

EXTENDED REPORT

Inhibition of c-kit tyrosine kinase by imatinib mesylate induces apoptosis in mast cells in rheumatoid synovia: a potential approach to the treatment of arthritis

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Background: Mast cells have been implicated in the pathogenesis of arthritis, but elucidation of their precise role has been hampered by a lack of efficient and selective inhibitors of their function.

Objective: To elucidate the role of mast cells in the pathogenesis of rheumatoid arthritis (RA) and to assess whether apoptosis of cultured and synovial tissue mast cells can be induced by inhibiting mast cell growth factor receptor, c-kit tyrosine kinase.

Methods and results: Double staining with tumour necrosis factor (TNF) α and tryptase antibodies showed the presence of TNF α positive mast cells in human rheumatoid synovial tissue. Selective activation of mast cells by anti-IgE resulted in production of TNF α in synovial tissue cultures. Inhibition of the c-kit tyrosine kinase with imatinib mesylate (1.0–10 μ mol/l) induced profound apoptosis in cultured mast cells as judged by typical apoptotic morphology, increased number of apoptotic nucleosomes, and activation of caspases 8 and 9. Importantly, imatinib also induced apoptosis of mast cells in explant cultures of synovial tissue obtained from patients with RA as judged by a TUNEL assay. Inhibition of c-kit tyrosine kinase was accompanied by significant reduction of TNF α production in synovial tissue cultures.

Conclusion: Mast cells may have a role in the pathogenesis of RA, and inhibition of c-kit may be a new means of inhibiting mast cell activity and of abrogating the contribution of mast cells to synovial inflammation in RA.

Increasing evidence suggests that mast cells have a role in the pathogenesis of rheumatoid arthritis (RA).¹ Thus, increased numbers of mast cells have been found in the human rheumatoid synovium, and their number correlates with the activity of the disease.² In addition, strong evidence has indicated that mast cells have an important role in the pathogenesis of experimental arthritis. In an autoantibody induced mouse model of arthritis, infusion of serum from an autoimmune mouse into normal mice containing mast cells induced erosive arthritis, whereas almost no signs of inflammation were seen in mast cell deficient mice (W/W^v).³ Susceptibility to autoantibody induced arthritis could be restored in the W/W^v mice by mast cell engraftment.³ The mechanisms by which mast cells may play a part in the pathogenesis of arthritis involve secretion of potent proinflammatory cytokines, such as tumour necrosis factor (TNF) α , which has been implicated in the pathogenesis of arthritis, as well as secretion of other mediators of inflammation and tissue destruction, such as matrix metalloproteinases.⁴

For the growth and survival of human mast cells, stem cell factor (SCF; also named c-kit ligand, steel factor, or mast cell growth factor) is essential.⁵ SCF is the ligand for the c-kit receptor (c-kit, CD117), which is a transmembrane glycoprotein and a member of the receptor tyrosine kinase subclass III family.^{6–8} c-Kit is expressed by haematopoietic progenitor cells, germ cells, and some human tumour cells, but of the mature immune system cells, only mast cells express c-kit in significant numbers.^{9–10}

Because c-kit tyrosine kinase is essential for the growth and survival of human mast cells, the inhibition of c-kit signalling might prevent mast cell activity in the synovium. Imatinib mesylate (also known as STI-571 or Gleevec) is a relatively specific inhibitor of tyrosine kinases, including

ABL, BCR-ABL, platelet derived growth factor (PDGF) receptor tyrosine kinases, and c-kit. Originally, imatinib mesylate was used in the treatment of chronic myeloid leukaemia^{11–12} and later also in the treatment of certain c-kit positive tumours.¹³ The results of a recent preliminary clinical study,¹⁴ and also a recent case report,¹⁵ suggest that imatinib mesylate may possess considerable antirheumatic efficacy. The mechanisms underlying the antirheumatic effects of imatinib mesylate are not known, but may involve the inhibition of mast cell activity. Therefore, we have studied the role of mast cells in RA and the effects of imatinib mesylate on cultured mast cells and on mast cells residing in synovial tissue.

METHODS

Cell culture

Human mast cell leukaemia cell line (HMC-1) cells express constitutively activated mutant c-kit polypeptide.^{16–17} HMC-1 cells were cultured in Iscove's modified Dulbecco's medium (Gibco, BRL) supplemented with 10% fetal calf serum (FCS; Gibco, BRL), 1% non-essential MEM amino acids (Gibco, BRL), 0.5% penicillin-streptomycin (Gibco, BRL), and 0.004% monothioglycerol (Sigma).

Mouse bone marrow derived mast cells (mBMMC) were obtained by culturing bone marrow cells from the femurs and tibias of BALB/c mice for 3 weeks in enriched culture medium (RPMI 1640 containing 10% FCS (Gibco, BRL), 1% non-essential MEM amino acids (Gibco, BRL), 0.5% penicillin-streptomycin (Gibco, BRL), and 0.004% monothioglycerol (Sigma, St Louis USA) supplemented with 50%

Abbreviations: ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; HuMC, human mast cells; IL, interleukin; mBMMC, mouse bone marrow derived mast cells; PDGF, platelet derived growth factor; RA, rheumatoid arthritis; SCF, stem cell factor; TNF, tumour necrosis factor; WCM, WEHI-conditioned media

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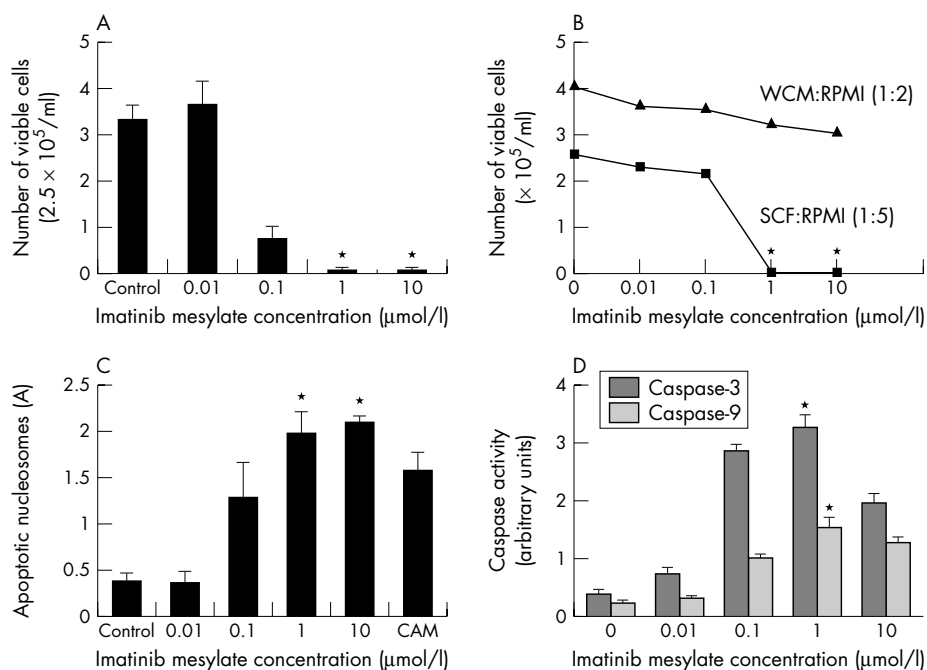


Figure 1 Imatinib induces apoptosis of cultured mast cells by inhibiting c-kit. (A) Human mast cell line (HMC-1) cells cultured with increasing doses of imatinib mesylate for 5 days. Imatinib was added on day 0 and trypan blue-excluding viable cells were counted on day 5. Mean (SEM) of four experiments. (B) Imatinib induces apoptosis of mBMMC only when cultured in the presence of SCF. mBMMC were cultured in the presence of SCF or IL3 (WEHI-conditioned media (WCM)) and imatinib (0.01–10 μmol/l) for 5 days, and the number of trypan blue excluding cells was assessed (n=4). (C) HMC-1 cells were incubated with the indicated concentrations of imatinib and the amount of apoptotic nucleosomes was measured 24 hours later with an ELISA. Mean (SEM) of four absorbance readings shown. Positive control, camphorcin (CAM) is a strong inducer of apoptosis. (D) Caspase activation by imatinib. HMC-1 cells were incubated with indicated concentrations of imatinib for 6 hours and the activity of caspase 3 and 9 was measured by a fluorometric assay. Mean (SEM) of three experiments. *p<0.05 compared with controls, results analysed with analysis of variance for repeated measurements.

WEHI-3B-conditioned medium.¹⁸ The human mast cells (HuMC) were derived from cord blood mononuclear cells as described previously.¹⁹ HuMC were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS. During the first 1–3 weeks the cells were cultured in the presence of 80 ng/ml human recombinant SCF, 50 ng/ml interleukin (IL) 6, and 10 ng/ml IL10 (R&D Systems), in the following 4–8 weeks in the presence of 80 ng/ml SCF, and after 8 weeks in the presence of 80 ng/ml SCF and 10 ng/ml IL4.

Cell viability and proliferation

Cell viability was assessed by directly counting the cells after trypan blue staining. HMC-1 cells were cultured at a density of 450 000 cells/ml and mBMMC at a density of 250 000 cells/ml. The cells were incubated at 37°C and trypan blue-excluding cells were counted 5 days after addition of imatinib mesylate.

Apoptosis assays and immunohistochemistry

The apoptosis of HMC-1 cells and HuMC was determined by measuring the amount of apoptotic nucleosomes after 20–24 hours' incubation at 37°C in the presence of different concentrations of imatinib mesylate with an enzyme linked immunosorbent assay (ELISA) assay kit according to the manufacturer's instructions (Roche Diagnostics). The enzymatic activities of caspases 3 and 9 were measured in HMC-1 cells after incubation of HMC-1 cells with imatinib mesylate for 6 hours using a fluorometric assay kit according to the manufacturer's recommendations (R&D Systems). The results were adjusted according to the protein content of the samples. Each experiment was carried out in triplicate. Apoptosis in cryosections of synovial tissue was examined

with the ApopTag apoptosis detection kit. Mast cells were identified by staining for tryptase using mouse antihuman mast cell tryptase (clone AA1, Dako) and mouse elite kit (Vector Laboratories) or Alexa-fluor (594 or 488) goat antimouse antibodies (Molecular Probes). TNFα was detected with polyclonal goat antibodies against TNFα (Santa Cruz) and Alexa-fluor (594) rabbit antigoat antibodies (Molecular Probes). Specificity was controlled by omitting the primary antibody.

Synovial tissue culture

Synovial tissue was obtained after informed consent during knee synovectomy or arthroplasty of seven patients fulfilling the revised criteria of the American College of Rheumatology.²⁰ The study was approved by the ethical committee of surgery of the Helsinki University Central Hospital. Fat, bone, cartilage, and fibrous tissues were removed and synovial tissue was cut into small pieces of approximately 2 mm³ each. Thereafter pieces were randomly picked, weighed, placed into wells of a 12 well plate (18–20 explants, approximately 300 mg tissue per well), and the exact total weight of synovial tissue in each well recorded. After washing with Hanks's buffered salt solution, the explants were transferred into 1.5 ml of Dulbecco's modified Eagle's medium. After incubation for 1 hour with imatinib mesylate, rabbit IgG antibody for human IgE (150 μg/ml/well) or control non-specific rabbit IgG (150 μg/ml/well; Dako) was added to activate the mast cells. Cultures were incubated at 37°C in 5% CO₂ for 20–24 hours. To verify the activation, a sample was taken for histamine assay after incubation for 1 hour and stored at –20°C. After incubation for 20–24 hours, the explants were embedded in the OCT compound containing mould, which was then frozen in

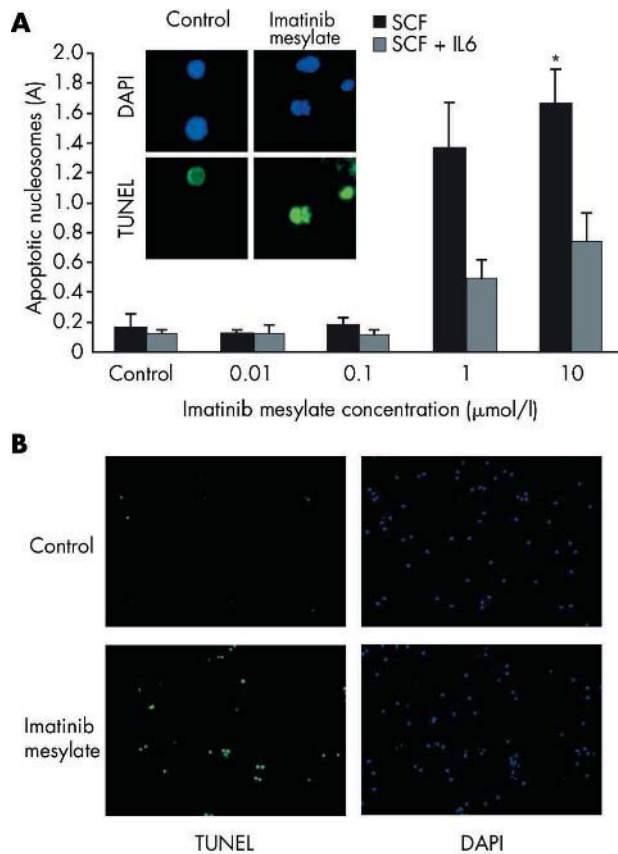


Figure 2 Imatinib induces apoptosis of non-malignant HuMC. (A) HuMC were cultured for 20–24 hours in the presence of either SCF or SCF and IL6. Thereafter, the amount of apoptotic nucleosomes was assessed with an ELISA. Data represent mean (SEM) amounts of apoptotic nucleosomes as absorbance readings of three experiments. * $p < 0.05$ compared with controls. The insert shows the typical apoptotic morphology of imatinib treated HuMC. (B) HuMC were cultured in SCF and with imatinib (10 µmol/l) for 24 hours. Cytospin slides were made and apoptosis was studied using the TUNEL assay. The green colour in the control panel and imatinib treated panel show the TUNEL positive cells. The blue colour (DAPI (4', 6'-diamino-2-phenylindole-dihydrochloride) stain) demonstrates the presence of equal numbers of cells. Results of two experiments are shown.

liquid nitrogen and stored at -70°C . Culture media were aliquoted and stored at -20°C for later analysis. The amount of $\text{TNF}\alpha$ released was measured in the supernatants by an ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

The overall significance of differences between experimental groups was analysed with Friedmann's non-parametric repeated measurements analysis of variance (InStat 3 for Macintosh, Graph Pad software Inc). The differences were considered to be significant for $p < 0.05$, in which case the differences between individual experiment groups were further tested with Dunn's multiple comparisons test. The data are shown as means (SEM).

RESULTS

Inhibition of c-kit tyrosine kinase induces apoptosis of cultured mast cells

In the presence of increasing concentrations of the c-kit inhibitor, imatinib mesylate, the number of cultured transformed human mast cell line cells (HMC-1) was significantly decreased (fig 1A). A considerable decrease in cell numbers

was already seen after 5 days of culture in the presence of imatinib mesylate (0.1 µmol/l), and at concentrations of 1 and 10 µmol/l imatinib mesylate only a few living cells were detected. Similarly, in the presence of an increasing concentration of imatinib mesylate, DNA synthesis, measured as the incorporation of [^3H]thymidine into HMC-1 cells, was decreased (data not shown).

To show that the effects of imatinib mesylate were mediated through c-kit, we next studied mBMMC. The viability of mBMMC can be sustained either with SCF (c-kit dependent pathway) or with IL3 (IL3 dependent pathway).²¹ mBMMC generated in IL3 containing WEHI-conditioned media (WCM) were taken and, after washing, half of the cells were further cultured in the presence of SCF and the other half in the presence of IL3 containing WCM; increasing concentrations of imatinib mesylate were added to both cultures. When the mBMMC were cultured in the presence of SCF and imatinib mesylate, there was a strong reduction in the cell number at 1 µmol/l and higher imatinib concentrations (fig 1B). In contrast, when mBMMC were cultured in the presence of IL3, imatinib mesylate did not significantly reduce the cell number, suggesting that imatinib mesylate specifically affected the c-kit dependent survival pathway. The experiment also shows that imatinib mesylate, in addition to inhibiting the c-kit in HMC-1 cells, inhibits the c-kit tyrosine kinase of normal mouse mast cells.

To verify that the observed reduction in cell number was caused by apoptosis, the amount of apoptotic nucleosomes in HMC-1 cells treated with imatinib mesylate was measured. A concentration of imatinib mesylate as low as 0.1 µmol/l induced a significant increase in the amount of apoptotic nucleosomes (fig 1C). The number of apoptotic nucleosomes at an imatinib concentration of 1 µmol/l was higher than in the presence of camptothecin (5 µg/ml), which was used as a positive control and which is a strong inducer of apoptosis (fig 1C). The induction of apoptosis in HMC-1 cells by imatinib mesylate was further verified using additional methods, including annexin binding and TUNEL staining, which confirmed the finding (data not shown). Furthermore, the inhibition of c-kit tyrosine kinase by imatinib mesylate (0.1 and 1 µmol/l) in HMC-1 cells resulted in significant activation of both caspase 3 and caspase 9 (fig 1D). Thus, inhibition of SCF/c-kit signalling in HMC-1 cells activates a metabolic cascade, which induces cell death by apoptosis.

Because c-kit is constitutively activated in HMC-1 cells owing to a dominant mutation,¹⁶ we wanted to verify that imatinib mesylate can induce apoptosis in normal human mast cells, in which the c-kit mediated cell survival pathway is dependent on the presence of SCF. Furthermore, it is not known whether the presence of other cytokines, besides IL3 in mouse mast cells, can rescue human mast cells from c-kit inhibitor induced apoptosis. When HuMC were cultured in the presence of SCF and an increasing concentration of imatinib mesylate, the number of apoptotic nucleosomes was significantly increased (fig 2A). The induction of human mast cell apoptosis by imatinib mesylate was further verified by studying nuclear fragmentation (insert in fig 2A), TUNEL staining (fig 2B), and annexin binding (data not shown). Interestingly, in the presence of another cytokine (IL6), in addition to SCF, HuMC are more resistant to apoptosis (fig 2A).

Mast cell activation increases levels of $\text{TNF}\alpha$ in synovial tissue

The above results show that imatinib mesylate mediated inhibition of c-kit tyrosine kinase can induce apoptosis in cultured mBMMC, HMC-1 cells, and huMC. However, in inflamed synovial tissues there are also other potential cytokine and growth factor pathways in addition to the

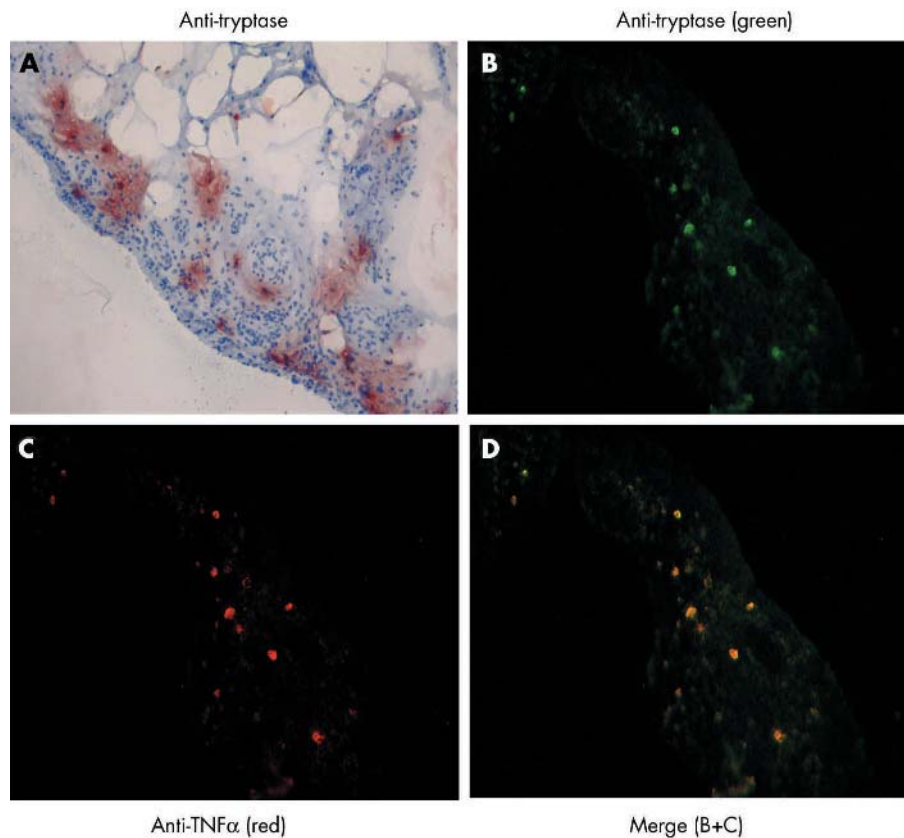


Figure 3 $\text{TNF}\alpha$ positive mast cells can be seen in the synovial tissue of control explant cultures. (A) Synovial tissue stained with anti-tryptase antibody shows mast cells in synovial tissue as brown staining cells. (B) Synovial tissue stained with anti-tryptase antibody and visualised with fluorescent label (green colour). (C) Synovial tissue stained with anti- $\text{TNF}\alpha$ antibodies (red). (D) When anti-tryptase and anti- $\text{TNF}\alpha$ stainings are merged, the resulting yellow colour shows the cells that are positive for both anti-tryptase (mast cells) and $\text{TNF}\alpha$. Representative stainings of three experiments are shown.

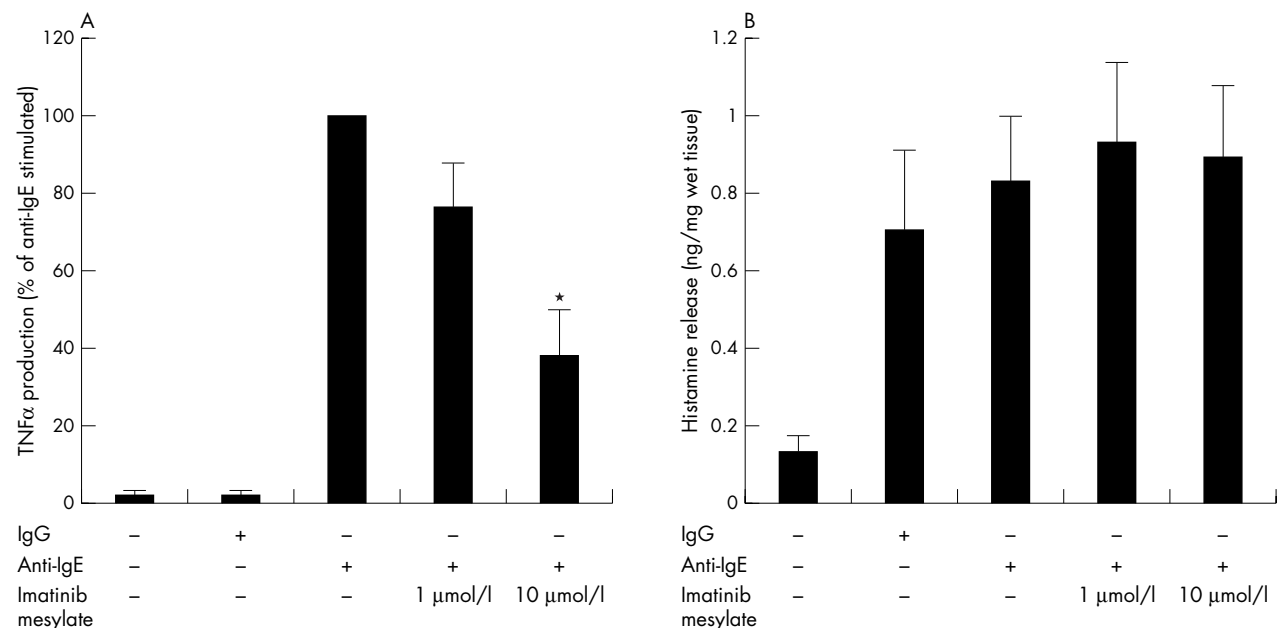


Figure 4 The specific activation of mast cells in synovial tissue results in the production of $\text{TNF}\alpha$, which is partly inhibited by imatinib. The indicated concentrations of imatinib were added to synovial tissue cultures. Mast cells were activated by adding anti-IgE. Addition of non-specific IgG was used as a control for the specificity of anti-IgE. (A) $\text{TNF}\alpha$ produced by the synovial tissue culture was measured 24 hours after activation of mast cells with an ELISA. Release of $\text{TNF}\alpha$ is shown as the percentage change from the $\text{TNF}\alpha$ produced by the IgE activated sample. (B) Histamine released from mast cells measured 1 hour after addition of anti-IgE. Mean (SEM) of results obtained from four patients is shown. * $p < 0.05$ compared with the activated control.

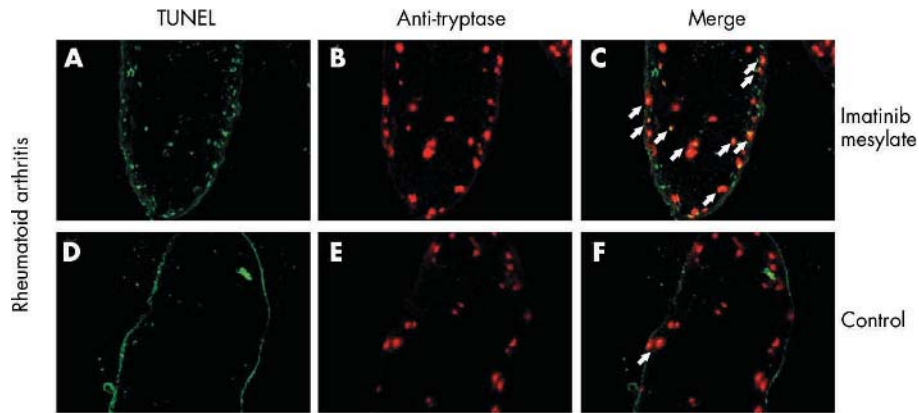


Figure 5 Inhibition of c-kit by imatinib induces apoptosis of mast cells in synovial tissue. Synovial tissue obtained from patients with RA was cultured in the presence of imatinib mesylate (1 $\mu\text{mol/l}$). The appearance of apoptotic cells was studied 24 hours later, using a TUNEL assay. The green colour indicates apoptotic cells and red the tryptase positive mast cells. When the anti-tryptase and TUNEL pictures are merged, the apoptotic mast cells appear as red cells with a yellow spot. The arrows indicate individual apoptotic mast cells. Synovial tissue from four separate patients with RA was analysed with a TUNEL assay, and representative samples are shown.

SCF/c-kit pathway, which can support the viability of tissue mast cells. Furthermore, during continuing inflammatory reactions, mast cells are likely to be activated, which renders them less susceptible to apoptosis. Therefore, we next studied synovial tissue obtained from the knee joints of patients with RA. The inflamed synovial tissue contained a large number of tryptase positive mast cells (brown-reddish stained (fig 3A) or green when stained with fluorescent secondary antibodies, fig 3B). The synovial tissue also showed TNF α positive cellular staining (fig 3C), which was colocalised to tryptase positive cells—that is, to mast cells (fig 3D). It is worth noting that the strong TNF α positive staining in mast cells, as compared with other TNF α producing cells, notably macrophages, is due to the ability of mast cells to store TNF α in their intracellular granules.

To study the effect of mast cell activation in synovial tissue, anti-IgE, a specific mast cell activator, was added to the synovial tissue in culture. Significant release of TNF α (0.65, 1.41, 14.3, and 26.0 pg/mg wet tissue) was seen in all the samples studied 24 hours after mast cell activation with anti-IgE, as compared with control synovial tissue, which did not release detectable amounts of TNF α (fig 4A). Importantly, in the presence of increasing amounts of imatinib mesylate, decreasing amounts of TNF α were released (fig 4A). One hour after the addition of anti-IgE, an increased amount of histamine was also detected in the incubation media, indicating mast cell activation. The amount of TNF α and histamine released upon mast cell activation correlated also with each other (Spearman's correlation, $r_s = 0.73$). However, in contrast with TNF α , imatinib did not inhibit IgE induced acute histamine release (fig 4B). Therefore we studied the histamine release also in cultured mast cells, and the results showed that imatinib had no clear dose dependent effect on IgE induced histamine release in huMC, or compound 48/80 induced histamine release in rat peritoneal mast cells (data not shown). The above results show that selective activation of synovial mast cells increases TNF α levels in synovial tissue, and this can be partly inhibited by imatinib mesylate, probably through induction of mast cell apoptosis.

Inhibition of c-kit tyrosine kinase results in apoptosis of synovial tissue mast cells

Finally, we studied whether imatinib mesylate could induce apoptosis of mast cells in synovial tissue. In untreated synovial tissue obtained from a patient with RA, only occasional apoptotic mast cells were observed (fig 5D-F). However, after incubation of the synovial tissue for 24 hours

in the presence of imatinib mesylate (1 $\mu\text{mol/l}$), several apoptotic cells could be seen, most of which were mast cells (fig 5A and B). Most mast cells in the rheumatoid synovial tissue were rendered apoptotic during the incubation with imatinib mesylate (fig 5C). These experiments show that inhibition of c-kit tyrosine kinase induces apoptosis not only in cultured mast cells but also in those mast cells which reside in RA synovial tissue.

DISCUSSION

TNF α has a central role in the pathogenesis of RA, which is demonstrated by the good clinical efficacy of anti-TNF α treatments.²² Here we show that mast cells, in addition to monocytes/macrophages, are also a significant source of TNF α in the synovial tissue. Indeed, selective IgE mediated activation of synovial mast cells resulted in the production and release of significant amounts of TNF α in synovial tissue. IgE mediated activation of mast cells was used here as it offers an opportunity to activate mast cells specifically, and the purpose of these experiments was to show that mast cells can bring about significant inflammatory response. In rheumatoid synovia it is more likely that mast cells are activated by mechanisms other than IgE mediated activation such as by immune complexes and complement components, C5a in particular.²³ The results of the present study and other immunohistochemical studies on synovial tissue²⁴ indicate that mast cells in RA synovium are in an activated state. Thus, irrespective of the way in which this activation occurs, these findings strongly support the view that mast cells do contribute significantly to the production and secretion of TNF α in RA, and, thus may have a significant role in the pathogenesis of this disease.

Inhibition of c-kit tyrosine kinase with imatinib mesylate induced apoptosis in HMC-1 cells, which suggests that the cells harbour the mutation at the codon 560 of the c-kit receptor, shown to be sensitive to imatinib.²⁵ Inhibition of c-kit by imatinib induced apoptosis also in normal cultured mouse and HuMC. The apoptosis was c-kit dependent, as no apoptosis occurred in mBMMC in the presence of IL3 only. Moreover, apoptosis occurred at low imatinib mesylate concentrations, corresponding to those typically found in patients treated with this drug.²⁶ The sensitivity of cultured mast cells to imatinib mesylate has been observed previously.^{27–29} However, here we show for the first time that apoptosis of mature mast cells cultured with cytokines other than SCF and, importantly, apoptosis of mast cells residing in synovial tissue, can be induced by inhibiting c-kit. This

finding is important as in synovial tissue, and in inflamed synovial tissue in particular, the mast cells are exposed to a myriad of cytokines and growth factors capable of supporting mast cell growth.

Recently, the results of a pilot clinical study suggested that imatinib mesylate may have significant antirheumatic activity.¹⁴ One of the patients who participated in the initial clinical study has now continued treatment with imatinib for 24 months and the disease activity of his RA has remained very low (Eklund *et al*, unpublished results). In addition, a recent case report described a patient with chronic myeloid leukaemia and concomitant RA whose RA symptoms clearly improved during imatinib treatment.¹⁵ The reason for this potentially antirheumatic activity of imatinib is not clear, but it might be related to the inhibition of the synovial PDGF receptor tyrosine kinase or the inhibition of c-kit in synovial tissue or it might be mediated by inhibition of some as yet unidentified tyrosine kinase.

We are currently studying the effect of inhibiting synovial PDGF receptor on the growth and proliferation of synovial cells. The only c-kit positive cells in synovial tissue have been shown to be mast cells.³⁰ Here we show that the function of synovial mast cells can be inhibited and their apoptosis induced by inhibiting c-kit. As mast cells are involved in many aspects of the inflammatory reaction, it is conceivable that inhibition of c-kit, resulting in inhibition of mast cell activity, might result in attenuation of the inflammatory reaction in arthritic joints.

If the imatinib mesylate induced apoptosis and depletion of tissue mast cells can also be accomplished in vivo, it might represent a new means of examining the significance of mast cells in RA. In addition, induction of mast cell apoptosis through inhibition of c-kit might be the basis for new targeted treatments of RA and of other diseases associated with inappropriate mast cell activity.

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