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Galectin-3 is induced in rheumatoid arthritis synovial fibroblasts after adhesion to cartilage oligomeric matrix protein

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Abbreviations: BSA, bovine serum albumin; CD29, integrin β1; CD51, integrin αV; CD61, integrin β3; COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; FACS, flow cytometry; FCS, fetal calf serum; FLI, fluorescence index; IL-1β, interleukin-1β; MFI, mean fluorescence intensity; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fibroblasts; TNF-α, tumor necrosis factor-α.
Abstract

Objectives. Galectin-3 is expressed in the synovial tissue of patients with rheumatoid arthritis (RA), particularly at sites of joint destruction. We explored the possibilities that galectin-3 is induced either by pro-inflammatory cytokines or by the adhesion to cartilage components.

Methods. Cell culture plates were coated with fibronectin, collagen I thru VI or cartilage oligomeric matrix protein (COMP) and the suspended cells were then added. The medium was changed after 1 hour at 37°C. Adherent cells were further incubated for 18 hours in the presence or absence of TNF-α or IL-1β. Cells were pretreated with murine IgG1, anti-CD29, -CD51, -CD61 (integrins) or -CD3 monoclonal antibodies and transferred to culture plates coated with COMP. Adherent cells were counted by light microscopy. The expression of intracellular galectin-3, or cell surface CD29, CD51 and CD61 were determined by flow cytometry before and after adhesion.

Results. Four times more RA-SF than OA-SF adhered to COMP. RA-SF presented more cell surface integrins and monoclonal antibodies against CD51 inhibited the adhesion to COMP by 80%. TNF-α reduced the expression of CD61 and the adhesion to COMP, but did not reverse the adhesion once it had taken place. The adhesion of RA-SF to COMP was found to increase the intracellular level of galectin-3. In contrast, intracellular galectin-3 decreased after exposure to TNF-α.

Conclusion. The increase of galectin-3 occurs after adhesion to COMP and the αVβ3 receptor (CD51/CD61) plays a pivotal role in this process.

Key words. Cartilage oligomeric matrix protein, galectin-3, CD51, synovial fibroblasts, rheumatoid arthritis
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by inflammation and progressive destruction of the affected joints (1). Galectins participate in cellular homeostasis. Both intracellular and extracellular activities of galectins have been described, with the former typically independent of lectin activity (2). Increased expression of galectin-3 has been associated with the development of tumors and inflammation (3). Intracellularly, galectin-3 is demonstrated as an inhibitor of apoptosis (2). Expression of galectin-3 has been reported in the synovial tissue of patients with RA, including at sites of joint destruction (4). Very little information is available regarding specific factors triggering the expression of galectin-3, with the exception that the promoter region contains a serum-responsive element (5). Pro-inflammatory cytokines did not stimulate the expression of galectin-3 mRNA in vitro (4). Therefore, our aim was to investigate whether the increase of galectin-3 in synovial fibroblasts (SF) occurs after these cells adhere to specific components of the extracellular matrix (ECM).

Integrins mediate the attachment of RA-SF to fibronectin-rich sites of cartilage (6). We analysed, in addition to fibronectin, the adhesion of SF to collagens and cartilage oligomeric matrix protein (COMP). COMP is a component of the hyaline cartilage and is produced by chondrocytes and SF (7). COMP fragments are markers of joint destruction (8, 9), synovial activation (7), and involved in autoimmune phenomena (10, 11).

Integrins are a family of proteins that can mediate both intercellular adhesion and cell adherence to ECM. They are composed of a larger alpha subunit and a smaller beta subunit. The increased expression of integrins on the surface of RA-SF has been associated with their enhanced binding to ECM (12). Previous functional data support the concept that integrin-mediated signalling events contribute to the invasive nature of RA-SF (13). Here we show that the up-regulation of galectin-3 in RA-SF occurs after cell adhesion to COMP via an integrin-dependent mechanism.
Material and methods

Patients
Synovial tissue specimens were obtained during joint arthroplasty from 9 RA patients and 4 osteoarthritis (OA) patients. All RA and OA patients fulfilled the respective criteria of the American College of Rheumatology (14, 15). In contrast to RA tissues, OA and normal samples had no lining cell hyperplasia and no subintimal cellular infiltration. The study was approved by the ethical committee of our institution (University Hospital Zürich).

Cell culture
Tissue specimens were washed with phosphate buffered saline, minced and digested enzymatically with 1.5 μg/ml Dispase II (Roche). The released cells were grown for 4-8 passages in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 0.5 μg/ml amphotericin B and 10 mM HEPES (Life Technologies). One week prior to the start of the experiments the cells were cultured in either DMEM containing 10% FCS or were serum starved in medium containing 0.5% FCS. Flow cytometric analysis (below) confirmed that after 4 passages more than 98% of the cells were fibroblasts.

Cytokines
RA-SF and OA-SF cultured in DMEM containing 10% or 0.5% FCS were incubated for 18 hours at 37°C with 10 ng/ml endotoxin-free recombinant human tumor necrosis factor-alpha (TNF-α) or 1 ng/ml interleukin-1beta (IL-1β) (Invitrogen). These concentrations were chosen, because they correspond to “physiological” levels, as it can be measured in RA synovial fluid. In the case of TNF-α, a higher dose (to e.g., 100 ng/ml) would increase the risk that the cells undergo apoptosis.

Adhesion assays
Serum starved SF cultures were used. Cell culture plates were coated with 100 ng/ml of purified human fibronectin (BD Biosciences), collagen type I thru VI (Center for Experimental Rheumatology, Zürich), or COMP (Center for Biochemistry, Cologne). Unreduced proteins were used for coating. Fibronectin and COMP were diluted in phosphate buffered saline (PBS), while collagens were diluted in 0.5 M acetic acid. After incubating the plate at 4°C for 24 hours, they were washed 3 times with PBS. The plates were blocked with 10 μg/ml heat-denatured BSA (Sigma, treated for 15 minutes at 85°C) (16). After an incubation for 1 hour at 37°C, the plates were washed with FCS-free DMEM.
RA-SF and OA-SF were detached using collagenases (Accutase, Omnilab), washed, suspended in FCS-free DMEM, and incubated for 30 minutes at 37°C in a tube rotator. The cells were then added to the coated culture plates (2 x 10^5 cells/well) in FCS-free DMEM containing 0 to 100 ng/ml TNF-α, and incubated at 37°C. The medium was changed (using DMEM containing 0.5% FCS) after 1 hour and the remaining adherent cells were incubated for an additional 18 hours at 37°C. A light microscope was used, by two independent observers, to count the cells at two time points: 30 minutes after changing the medium (total incubation time: 1.5 hour) and at end of the incubation period. After the 18-hour incubation period the intracellular level of galectin-3 protein...
was determined by FACS and the level of galectin-3 in the cell supernatant was measured by ELISA.

**Blocking experiments**

Serum starved SF cultures were used to explore the mechanisms underlying the adhesion of RA-SF to COMP. RA-SF and OA-SF were pre-treated with 1 microg/ml murine IgG1 (as negative control) or monoclonal antibodies against human CD29, CD51, CD61 or CD3 (BD Transduction Lab.) Culture plates were coated with 100 ng/ml COMP and blocked with heat denatured BSA. Cells were treated with collagenases (Accutase), washed, and suspended in FCS-free medium. The cells were then pre-treated with monoclonal antibodies, as described above, for 10 minutes at 22-24°C in a tube rotator. The pre-treated cells were transferred to the coated culture plates and incubated for 1 hour at 37°C. The culture medium was changed and the adherent cells were incubated for an additional 30 minutes (total: 1.5 hour), then counted by two independent observers using a light microscope.

**TNF-α and integrin expression**

A final experiment was carried out to determine if the expression of CD51, CD29 and CD61 changed in the presence TNF-α. Serum starved RA-SF and OA-SF cultures were treated with collagenases (Accutase), washed, and suspended in FCS-free medium. The expression of CD29, CD51 and CD61 on both cell types was measured by FACS. The cells were then incubated for 30 minutes at 37°C in medium containing 0 or 100 ng/ml TNF-α.

**Flow cytometry**

Prior to staining, single cell suspensions were obtained by treating cultures with collagenases (Accutase). The cells were resuspended in DMEM with 0.5% FCS, then incubated for 20 minutes at 37°C in a tube rotator. Control analysis was performed using FITC-conjugated anti-fibroblast monoclonal antibodies Thy-1 (clone AS02, Dianova) and anti-CD45 HLe-1 (Becton Dickinson) to confirm that, after 4 passages, more than 98% of the cells were fibroblasts, and to exclude the presence of contamination by a small population of monocytes that would influence the results. Gating was set on single cells, without debris or large cell aggregates. The expression of CD51, CD61 and CD29 on the cell surface was detected indirectly by purified monoclonal antibodies (IgG1, BD Transduction Lab.) and a PE-conjugated rat anti-murine IgG1 (BD Biosciences). PE-conjugated murine IgG1 alone and PE-conjugated CD45 HLe-1 monoclonal antibodies were used as negative controls. The fluorescence positive marker was set at <2% using the negative control antibodies. The intracellular level of galectin-3 protein in collagenase-treated and permeabilized cells (with BD permeabilizing solution) was determined by FACS using PE-conjugated murine monoclonal anti-galectin-3 antibodies (BD Biosciences). PE-conjugated murine IgG1 served as the negative control.

**ELISA**

The levels of galectin-3 were measured by ELISA in cell culture supernatant using a previously described protocol (4). Briefly, plates were coated with affinity purified goat anti-galectin-3 antibodies. Samples were added to the wells for 2 h at 37°C, followed by
the addition of affinity purified rabbit anti-galectin-3 antibodies and a further incubation for 1 h. After washing, goat anti-rabbit IgG-HRP was added and incubated for 1 h. Tetramethylbenzidine containing 0.001% hydrogen peroxide was used as substrate and optical density was read at 450 nm.

**Statistics**
The following equations were used for analysis: Fluorescence index (FLI) = %positive cells x mean fluorescence of positive cells; FLI% = FLI of the given culture x 100 / FLI obtained by cells cultivated in 0.5% FCS; and dFLI% = FLI% after – FLI% before adhesion. For statistical interpretation, Mann-Whitney U-test and Spearman’s rank correlation test were used.
Results

Adhesion to COMP
Figure 1 shows the significant results of the first adhesion assay. In the absence of TNF-α in the culture medium, 4 times more RA-SF than OA-SF adhered to COMP (p < 0.001, n = 8 RA-SF and 3 OA-SF). For RA-SF, this corresponds to 39 ± 18% of the maximum adhesion obtained in culture plates coated with fibronectin, blocked with heat-denatured BSA, and incubated in TNF-α-free medium. The number of cells per well counted after a total incubation time of 1.5 hours was equal to the number at the conclusion of the 18 hour incubation period. The number of adherent cells / well not coated with COMP (negative control) were < 20 and subtracted from the result. The intra-individual coefficient of variation in the adhesion assay was 7%.

Figure 2 demonstrates that when simultaneously adding RA-SF with varying doses of TNF-α to culture plates coated with COMP, there is a dose-dependent reduction in the number of cells adhering to COMP, reaching 78% inhibition (p < 0.05, n = 3). When the culture medium is substituted 1 hour after cell adhesion to COMP with fresh medium containing 10 or 100 ng/ml TNF-α, the number of adherent cells counted 18h later remained the same.

Inhibition of the adhesion to COMP by blocking CD51
Figure 3 shows that monoclonal antibodies against CD51 significantly blocked the adhesion of RA-SF to COMP by at least 80% (p < 0.001, n = 6 RA-SF). In a second set of experiments, pre-treatment of RA-SF with anti-CD29 or anti-CD61 monoclonal antibodies was less efficient than anti-CD51. Pre-treatment with murine IgG1 or anti-CD3 monoclonal antibodies had no effect on RA-SF adhesion to COMP (data not shown).

Modulation of galectin-3 by ECM components
Figures 4 and 5 present the changes in the intracellular expression of galectin-3 protein in RA- and OA-SF (n = 5 and 3), under normal culture conditions, after one week of serum starvation and 18 hours after cell adhesion to COMP, all in the presence or absence of 10 ng/ml TNF-α. The FACS results are presented as the mean ± SD change in FLI%.

There was a distinct contrast in the change of intracellular galectin-3 expression between RA- and OA-SF upon cell adhesion to collagen type VI (data not shown) or more obviously COMP (Figure 4 and 5). The adhesion of RA-SF to COMP induced a significant increase in the expression of intracellular galectin-3 (255 ± 67%, p < 0.005). This experiment was repeated with SF samples from 2 additional patients (1 OA and 1 RA) in order to confirm this finding. Representative examples of the FACS analysis (intracellular galectin-3) before and after RA- and OA-SF adhesion to COMP are shown in figure 5. No significant change of galectin-3 in the cell culture supernatant was detected after attachment to COMP (data not shown). In contrast to the response to collagen type VI and COMP, the expression of galectin-3 decreased after cell adhesion to fibronectin and collagen type III (data not shown).

Figure 6 show that, in both OA-SF and RA-SF, serum starvation and TNF-α decreased the intracellular level of galectin-3 by 20% to 50% (p < 0.005). However, serum starvation does not affect the level of galectin-3 in the presence of TNF-α.
There was no change in galectin-3 expression when either RA- or OA-SF were transferred to fresh culture medium simultaneously with IL-1β, or after cell adhesion to collagens type I, II, IV and V.

**Decrease of CD61 expression by TNF-α**
The expression of CD51, CD29 and CD61 on RA- and OA-SF was measured before and after a 30-minute incubation with 10 ng/ml TNF-α (data not shown).
Regarding OA-SF, CD29 and CD51 were undetectable before stimulation in 2 of the 3 fibroblast cultures. In the positive OA cell culture (1 of 3), the 30 minute incubation with TNF-α slightly increased the expression of CD51 by 20-24%. In all 3 OA cultures TNF-α increased CD29 expression by 1.5 to 3-fold (as shown by the mean fluorescence intensity or the FLI).
RA-SF expressed more cell surface integrins than OA-SF. In RA-SF, both CD51 and CD29 increased (2 to 3 fold) further upon stimulation with TNF-α. The most important finding, particularly regarding an explanation for the results of the adhesion assays above, is the inhibition of the expression of CD61 during the short incubation with TNF-α. Both OA-SF and RA-SF showed a strong down-regulation (by 37-98% and 29-61% respectively) of CD61.
Discussion

In RA, about 39% of SF adhered to COMP, 4-times more than in OA, indicating that a specific receptor-ligand interaction may occur in RA. COMP is an important component of human hyaline cartilage (7). The adhesion of RA-SF to COMP is reduced by a pre-treatment with TNF-α. It can be argued that TNF-α induced cell death and therefore reduced the number of adherent cells. However, the small concentration used in those experiments was low and should not induce apoptosis in such a short time. The number of adherent cells after 1 and 18 hours incubation periods did not differ significantly, demonstrating that TNF-α-induced cell death is only a marginal phenomenon in our assay.

The possibility that integrins are involved in the adhesion of RA-SF to COMP was explored. In accordance to reports by others (12), our results indicated that an interaction between COMP and CD51 is essential for the adhesion process of RA-SF. This suggests a role for this system in cell attachment to the hyaline cartilage. CD51 also mediate e.g. human chondrocyte adhesion to cartilage (17). The constitutive expression of CD51 is about 2-times higher in RA. Of course, RA-SF also expressed other α integrin subunits, e.g. α6 (18), and it can be speculated that integrin subunits are differently regulated under inflammatory conditions.

An explanation was found for the TNF-α-induced decrease of cell adhesion to various ECM components. FACS was used to measure the expression of integrin subunits on SF cell membranes, before and after a 30 minute incubation with TNF-α, i.e. corresponding to the treatment prior to the attachment assay. In both OA- and RA-SF, the short incubation with TNF-α modestly increased the expression of CD29 on the cell surface. Similarly, CD51 was increased in RA-SF. In contrast, CD61 is down-regulated in both RA- and OA-SF, suggesting that it is the rate limiting factor regarding the adhesion to COMP after pre-treatment with TNF-α. CD61 might not be the only integrin that is down-regulated by TNF-α, since this also is observed for α6 and β4 (19).

The β3 integrin subunit (CD61) forms a complex with the αV subunit (CD51) to form the vitronectin receptor. Our experimental data suggests that the αV and β3 integrins, independent of one another, or as a complex, are major switches regulating the attachment capability of these cells. The αVβ3 integrin complex has been reported by others to bind to thrombospondin-1 (TSP-1) (20), a molecule with high amino acid homology to COMP (also known as thrombospondin-5). In fibroblasts, an autocrine loop of the integrin αVβ3 / CD47 receptor complex and TSP-1 is identified as the molecular coupling device between mechanical loading and apoptosis. Recently, the CD47 / TSP-1 interaction has been proposed to be a key component of a SF / T cell regulatory circuit that perpetuates the inflammatory process in the RA joint (21). TSP-1 can increase the production of growth factors (22) and this can be partially blocked by anti-αVβ3 antibodies indicating that it may be mediated in part by TSP-1 binding to those integrins. COMP was found to stimulate the intracellular accumulation of galectin-3 in RA-SF. Increased levels of galectin-3 are detected in the RA synovial fluid (4); however, in vitro, galectin-3 was not released from RA-SF after attachment to COMP. Thus, it would attractive to speculate on a lectin-independent intracellular role (2) upon attachment, but this has to be verified experimentally. In RA, galectin-3 was found throughout the synovial tissue and in certain patients prominently at sites of joint destruction (4). This clearly suggest an in vivo induction by multiple factors, including attachment.
TNF-α reduced the expression of galectin-3 within 18 hours in both “floating” OA- and RA-SF. The effect of TNF-α is dose- and time-dependant. In RA, it can show high inter-individual variations; a modest and transient accumulation (instead of a decrease) of intracellular galectin-3 can occur, while galectin-3 gene translation and protein secretion remain unchanged (4). Not only the stage of the disease, but also the localisation of the biopsy and the “imprinting” of former therapies can bias the results. Taken together, our data suggested that the obvious induction of galectin-3 in situ is not the result of pro-inflammatory cytokines, but rather appears following the adhesion to certain ECM components.

Three hypotheses regarding the origin of adhesive RA synovial cells can be considered: 1) cells of synovial lining come directly into contact with cartilage and bone and invade these structures, 2) collagenase-producing synovial cells detach from the tissue, reach the synovial fluid and attach to cartilage and bone, or 3) precursor cells originate from e.g. the bone marrow and differentiate into highly destructive fibroblast-like cells in the joint cavity. Indeed, a subpopulation of activated mononuclear cells floating in the RA synovial fluid can differentiate into fibroblast-like cells and they have the potential to destroy human cartilage (23). TNF-α inhibits the adhesion process of floating cells by reducing vis-à-vis the expression of CD61, but does not reverse the cellular adhesion to COMP once it has taken place. After attachment, the cells can either destroy the ECM or proliferate (24). Based on our data, it would be predicted that in a florid RA, TNF-α decreases the adhesion of SF to cartilage; alternatively, other receptor-matrix interactions might exist during this condition allowing the attachment of SF and triggering the destruction. However, inflammation and destruction are dissociated phenomenon (1); it is possible that SF attached before the first clinical signs of inflammation and/or during clinically more “quiet” phases. It is important to note that one should be very cautious when transferring the in vitro data from samples of late stage disease to the in situ situation.

The proliferation of synovial fibroblasts in response to growth factors requires a second signal generated after adhesion to an ECM component (25). It is possible that the adhesion process protects the cells from TNF-α-induced apoptosis occurring locally in the presence of high concentrations of this cytokine. Since intracellularly, galectin-3 is an anti-apoptotic molecule (2, 26), it is tempting to speculate that its up-regulation after the adhesion of RA-SF to cartilage components could induce a transient resistance to apoptosis at sites of joint destruction. Thus, CD51 (27) and possibly CD61, as well as galectin-3, could represent promising therapeutic targets for the treatment of RA.
Legend to Figures

**Figure 1.** Number of synovial fibroblasts from patients with osteoarthritis (OA-SF) or rheumatoid arthritis (RA-SF) adhering to plates coated with cartilage oligomeric matrix protein (COMP). Four times more RA-SF than OA-SF adhered to COMP.

**Figure 2.** Number of rheumatoid arthritis synovial fibroblasts adhering to plates coated with COMP and dose-dependent decrease of adhesion induced by TNF-α.

**Figure 3.** Number of synovial fibroblasts (SF) from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) adhering to plates coated with COMP and inhibition of adhesion by pre-treatment with anti-CD51 monoclonal antibodies.

**Figure 4.** Galectin-3 in synovial fibroblasts (SF) obtained from patients with osteoarthritis (OA) or rheumatoid arthritis (RA) upon adhesion to culture plates coated with or without cartilage oligomeric matrix protein (COMP), in the presence or absence of TNF-α. The binding assay was performed in cells adapted to 0.5% FCS. Galectin-3 was highly significantly increased in RA-SF.

**Figure 5.** Galectin-3 in synovial fibroblasts (SF) obtained from patients with osteoarthritis (OA) or rheumatoid arthritis (RA) upon adhesion to culture plates coated with or without cartilage oligomeric matrix protein (COMP). The line represents the 1% marker of negative IgG controls. Galectin-3 was induced in RA-SF, but not in OA-SF.

**Figure 6.** Galectin-3 in synovial fibroblasts (SF) obtained from patients with osteoarthritis (OA) or rheumatoid arthritis (RA) upon adhesion to culture plates. Differences between cell cultures maintained in 10 and 0.5% FCS and effect of 10 ng/ml TNF-α during the adhesion assay. Serum starvation and TNF-α decreased the expression of galectin-3.
References

Figure 1

Adhesion to COMP

Number of adherent cells / well

OA
RA
Synovial fibroblasts

p<0.001
TNF-α and Adhesion to COMP

Number of adherent cells / well

TNF-α (ng/ml)

P<0.05

Figure 2
Figure 3

CD51 and Adhesion to COMP

Coating:
BSA
COMP
Pre-treatment:
Mouse
IgG1
Mouse
IgG1
Anti-CD51

Number of adherent cells / well

OA
RA

p<0.05
p<0.001
Galectin-3 (FLI %)

- 0.5% FCS
- TNF-α
- COMP

Figure 4

NS (RA: p<0.001)

NS (RA: p<0.005)

p<0.001
Before adhesion

After adhesion to COMP

Intracellular galectin-3 (FL2)

Figure 5
Galectin-3 (FLI%)

OA
RA

10% 0.5% 10% 0.5% FCS

p<0.001
p<0.001
p<0.001
p<0.005

Figure 6
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