Regulation by transforming growth factor-β1 of class II mRNA and protein expression in fibroblast-like synoviocytes from patients with rheumatoid arthritis

Alexander Navarrete Santos, Astrid Kehlen, Wolfgang Schütte1, Jürgen Langner and Dagmar Riemann

Institute of Medical Immunology, Martin Luther University Halle-Wittenberg, Strasse der Odf 6, D-06097 Halle, Germany
1Clinical Department of Internal Medicine, Hospital Martha-Maria Halle-Dolau, Rontgenstrasse 12, D-06120 Halle, Germany

Abstract

Transforming growth factor (TGF)-β1 is an immunosuppressive cytokine that modulates the expression of class II histocompatibility antigens on human cells. Aberrant HLA class II expression on synovial lining cells of rheumatoid arthritis synovial membrane has been described, and the extent and intensity of class II expression on the cells was claimed to be linked with the severity of the disease. In this study, the effects of TGF-β1 on HLA class II antigen expression in fibroblast-like synoviocytes (SFC) from rheumatoid synovectomy tissues were determined by flow cytometric analysis and quantitative RT-PCR. We found that pre-incubation of cells with TGF-β1 was able to down-regulate IFN-γ-induced DR protein expression in SFC. TGF-β1, additionally, down-regulated IFN-γ-stimulated class II transactivator (CIITA) and DRB mRNA expression. The constitutive expression of CIITA mRNA was completely abolished and the constitutive expression of DRB mRNA was decreased after treatment of SFC with TGF-β1 for 24 h. Addition of the TGF-β inhibitor decorin to SFC for 24 h before TGF-β1/IFN-γ treatment was able to reduce the down-regulatory effect of TGF-β1 on DR antigen expression induced by IFN-γ. Using competitive RT-PCR, we found that SFC constitutively expressed decorin mRNA and that treatment of cells with TGF-β1 for 24 h reduced the constitutive expression of decorin mRNA by 65%. Our results show that TGF-β1 is able to reduce the expression of HLA class II mRNA and protein, and suggest a tight regulation between TGF-β1 and decorin in SFC of the rheumatoid synovium.

Introduction

Rheumatoid arthritis, a chronic inflammatory autoimmune disease of unknown etiology, is characterized by excessive growth of the synovial membrane in diseased joints, causing destruction of cartilage and bone.

Transforming growth factor (TGF)-β, predominantly as type 1, is known in latent and active forms in rheumatoid arthritis synovial fluid (1–5), and has potent activities on cell growth, motility and differentiation (for review see 6). The important role of TGF-β1 in inflammation has been clearly demonstrated in knockout mice lacking the TGF-β1 gene after homologous recombination. These mice showed multifocal inflammatory disease accompanied by overexpression of proinflammatory cytokines such as tumor necrosis factor-α, IFN-γ and MIP-1α (7). Isolated synovial cells from joints with rheumatoid arthritis were shown to express TGF-β1 mRNA and to secrete TGF-β1 protein (3,4). Additionally, TGF-β1 was detected in the synovial tissue and cartilage/pannus junction from patients with rheumatoid arthritis (8). The presence of TGF-β1 has been proposed to account for most of the immunosuppressive activities in synovial fluids from rheumatoid arthritis patients (9).

An important property of TGF-β1 as an immunosuppressive cytokine could be its capacity to down-regulate HLA class II expression in different cell types (10,11). However, rheumatoid arthritis synovium contains various cell types strongly expressing HLA-DR antigens (12,13), and the extent and intensity of HLA class II expression on the cells of the synovial...
membrane has been correlated with severity of disease (14–16). The presence of both active TGF-β1 and an increased expression of HLA class II molecules in the rheumatoid arthritis joint seems to be contradictory, although up to now there is no data available on the effects of TGF-β1 on HLA class II antigen expression in fibroblast-like synoviocytes (SFC). Furthermore, the mechanisms by which TGF-β is activated physiologically in the inflamed joint are not well understood. Newly synthesized TGF-β can be targeted to the extracellular matrix by a second molecule, the latent TGF-β1. Latent TGF-β can be targeted by a number of soluble or matrix molecules, including α2-macroglobulin, heparin, fucoidan and decorin (17–19). Among others, decorin could play a role in the regulation of TGF-β1 activity in SFC, since the presence of decorin in diseased cartilages and in cultured synoviocytes has been demonstrated (20).

The objective of this study was to obtain more information about the effects of the immunosuppressive cytokine TGF-β1 on HLA class II antigen expression in SFC of rheumatoid arthritis pannus. Furthermore, the possible interaction between TGF-β1 and decorin with respect to the expression of class II molecules in SFC was studied.

**Methods**

**Preparation and culture of cells**

SFC cultures were prepared and cultured as described previously (21). Briefly, all cells were grown in RPMI 1640 with 10% FCS and antibiotics, and used at confluence at the fourth to sixth passage. Cells were not positive for the macrophage-specific antigen CD68 (EMB 11; Dako). To induce an increase of HLA class II and class II transactivator (CIITA) mRNA and protein, cells were cultured with rIFN-γ (100 U/ml). The concentration of TGF-β1 and TGF-β2 used in the experiments was 10 ng/ml; in the case of the combination of IFN-γ and TGF-β, TGF-β was present in culture continuously. Decorin was used at a concentration of 5 µg/ml (Sigma, Deisenhofen, Germany). IFN-γ and TGF-β1 were supplied by Pharma Biotechnology (Hannover, Germany). TGF-β2 was purchased from R & D Systems (Wiesbaden, Germany). The use of joint pannus material as a source of SFC was performed with the permission of the local ethics committee.

**Quantitative RT-PCR for CIITA mRNA and DRB mRNA**

The quantification of CIITA mRNA and DRB mRNA was performed as described (22). Briefly, 1 µg of total RNA from SFC isolated as described by Chomczynski and Sacchi (23) was mixed with an aliquot of given dilutions of standard cRNA (30–0.006 ng for DRB mRNA and 5–0.001 ng for CIITA mRNA). RT was performed using SuperScript II RT (Life Technologies Eggenstein, Germany) under the following conditions: 94°C denaturation, 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 35 cycles, and then a final extension step of 5 min at 72°C. For the determination of TGF-β1 mRNA expression, 1 µg of total RNA isolated from SFC was transcribed to complementary DNA with 10 pmol of the 3′ antisense primer for TGF-β1. An exogenous internal standard supplied by Clontech (TGF-β1 amplifier set; Clontech, Heidelberg, Germany) was used in a competitive PCR, during which one set of primers was used to amplify both the target cDNA and a DNA fragment. A dilutions series of the competitor was added to a constant amount of target cDNA. The following controls were performed to monitor DNA contamination: (i) complete assay with Taq polymerase but without template and RT; and (ii) complete assay with Taq polymerase and RNA template, but without RT. Following amplification, samples of the PCR products were resolved by gel electrophoresis, and the yields of amplified competitor and target products were quantified by scanning densitometry and the data were analyzed with ScanPack 2.0 software (Biometra).

**Construction of a decorin internal DNA standard for use in competitive RT-PCR**

An internal decorin standard for use in competitive RT-PCR was constructed by deletion of a decorin PCR product (881 bp). This decorin PCR amplificate includes two restriction sites for the Ddel enzyme (631 and 810 bp). After restriction and gel analysis, three fragments (631, 179 and 71 bp) arise. The 631 and 71 bp fragments were cut out from the gel and cleaned using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Following the ligation of both fragments using the Ready To Go T4 DNA Ligase system (Pharmacia, Freiburg, Germany), 1 µl of the reaction was PCR amplified (55°C annealing, 10 cycles). The product from this amplification is 179 bp shorter than the initial decorin PCR product and can be amplified with the same pair of primers. This primer pair expands several exons (the 5′ sense primer [AGGGGTCCTGT-GGCAATT] expands the first exon and the 3′ antisense primer [TCAGATGAGGC-TGGTTGG] expands the seventh exon of the decorin gene) avoiding amplification of contaminating chromosomal DNA. The PCR product was analyzed by gel electrophoresis, cleaned as described above and used as the decorin internal standard in competitive RT-PCR.

**cDNA synthesis and competitive decorin RT-PCR**

For the cDNA synthesis 1 µg of total RNA isolated from SFC with or without TGF-β1 treatment was reverse transcribed using SuperScript II RT (Life Technologies Egggenstein, Germany) and 50 pmol random primer in a total volume of 20 µl as above. Then, 1 µl of the cDNA synthesis was combined with variable amounts of internal decorin standard and amplified using the following program: 3 min at 94°C initial denaturation, 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for 30 cycles with a final extension step for 5 min at 72°C. Reaction products (10 µl) were analyzed on a 1.2% agarose gel and visualized by staining with ethidium bromide. The relative amounts of decorin and internal standard products were calculated after...
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densitometric analysis using ScanPack 2.0 software (Biometra).

Immunofluorescence staining

For the flow cytometric analysis of HLA class II proteins, cells were detached by use of EDTA (0.2 mM) and trypsin (0.05 mg/ml), washed twice with PBS at 20°C, and incubated with the primary antibody (HLA-DR; IgG2a, HLA-DP; IgG1, HLA-DQ; IgG1; Becton Dickinson Heidelberg, Germany) at 4°C for 40 min. After two washing steps with PBS containing 0.1% sodium azide, a second incubation step with phycoerythrin-labeled goat anti-mouse IgG (Dianova, Hamburg, Germany) at 4°C for 30 min was carried out before fixation of cells with 1% paraformaldehyde in PBS. Flow cytometric analysis was performed on a FACScan (Becton Dickinson) using Lysis software. Results are given as relative increase (rFI) in mean fluorescence intensity (MFI) according to TeVelde (24): rFI = [(MFI of test sample –MFI of negative control)/MFI of negative control].

Statistical analysis

Data are expressed as range or as mean ± SD. The Mann–Whitney rank sum test was used to determine whether two experimental values were significantly different (P < 0.01).

Results

Effect of TGF-β1 on the expression of HLA class II proteins in SFC

The effects of TGF-β1 on the IFN-γ-induced expression of DR, DP and DQ in SFC are summarized in Fig. 1. SFC were incubated in the presence of TGF-β1 and/or IFN-γ, and the expression of DR, DP and DQ protein was measured by flow cytometry. IFN-γ had only a small effect on the induction of DQ protein expression in SFC, as already known (21). We found that TGF-β1 was able to decrease the IFN-γ-stimulated DR, DP and DQ protein. A pre-incubation of SFC with TGF-β1 for 3 h before the addition of IFN-γ for 72 h was sufficient to reduce the IFN-γ-stimulated DR protein expression by 59 ± 6%. Figure 2 demonstrates the time course of the IFN-γ-up-regulation of HLA-DR protein with or without TGF-β1 pretreatment of cells. A 3 h pretreatment of SFC with TGF-β1 caused a diminution of the IFN-γ-induced increase in DR expression after 48–72 h. Using flow cytometry and fluorescence microscopy, 5–30% of SFC were found to express DR protein constitutively in a weak but clear intensity. A 24 h to 3 day incubation of SFC with TGF-β1 was not able to significantly down-regulate this basal DR expression (data not shown).

Regulation of CIITA and DRB mRNA expression in SFC by TGF-β1

To determine whether down-regulation of IFN-γ-induced HLA class II expression by TGF-β1 occurs at the transcriptional level, the effect of TGF-β1 on IFN-γ-stimulated CIITA and DRB mRNA expression was assessed by competitive RT-PCR. SFC were incubated in the presence of TGF-β1 and/or IFN-γ alone for 24 h. TGF-β1 was capable to down-regulate both IFN-γ-stimulated CIITA mRNA expression (88 ± 3% inhibition, Fig. 3) and DRB mRNA (74 ± 20% inhibition). In previous experiments, SFC were found to express constitutively CIITA and class II mRNA (22), therefore it was of interest to study whether TGF-β1 also down-regulated the constitutive
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Fig. 3. Effect of TGF-β1 on IFN-γ-stimulated CIITA mRNA expression in SFC. SFC were cultured in the presence of IFN-γ alone (100 U/ml) or with IFN-γ/TGF-β1 for 24 h and the expression of CIITA was analyzed by competitive RT-PCR. TGF-β1 down-regulates IFN-γ-stimulated CIITA mRNA expression (lanes 6–10) in comparison with IFN-γ alone (lanes 1–5). M: 100 bp ladder. IS: internal standard.

Fig. 4. Effect of TGF-β1 on constitutive CIITA mRNA expression in SFC. (Upper panel) SFC were cultured in the presence of TGF-β1 (10 ng/ml) for different periods of time; the expression of CIITA was analyzed by competitive RT-PCR. (Lower panel) After 24 h incubation with TGF-β1, SFC do not express CIITA mRNA (lanes 6–10) as compared to controls (lanes 1–5). M: 100 bp ladder. IS: internal standard.

expression of CIITA and DRB. The levels of basal DRB transcripts were reduced by 49.1 ± 14% in SFC treated for 24 h with TGF-β1. CIITA mRNA transcripts began to decrease after 4 h TGF-β1 incubation, disappearing completely after 16–24 h (Fig. 4). Furthermore, down-regulation by TGF-β1 of CIITA mRNA was detectable in concentrations down to 0.5 ng/ml. TGF-β2 was similarly active in down-regulating CIITA mRNA expression (data not shown).

Constitutive expression of TGF-β1 and decorin mRNA in SFC
Using competitive RT-PCR, we detected high levels of constitutively expressed TGF-β1 mRNA in SFC (26.4 ± 13 pg/µg total RNA) as already described by several authors (4,7). Since the TGF-β-binding protein decorin could be involved in the regulation of TGF-β1 activity, we determined decorin mRNA expression in SFC by competitive RT-PCR. Figure 5 shows the results of one out of five experiments. SFC were able to express constitutively decorin mRNA and addition of TGF-β1 to SFC resulted in a statistically significant down-regulation of decorin mRNA (65.3 ± 25% inhibition).

Effect of decorin on the DR expression in SFC
To study whether decorin was able to abrogate the down-regulation of IFN-γ-induced DR protein expression by TGF-β1, SFC were incubated in the presence of decorin 24 h before TGF-β1/IFN-γ treatment. Decorin partially prevented TGF-β1-induced inhibition of the increase in DR protein expression caused by IFN-γ. The levels of IFN-γ-induced DR antigen expression in decorin/TGF-β1-treated SFC were 20% higher as compared with DR antigen expression in SFC treated only with TGF-β1 (Fig. 6). However, addition of decorin to SFC at the same time as TGF-β1 could not prevent TGF-β1-induced inhibition of the IFN-γ effect on class II expression. Furthermore, decorin was not able to alter the basal DR expression in SFC nor to elevate the IFN-γ-induced DR expression (data not shown).
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Fig. 6. Representative histograms showing the effect of TGF-β1 and decorin on the IFN-γ-induced HLA-DR expression of SFC. SFC were stained with the isotype control (control) or with mAb specific for HLA-DR. SFC were cultured with medium alone (basal) or IFN-γ for 3 days. TGF-β was given 3 h before the addition of IFN-γ and decorin was given 24 h before TGF-β-treatment.

Discussion

The expression of class II MHC genes has been described to be subject to tight developmental and tissue-specific regulation (25). Certain cell types such as macrophages or endothelial cells, keratinocytes and others become competent for antigen presentation after induction of class II MHC genes. It is accepted that class II MHC antigens expressed on antigen-presenting cells are intimately involved in the process of immune recognition and abnormal expression of these antigens may have a significant impact on the development of autoimmune diseases such as rheumatoid arthritis. Augmented MHC class II protein expression was observed in the rheumatoid arthritic synovium, although IFN-γ has been found within the synovial fluid only in negligible amounts (8). The ability of class II molecules to act as receptors for several microbial products including superantigens from staphylococci, streptococci and Mycoplasma arthritidis may have an important implication in the development of disease. Class II protein expression facilitates the ability of SFC to act as accessory cells (26). Furthermore, class II molecules have signal transducing properties, e.g. ligation of these molecules induces IL-8, RANTES and MCP-1 gene expression in SFC (27).

High levels of TGF-β1 mRNA and protein were detected in rheumatoid synovials (3–5). Since the absence of TGF-β1 can induce a systemic autoimmune disease (28), the presence of TGF-β1 in the inflamed joint could be associated with anti-inflammatory actions. In addition to its effect on class II expression, TGF-β can limit cartilage destruction by inhibiting matrix degradation and by promoting deposition of matrix components, processes which are crucial to tissue repair (6). Kuruvilla et al. (29) showed that TGF-β1, in animal models of rheumatoid arthritis, has powerful anti-inflammatory effects, mimicking in some respects the beneficial effects of immunosuppressive drugs, but without discernible adverse effects.

Although it is known that the immunosuppressive cytokine TGF-β1 regulates the expression of HLA class II molecules in various cells, up to now no data has been available on the effect of this cytokine on class II expression in SFC. We show for the first time that TGF-β1 can down-regulate IFN-γ-induced HLA class II expression also in SFC. Furthermore, TGF-β1 down-regulates IFN-γ-stimulated up-regulation of CIITA and DRB mRNA. CIITA is a general regulator required for both constitutive and IFN-γ-inducible class II transcription and protein expression (30), thus the conclusion seems reasonable that the down-regulation of class II expression in SFC by TGF-β1 is mediated by a reduction of transcription of the CIITA gene. Our results are in agreement with the studies of Lee et al. and Nandan and Reiner (31,32) who showed that TGF-β suppresses IFN-γ-induced class II MHC gene expression in human tumor cell lines by inhibiting CIITA mRNA expression. Whereas TGF-β1 completely abolishes the constitutive expression of CIITA mRNA, it only down-regulates the constitutive expression of DRB mRNA in SFC. One reason could be the different half-life time of CIITA mRNA (3–4 h, unpublished data) and DRB mRNA (14–16 h (22)). The long half-time of class II proteins could similarly be one reason for our result that TGF-β1 could not significantly decrease the low basal DR protein expression in cultured SFC.
Since TGF-β1 can down-regulate class II expression in SFC, the question arises why a strong MHC class II expression can be found in vivo within the joint together with high TGF-β concentrations, and why SFC constitutively express CIITA and DRB mRNA in the presence of endogenous TGF-β1. One possible, although unlikely, explanation could be a disturbed TGF-β activation. TGF-β is secreted by virtually all cell types in a latent form which must be activated prior to interaction with its receptor (for review see 33). Latent TGF-β can be activated, for example, by proteolysis of the latent complex with plasmin and cathepsin D (34). SFC of rheumatoid arthritis synovium express transformation-associated proteinases, such as cathepsin D (35), which could activate TGF-β. Indeed, biologically active TGF-β can be detected within the conditioned medium of SFC, although in small concentrations (4). Since the addition of neutralizing mAb to TGF-β causes a dose-dependent reduction in rheumatoid arthritis SFC growth (4), one can implicate that synovial cell growth is regulated by endogenously activated TGF-β. Another explanation for up-regulated class II expression in the presence of high levels of TGF-β would be the presence of inhibitors of active TGF-β in SFC or within the inflamed joint. Decorin, a leucine-rich proteoglycan, may play essential biological roles during inflammation through its ability to bind extracellular matrix constituents and growth factors (19). Decorin could operate as a reservoir of TGF-β by binding of the active protein (19). Cartilages from patients with rheumatoid arthritis contain more decorin than normal adult cartilages (20). This function of decorin is achieved through its core protein sequestering the growth factor in the extracellular milieu from receptors expressed on the cell surface (17). The high affinity of decorin for TGF-β could lead to the removal of TGF-β from the cellular microenvironment, thereby neutralizing its biological activity. Consistently, we could demonstrate a diminished action of TGF-β1 as inhibitor of the class II induction caused by IFN-γ after pre-incubation of SFC with decorin. Our observation of the down-regulation of decorin mRNA by TGF-β1 in SFC corresponds to findings in both skin and gingival fibroblasts (36), and implicates a tight interaction between both the molecules in SFC. The result of Kahari et al. (37) that dexamethasone increases decorin mRNA and protein in skin fibroblasts and also prevents the TGF-β elicited down-regulation of decorin mRNA levels could be similarly of biological importance for the treatment of arthritis, and work is in progress to investigate the effects of glucocorticoids on TGF-β as well as decorin production in SFC.

It has been claimed that joint destruction is related to the presence of transformed-appearing synovial lining cells attached to cartilage and bone at the site of early destruction (38). Therefore, our earlier observation that SFC from rheumatoid arthritis pannus express constitutively high amounts of CIITA and class II mRNA (22), whereas skin fibroblast are negative, may be of special importance. The question arises whether SFC are simple fibroblasts responding temporarily to local signals or whether they are differentiated in terms of an altered repertoire of responses. Ritchlin et al. observed that the modulated phenotype of SFC included elevation in the steady-state levels of mRNA for IL-6, cathepsin D and stromelysin, the cytoskeletal component vimentin as well as MHC class II molecules (35). The authors discuss this pattern as an intrinsic characteristic of SFC as it was found to persist through multiple passages of the cells in tissue culture in the absence of an additional ongoing inflammatory stimulus. Since antisense experiments have provided functional evidence that c-fos, one of the oncogenes elevated in SFC (39), can be a trans-activating factor controlling transcription of class II genes (40), the high basal expression of class II mRNA in SFC could be associated with the transformed state of cells. The ectopic class II expression by SFC could support an aberrant T cell activation observed also in the rheumatoid synovium. Our experiments show for the first time that CIITA mRNA can be down-regulated by TGF-β1 in the tumor-like growing SFC. Intervention in the expression of class II genes in SFC seems to be a promising therapeutic strategy for rheumatoid arthritis.

We conclude that TGF-β can diminish the expression of CIITA as well as class II molecules in SFC of patients with rheumatoid arthritis. The down-regulation of HLA class II expression by TGF-β1 can be related to the immunosuppressive property of this cytokine and points to a beneficial role of the cytokine in the treatment of rheumatoid arthritis. The constitutive expression of CIITA mRNA as well as of class II mRNA in SFC makes these cells a useful model for studying the regulation of class II expression, the functional consequences of class II molecules as well as of ways of preventing the expression of class II proteins as a therapeutic approach in rheumatoid arthritis.

**Abbreviations**

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<tr>
<td>CIITA</td>
<td>class II transactivator</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>SFC</td>
<td>fibroblast-like synoviocytes</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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**References**

7. Shull, M. M., Ormsby, I., Kientz, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D.,
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