on its expression in the synovium in situ are available, whereas the expression of β1 is increased in the rheumatoid synovium.[4 5] Furthermore, tenascin is abundant in synovial membranes with chronic synovitis compared with normal synovial membranes.[32 33] This observation might explain the increased expression of the corresponding integrin receptors in rheumatoid synovial lining cells in situ and in rheumatoid FBS in vitro. In vitro, in response to integrin derived signals the attachment to tenascin has been shown to regulate the collagenase gene expression.[34] Thus an increased adhesion capacity to tenascin of synoviocytes may contribute to the remodelling process in the rheumatoid synovium in vivo.

In all, our results indicate that in cultured RA-FBS, enhanced expression of integrin receptors for collagen type IV, fibronectin, laminin, and tenascin correlates with an increased attachment to these substrates. Furthermore normal and rheumatoid FBS utilise β1 integrins as receptors for these ECM proteins, as evidenced by blocking studies. β1 Integrins have been shown not only to mediate the attachment to the ECM proteins, but also to transmit signals into cells leading to mitosis and upregulation of the gene expression for lytic enzymes in fibroblasts[34] and FBS.[35] Thus the increased adhesive interaction of RA-FBS to ECM proteins through β1 integrins may be a potential trigger for the cellular activity of FBS.

In conclusion, it is tempting to speculate that the tight interaction of FBS with the ECM proteins through the β1 integrin receptors might play a role in extracellular matrix remodelling and synovial damage in the rheumatoid process in vivo. Since differences in integrin profile of rheumatoid synoviocytes persist under in vitro conditions, this in vitro model can be used in the future to explore the factors involved in the regulation of integrin expression and function.

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