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Imatinib mesylate inhibits platelet derived growth factor stimulated proliferation of rheumatoid synovial fibroblasts ☆

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Abstract

Synovial fibroblast is the key cell type in the growth of the pathological synovial tissue in arthritis. Here, we show that platelet-derived growth factor (PDGF) is a potent mitogen for synovial fibroblasts isolated from patients with rheumatoid arthritis. Inhibition of PDGF-receptor signalling by imatinib mesylate (1 μM) completely abrogated the PDGF-stimulated proliferation and inhibited approximately 70% of serum-stimulated proliferation of synovial fibroblasts. Similar extent of inhibition was observed when PDGF was neutralized with anti-PDGF antibodies, suggesting that imatinib mesylate does not inhibit pathways other than those mediated by PDGF-receptors. No signs of apoptosis were detected in synovial fibroblasts cultured in the presence of imatinib. These results suggest that imatinib mesylate specifically inhibits PDGF-stimulated proliferation of synovial fibroblasts, and that inhibition of PDGF-receptors could represent a feasible target for novel antirheumatic therapies.

Keywords: Imatinib mesylate; Rheumatoid arthritis; Synovial fibroblast

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the excessive growth of synovial membranes, leading to progressive destruction of the affected joint [1]. The pathophysiological mechanisms leading to proliferation of synovial cells and invasive growth of synovial tissue are not fully understood [2,3]. Rheumatoid synovial fibroblasts are the key cell type in the growth of the pathological synovial tissue in arthritis. Thus, inhibition of their proliferation represents a lucrative target for antirheumatic therapies.

Platelet-derived growth factor (PDGF) is an important mitogen for fibroblasts including synovial fibroblasts [4,5]. It binds and triggers the PDGF-receptor (PDGFR) α and β chains. Both PDGF and PDGFRs are upregulated in RA synovial membrane suggesting a role for PDGF in synovial fibroblast proliferation in RA [6–8]. Elevated levels of PDGF have also been detected in synovial fluid of RA patients [9]. In addition to promoting proliferation, PDGF has been shown to increase the expression of proinflammatory cytokines and matrix metalloproteinase-1 in synovial fibroblasts [8,10].

Imatinib mesylate, hereafter referred to as imatinib, is a selective tyrosine kinase inhibitor. Kinases inhibited by imatinib include PDGF-receptors, KIT, and macrophage c-fms receptor [11,12] which all have been implicated in the pathogenesis of RA. In accordance with this, recent preliminary clinical observations suggest that imatinib may indeed have significant antirheumatic activity [13,14]. We have shown that imatinib induces selective apoptosis in synovial mast cells by inhibiting the KIT tyrosine kinase signalling pathway [15]. As mast cells are highly proinflam-

☆ *Abbreviations:* RA, rheumatoid arthritis; PDGF, platelet derived growth factor; FCS, fetal calf serum.

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matory cells, their depletion in synovial tissue could be one factor explaining the antirheumatic effect of imatinib. Since, imatinib is a potent inhibitor of also PDGF-receptor tyrosine kinases the aim of this study was to elucidate whether imatinib could inhibit the growth and induce apoptosis of rheumatoid synovial fibroblasts.

Materials and methods

Culture of synovial fibroblasts. Human synovial fibroblasts were isolated by collagenase treatment (Sigma, St. Louis, USA) from synovial tissue obtained from knee replacement surgeries of seven patients fulfilling the ACR revised criteria for RA. The study was approved by the Ethics Committee of the Surgical Unit of the Helsinki University Central Hospital. Synovial fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, UK) supplemented with 10% FCS (Gibco BRL, UK), 50 IU/ml penicillin, 50 μg/ml streptomycin (Gibco BRL, UK), and incubated at +37 °C in 5% CO_2-atmosphere. Experiments were performed with synovial fibroblasts from third to sixth passage.

Immunohistochemistry was used to characterize the cultured synovial cells and to estimate the amount of contaminating macrophages. Cells (7.5×10^4) were cultured on coverslips for 48 h and fixed with paraformaldehyde (1%) and methanol. Non-specific binding was blocked with 1% milk (Thy-1 staining) or 3% BSA buffer (CD68 staining) before staining the cells with monoclonal antibody for fibroblast-specific Thy-1 (Dianova, Germany) or macrophage-specific CD68 (Dako, Denmark) antigens. After overnight incubation at +4 °C with the primary antibodies, cells were incubated for 1 h with HRP-coupled rabbit-anti-mouse antibody (Dako, Denmark) and stained with AEC. Finally, the cells were stained with haematoxylin (Dako, Denmark).

Isolated synovial tissue cells had typical appearance of fibroblasts and were positive for the Thy-1 antigen (Fig. 1). No more than 10% of the cells in cultures were macrophages (positive for CD68 antigen).

Proliferation assay. Cell proliferation was measured by BrdU assay (Roche Molecular Science, Germany). Synovial fibroblasts were seeded (1×10^5 cells/well) and starved for 48 h in DMEM. PDGF (50 ng/ml, recombinant PDGF-AB, R&D Systems, UK), FCS 2%, and imatinib mesylate or PBS were added and 24 h later the BrdU assay was started. When anti-PDGF antibodies (100 μg/ml, R&D Systems, UK) were used, they were incubated with the cell culture media containing either PDGF or FCS for 2 h, after which the media were added to the cells. All the experiments were performed with duplicate samples. Results are expressed as per cent of BrdU uptake by control cells cultured in DMEM supplemented with either PDGF (50 ng/ml) or FCS (2%).

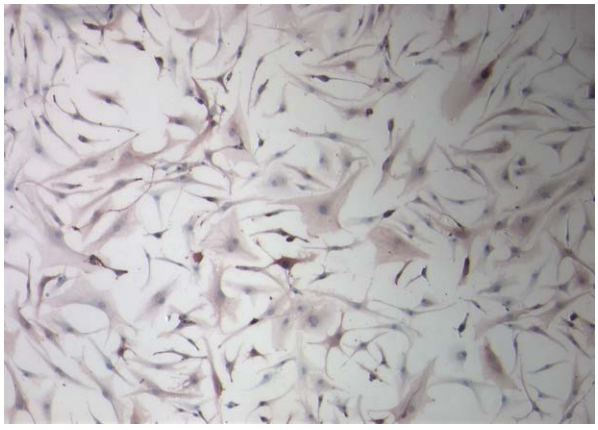

Fig. 1. Immunohistochemistry of isolated cultured synovial tissue cells. Cells (seeded at 7.5×10^4) were stained with monoclonal antibody against fibroblast-specific Thy-1 antigen. Most of the cells have a typical appearance of fibroblasts and are positive for the Thy-1 antigen. The photograph shown is a representative of three independent experiments with synovial fibroblasts from three patients.

Apoptosis assay. Cell apoptosis was studied with Cell death detection Elisa plus (Roche Molecular Science, Germany). Synovial fibroblasts were seeded (2×10^4 cells/well) and cultured with or without imatinib. Camptothecin was used as a positive control for apoptosis. Cell apoptosis was also assessed by TUNEL assay (Chemicon, Temecula, US) after incubation of cultured synovial fibroblasts (7.5×10^4) on coverslips for 48 h with or without imatinib and growth factors. In TUNEL assay, human mast cell line (HMC-1) cells treated with 1 μM imatinib were used as positive control as they have been shown to undergo apoptosis under the treatment [16].

Statistics. Results are expressed as means ± SEM. One-way ANOVA/Tuckey's test was used to assess significance between treatments. A value of $p < 0.05$ was considered to be significant.

Results

PDGF stimulates the proliferation of synovial fibroblasts

First, we confirmed the previous findings that PDGF stimulates the proliferation of cultured rheumatoid synovial fibroblasts. As demonstrated in Fig. 2, PDGF stimulated the proliferation of synovial fibroblasts in a dose-dependent manner. In the presence of 50 ng/ml PDGF the uptake of BrdU was 5.1 ± 0.32-fold higher when compared to medium alone.

Inhibition of PDGFR-signalling completely inhibits the PDGF-induced proliferation

Next, we studied the effect of blocking the PDGFR-signalling on PDGF-induced proliferation. As demonstrated in Fig. 3, inhibition of PDGFR signalling by imatinib completely abrogated the PDGF-induced proliferation of synovial fibroblasts. A significant and dose-dependent inhibition of BrdU uptake was observed by imatinib in the 0.01–1 μM concentration range. Complete inhibition was evident in the presence of 1 μM imatinib. Similar extent of inhibition of synovial fibroblast proliferation as with 1 μM imatinib was observed when PDGF in the medium was neutralized with anti-PDGF antibodies.

Inhibition of PDGFR-signalling partly inhibits serum-stimulated proliferation of synovial fibroblasts

In serum, like in synovium, there are a large number of growth factors capable of stimulating the proliferation of synovial fibroblasts. Therefore, the important question is, whether the inhibition of PDGFR-signalling would inhibit the proliferation of synovial fibroblasts also in the presence of serum. As demonstrated in Fig. 4, also FCS significantly stimulated the proliferation of synovial fibroblasts. The extent of PDGF- and serum-induced proliferation was throughout the experiments of similar magnitude. As shown in Fig. 4, imatinib inhibited also the serum-stimulated uptake of BrdU by synovial fibroblasts in a dose-de-

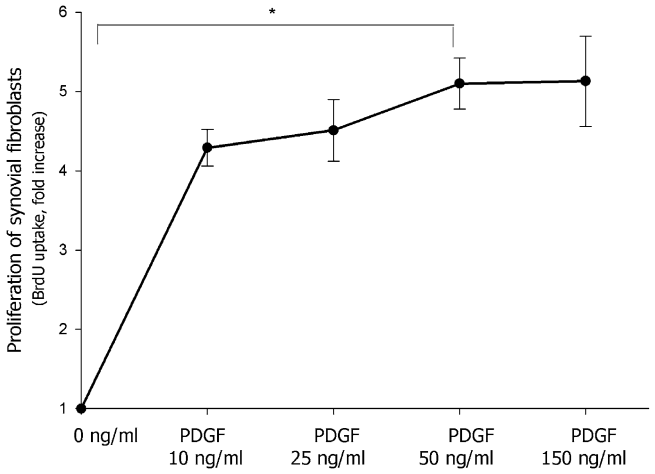

Fig. 2. Platelet-derived growth factor induces proliferation of rheumatoid synovial fibroblasts. Synovial fibroblasts were isolated by collagenase treatment from the synovial tissue of RA patients. Cells were stimulated with increasing concentrations (10–150 ng/ml) of PDGF and cell proliferation was determined with BrdU assay. Control cells were grown in DMEM without growth factors. From each patient duplicate cultures of synovial fibroblasts were analyzed. Means ± SEM of synovial fibroblasts from two patients are shown. *$p < 0.05$.

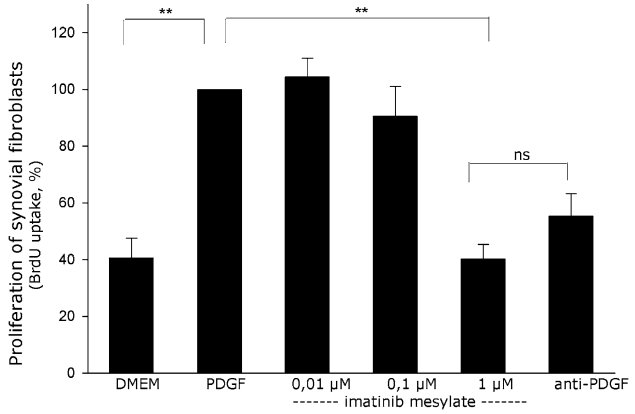

Fig. 3. Imatinib mesylate inhibits PDGF-induced proliferation of cultured rheumatoid synovial fibroblasts. Synovial fibroblasts were isolated by collagenase treatment from the synovial tissue of RA patients. Cells were stimulated with 50 ng/ml PDGF in the presence of the indicated concentrations of imatinib mesylate. Cell proliferation was determined with BrdU assay. The concentration of anti-PDGF antibody was 100 µg/ml. From each patient duplicate cultures of synovial fibroblasts were analyzed. Means ± SEM of synovial fibroblasts from six patients are shown. **$p < 0.01$; ns, non significant.

pendent manner. However, only a partial inhibition of synovial fibroblast proliferation was evident by 1 µM imatinib, a concentration known to block completely the PDGFR tyrosine kinase. These results are compatible with the notion that in serum there are, in addition to PDGF, also other factors that stimulate synovial fibroblast proliferation. However, the results also suggest that PDGFR-signalling contributes significantly to the serum-induced proliferation of synovial fibroblasts.

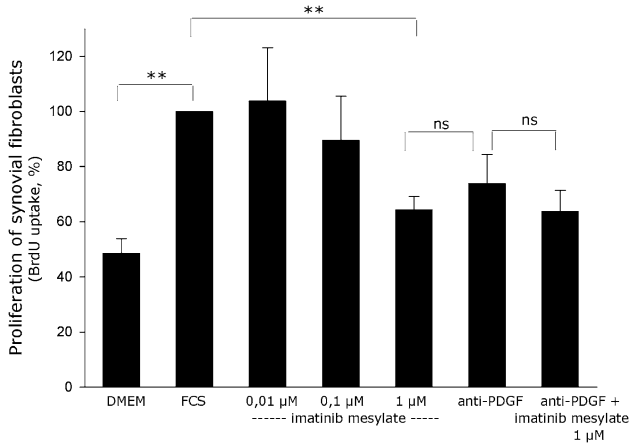

Fig. 4. Imatinib mesylate inhibits serum-induced proliferation of synovial fibroblasts through inhibition of PDGF-signalling pathway. Synovial fibroblasts were isolated by collagenase treatment from the synovial tissue of RA patients. Cells from passages 3 to 6 were stimulated with 2% FCS in DMEM in the presence of indicated concentrations of imatinib. Anti-PDGF antibody (100 µg/ml) was added to FCS-containing culture media 2 h prior to adding the media to cells. Cell proliferation was determined with BrdU assay. From each patient duplicate cultures of synovial fibroblasts were analyzed. Means ± SEM of synovial fibroblasts from four patients are shown. **$p < 0.01$; ns, non significant.

In rheumatoid synovial fibroblasts imatinib inhibits only PDGFR-signalling

The experiments above suggest that imatinib completely inhibits PDGF-induced proliferation and partly the serum-induced proliferation of synovial fibroblasts. However, it was not clear whether imatinib could inhibit, in addition to PDGFR-tyrosine kinase, also some other, perhaps yet unknown, growth factor receptor tyrosine kinase(s) supporting the proliferation of synovial fibroblasts. To study this, we compared the effect of imatinib with the effect of neutralizing PDGF with antibodies. As shown in Fig. 4, also in the presence of serum the inhibition of BrdU uptake by 1 µM imatinib was comparable to the inhibition observed with anti-PDGF antibodies (64 ± 5% vs. 74 ± 11%, respectively). Importantly, if imatinib was added to media in which PDGF had been neutralized with anti-PDGF antibodies, no additional inhibition of BrdU uptake was observed. This suggests that imatinib specifically prevents the action of PDGF in serum, leaving signalling pathways stimulated by other growth factors present in serum intact.

Imatinib does not induce apoptosis in synovial fibroblasts

Finally we addressed the important question whether inhibition of PDGFR-signalling induces apoptosis in synovial fibroblasts. No indication of apoptotic cell death was observed upon addition of imatinib whether studied by quantitation of apoptotic nucleosomes (Fig. 5) or by the number of TUNEL positive cells (Fig. 6). These results

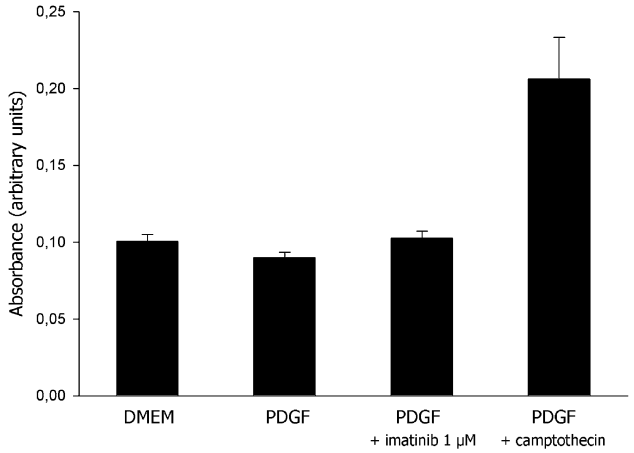

Fig. 5. Inhibition of PDGF-receptor signalling does not induce apoptosis in cultured synovial fibroblasts. Synovial fibroblasts were cultured in the presence of PDGF (50 ng/ml) and 1 μM concentrations of imatinib mesylate. Camptothecin (5 μg/ml) was used as a positive control. Twenty four hours later the amount of apoptotic nucleosomes was analyzed with cell death detection ELISAplus. Means ± SEM of two different experiments are shown.

were independent of whether synovial fibroblasts were cultured in the presence of PDGF, FCS, or in medium alone (data not shown).

Discussion

In the present study, we show that PDGF stimulates significantly the proliferation of rheumatoid synovial fibroblasts, which is in agreement with the previous findings on cultured synovial fibroblasts [4,8]. PDGF receptors have been found to be upregulated in chronic synovial inflammation, suggesting a role for PDGF in RA [6–8]. PDGF enhances the expression of proinflammatory cytokines IL-1β and IL-8, the expression of matrix metalloproteinase-1, and also the activity of nuclear factor κB in rheumatoid synovial fibroblasts [8,10]. Furthermore, elevated levels of PDGF have been detected in the synovial fluid of RA patients [9]. Taken together these findings strongly suggest that PDGF plays a role in the pathogenesis of RA.

Inhibition of synovial fibroblast proliferation is considered as an attractive therapeutic approach in the treatment of RA [3]. In the present study we show for the first time that, by inhibiting the PDGFR-signalling by imatinib mesylate, the PDGF-stimulated proliferation of synovial fibroblasts can be completely inhibited. This is important as the growth of synovial fibroblasts is invasive and it has been shown to be anchorage-independent especially in the presence of PDGF or high concentrations of serum [16]. The inhibition of PDGF-stimulated proliferation by 1 μM

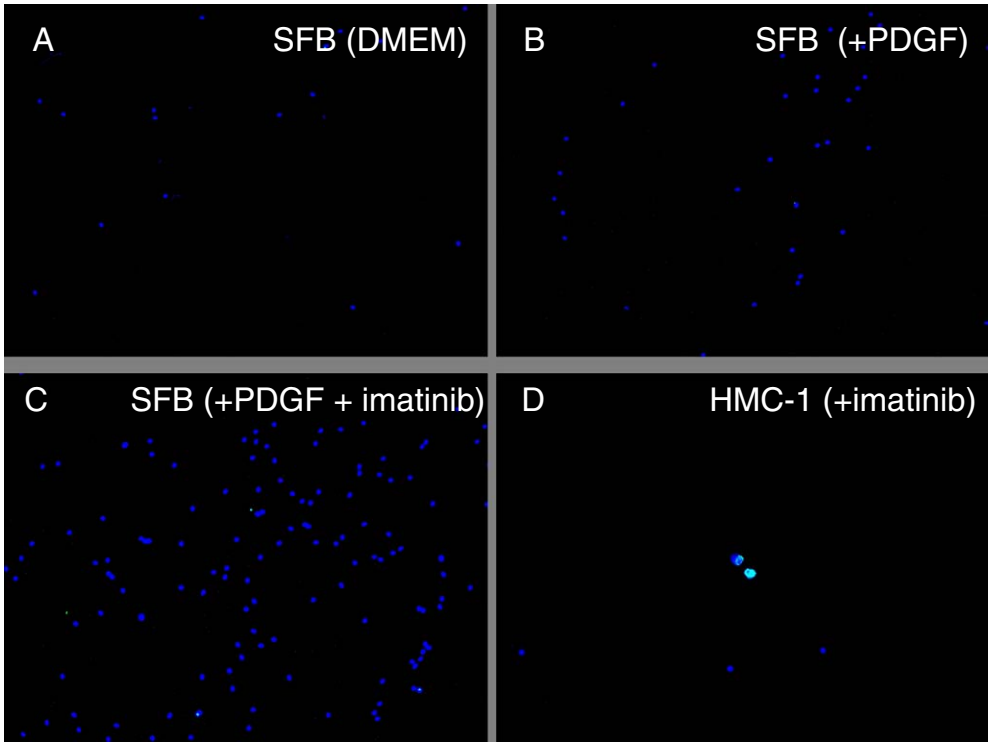

Fig. 6. Inhibition of PDGF-receptor signalling does not result in appearance of apoptotic cells. Synovial fibroblasts were grown either in DMEM without growth factors (A) or in DMEM supplemented with PDGF (B,C). Human mast cell line (HMC-1) cells were used as a positive control (D). Imatinib (1 μM) was added to the culture media (C,D). Apoptosis was studied 24 h later using the TUNEL assay on the coverslips covered with synovial fibroblasts or on cytospin slides (positive control). The green colour shows that TUNEL-positive cells are present only in the positive control. The blue colour (DAPI stain) stains the nuclei of the synovial fibroblasts. The photographs shown are representatives of two-independent experiments. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

imatinib was comparable to the inhibition observed when PDGF was neutralized by using anti-PDGF antibodies. Importantly, imatinib also partly inhibited the serum-stimulated proliferation of synovial fibroblasts. This was not obvious, as in serum, like in synovial tissue, there are a myriad of growth factors other than PDGF, which can promote the proliferation of synovial fibroblasts. Thus these findings suggest also that PDGFR-signalling may be a significant factor in the proliferation of synovial fibroblasts.

Similar extent of inhibition as with imatinib mesylate was observed when PDGF present in serum was neutralized with anti-PDGF antibodies. Noteworthy, no additional inhibition of serum-stimulated proliferation of synovial fibroblasts was observed when imatinib was added in addition to anti-PDGF antibody. These results suggest that the inhibitory effect of imatinib, at least on serum-stimulated proliferation of synovial fibroblasts, is specifically mediated via inhibition of PDGFR-tyrosine kinase and the proliferation-stimulating PDGF-signalling. Thus imatinib appears not to inhibit any additional known or yet unknown tyrosine kinases in synovial fibroblasts.

Inhibition of PDGFR-signalling did not induce apoptosis of synovial fibroblasts. This was not surprising in the presence of serum as in serum there are several other growth factors which can support the viability of synovial fibroblasts. Furthermore, rheumatoid synovial fibroblasts are known to be more resistant to apoptosis than fibroblasts from other origins [3]. This may also explain why apoptosis was not observed when PDGF-signalling was blocked in the presence of PDGF or when synovial fibroblasts were grown in DMEM without any growth factors.

Imatinib inhibits PDGF-receptor, KIT, and c-fms-tyrosine kinases, which all are considered to play a role in the pathogenesis of RA. We have previously shown that imatinib induces a profound apoptosis of cultured and synovial tissue mast cells by inhibiting KIT tyrosine kinase [15]. As mast cells are powerful immune system cells, their apoptosis in synovium may result in the alleviation of inflammatory reaction characteristic for synovium in RA. Here, we show that imatinib also inhibits proliferation of synovial fibroblasts stimulated by PDGF or serum which is also likely to result in an antirheumatic effect. Inhibition of two types of synovial cells, i.e., mast cells and synovial fibroblasts, both involved in the pathogenesis of RA, might be a feasible approach for novel antirheumatic therapies.

Acknowledgments

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