Induction of Rheumatoid Arthritis and Response to Tyrosine Kinase Inhibitors

Emad Y. Moawad

Dept. of Engineering, Ain Shams University, Cairo, Egypt
*Corresponding Author: emadmoawad@hotmail.com

Abstract The goal of this work is to determine the role of the autoimmune cells in Rheumatoid arthritis (RA) induction and the immunomodulatory mechanism of therapy with Tyrosine Kinase Inhibitors (TKIs) in RA attenuation. B cells were isolated from naive DBA/1 mouse spleens and stimulated for 72 hours with LPS as a positive control forming a mouse model of RA in the presence or absence of 1–5 μ M imatinib. B cell staging were assessed by adding 1 μ Ci of [3 H] thymidine for measuring proliferation in the final 18 hours of the stimulation, and a Beta plate scintillation counter was used to quantitate incorporated radioactivity. Samples of C1.MC/57.1 mast cells were stimulated with 100 ng/mL of Self Cell Factor (SCF) as a positive control of a mouse model of RA in the absence or presence of 1-5 μ M of imatinib. Tumour Necrotic Factor (TNF) levels in culture supernatants from C1.MC/57.1 mast cells were measured by ELISA. The histologic grade (H_G) and the level of TNF of the mouse model of RA were 1/10 and 10 times respectively those in the control one. This inverse proportion clarifies that RA disease is the result of big increase in TNF level perpetuating local inflammation and joint destruction leads to a major decrease in H_G with the same ratio. The addition of 1 and 5 μ M doses of imatinib increased H_G by 200% and 300% respectively while decreased TNF level to be 12.5% and 10% respectively of that in the mouse model of RA restoring rate of TNF level of normal tissue. This demonstrates that effective mitigation of symptoms of RA is the result of a significant increase in H_G because of the cell cycle arrest resulting from the treatment of TKIs which leads to a significant reduction in the level of TNF but with a different ratio to increase H_G unlike happened in incidence of RA.

Keywords Platelet-derived Growth Factor; Tumour Necrotic Factor; Histologic Grade

principally attacks flexible (synovial) joints. The exact mechanisms by which innate immune cells contribute to RA progression are not known yet. Mast cells, macrophages, and B cells contribute to RA pathogenesis and progression inducing synovial inflammation and joint destruction [1]. There is compelling evidence that number of mast cells in the human rheumatoid synovium is strongly correlated with the activity of RA [2]. In addition it is evident that macrophages infiltrate the synovium which is characteristically associated with the over-production and activity of TNF- α and other proinflammatory cytokines that potentiate inflammation in RA, transforming growth factor β (TGF β) and platelet derived growth factor (PDGF) [3,4]. To date there is no good immunotherapy to treat or prevent the development of autoimmune diseases but only for attenuating symptoms and inhibiting disease progression. A recent study in an autoantibody induced mouse model of arthritis showed that Tyrosine Kinase Inhibitors (TKIs) inhibit a select set of tyrosine kinases that are directly implicated in the pathogenesis of RA [5]. Thus, TKIs is a one of those therapies that can treat autoimmune diseases inhibiting signaling pathways implicated in RA, including those mediated by the tyrosine kinases c-Fms and platelet-derived growth factor receptor (PDGFR) [6, 7]. However, yet TKIs immunomodulatory mechanism of action is not fully understood. Therapy with TKIs modulates cytokine levels, inhibits T-cell activation and proliferation directly affecting the histologic grade (H_G) [8, 9].

Recently, Moawad improved models of clinical and pathology based staging of the cellular kinematics' alterations enables to estimate the energy yield of drug doses and thus expecting the ability of those doses to inhibit the cell cycle progression in order to administer the appropriate dose and introduced an easy method allow more frequent monitoring to therapy response[8-10]. In an attempt to understand the immunological basis of RA, current approach tests the ability of TKIS to attenuate RA symptoms in a mouse model of RA.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease affects many tissues and organs, but

2. Methods and Materials

Experiments were performed to determine the ability of imatinib to inhibit each of B cell proliferation and

proinflammatory cytokines (TNF- α) production in vitro as conducted and described by Paniagua et al [11]; Six- to eight-week-old male DBA/1 mice (The Jackson Laboratory) were housed at Stanford University and experiments performed under protocols approved by the Stanford University Committee of Animal Research and in accordance with NIH guidelines. B cells were isolated from naive DBA/1 mouse spleens by negative selection with MACS beads (Miltenyi Biotec). Isolated B cells were stimulated for 72 hours with LPS (5 μ g/ml; Sigma-Aldrich) in the presence or absence of 1–5 μ M imatinib. For measurement of B cell proliferation, after 48 hours B cells were pulsed with 1 μ Ci [3 H] thymidine (ICN Pharmaceuticals) for the final 18 hours of the stimulation, and a Beta plate scintillation counter (PerkinElmer) was used to quantitate incorporated radioactivity. For cytokine analysis; mouse mast cell line C1.MC/57.1 [12, 13] were serum starved for 6–8 hours, pre incubated with imatinib for 2 hours, and stimulated for 10 minutes with SCF (100 ng/mL; Pepro Tech) in the presence of 0–5 μ M imatinib, and after 48 hours culture supernatants were collected and analyzed for TNF- α using a bead-based cytokine assay. Pure imatinib was utilized for both of the in vitro B cell proliferation assays and mast cell cytokine release measurement. The induction of apoptosis by imatinib mesylate was investigated by annexin V (a marker for early apoptosis) or propidium iodide (a marker for cell death) using flow cytometry analysis as conducted by Juurikivi et al. [14].

3. Results and Analysis

Data and results as shown by Paniagua et al [11]; Imatinib inhibited LPS-stimulated B cell proliferation in a dose-dependent fashion ($P < 0.001$) for concentrations of 1 μ M and higher (Table 1).

The incorporated radioactivity was quantified with Betaplate scintillation counter. The percentage of Labeled index(%Li) shown in table 1 represent the mean cpm $\pm$ SEM of quadruplicates and are representative of 3 independent experiments at statistical significance of $P < 0.05$ by Student's t test, compared with cells stimulated in the absence of inhibitor. Further, TNF- α production by SCF-stimulated C1.MC/57.1 mast cells was significantly reduced by imatinib at a concentration of 1 μ M, and restored the normal rate of control sample at 5 μ M as shown in Table1.

As shown by Juurikivi et al [14]; the induction of apoptosis by imatinib mesylate was verified as determined by flow cytometry analysis at an imatinib concentration higher than 1 μ M ($P < 0.001$) [14].

Imatinib Inhibits in Vitro Proliferation of B Cells from Naive DBA/1 Mouse Spleens

Labeled index (Li) of ^3H -TDR incorporation in LPS-stimulated B cell treated groups was decreased in dose dependent manner due to proliferation inhibition induced by imatinib mesylate doses where number of cells was constant in treated groups at 1 μ M dose of imatinib while for higher doses, the number of cells in treated groups was decreased in dose dependent manner due to apoptosis induction as shown in table 1. Thus, H_G of the control, +ve control and treated samples with imatinib of LPS-stimulated B cell groups were identified according to Moawad pathologic staging system in cases of constancy of number of cells or apoptosis induction as follows [15-18]:

The released energy of 1 μ Ci/well (0.106032443mL) of Tritiated thymidine (half-life time = 12.32 Years, decay energy = 0.01859 MeV [19]) per mL during the incubation of 18 hr is equivalent to:

$$E_{^3\text{H-TDR}} = \frac{1 \text{ mL} \times 10^{-6} \times 3.7 \times 10^{10}}{0.106032443 \text{ mL}} \times \frac{12.32 \times 3.16 \times 10^7}{\ln 2} \times \left(1 - e^{\frac{-\ln 2 \times 18 \times 60 \times 60}{12.32 \times 3.16 \times 10^7}} \right) \times 0.01859$$

$$\Rightarrow E_{^3\text{H-TDR}} = 4.20331840 \times 10^8 \text{ MeV/mL.}$$

Thus, according to Moawad pathologic model for cellular cancer staging in case of constancy in number of cells:

$$H_G = U\% \times E_{^3\text{H-TDR}} \text{ MeV (equation 1) [8, 9]}$$

While in case of apoptosis induction:

$$H_G = \frac{U\% - \% \text{Apoptosis}}{1\% \text{Apoptosis}} \times 100\% \times E_{^3\text{H-TDR}} \text{ (equation 2) [10]}$$

, where ($U\% = 1 - \text{Li}$) is the unlabeled fraction of the detected cells by ^3H -TDR, ($E_{^3\text{H-TDR}}$) is the energy of the used Tritiated Thymidine, and H_G is the histologic grade of the detected tissue.

Thus from Eq 2, H_G of the control, +ve control and treated samples of LPS-stimulated B cell groups were identified as shown in table 2.

Table 1 shows Labeled index of ^3H -TDR incorporation in groups of B cells from naive DBA/1 mouse spleens were stimulated with 5 $\mu\text{g/ml}$ LPS (+ve control) in the presence of 1, 2.5 and 5 μM doses of imatinib, and TNF level in C1.MC/57.1 mast cells treated by 1, 2.5 and 5 μM doses of imatinib.

Imatinib dose (μM)	Labeled Index (%Li)	% of Apoptosis	TNF level (pg/mL)
0(Control)	1%	0	120
0 (+ve Control)	90.1%	0	1200
1	69.6%	0	150
2.5	42%	34.266%	135
5	10%	83.127%	120

Table 2. shows the histologic grade (H_G in MeV) of C1.MC/57.1 mast cells treated groups, the corresponding alterations in H_G induced by imatinib doses, and Energy of the used imatinib doses.

Imatinib dose (μM)	Histologic grade (H_G in MeV)	$H_G - H_{G, +ve\ control}$ (in MeV)	Energy of imatinib dose ($E_{\text{Imatinib Dose}}$ in MeV)
0(Control)	4.16128522×10^8		0
0 (+ve Control)	4.16128522×10^7		0
1	1.27780879×10^8	8.61680272×10^7	8.61680272×10^7
2.5	1.51768005×10^8	1.10155153×10^8	1.10155153×10^8
5	1.71216780×10^8	1.29603928×10^8	1.29603928×10^8

Accordingly, percentage of decrease in Li by ^3H -TDR incorporation of $E_{^3\text{H-TDR}}$ expresses the increase in H_G than $H_{G, +ve\ control}$ as a result of the induced Cell Cycle Arrest (CCA) by the studied drug as hypothesized and confirmed by Moawad model [15- 18].

i.e. Energy of the imatinib dose = $H_G - H_{G, +ve\ control} = \%$ decrease in Li by ^3H -TDR incorporation $\times E_{^3\text{H-TDR}}$ (Eq 3)

Thus from Eq 3, energy yield by 1, 2.5 and 5 μM doses of imatinib mesylate that equivalent to alterations induced in the histologic grades ($H_G - H_{G, +ve\ control}$) were as follows:

($H_G - H_{G, +ve\ control}$) induced by 1 μM dose of imatinib mesylate = $(90.1\% - 69.6\%) \times 4.20331840 \times 10^8$

= 8.61680272×10^7 MeV/mL, while ($H_G - H_{G, +ve\ control}$) induced by 2.5 μM dose of imatinib mesylate = $(90.1\% - \frac{42\%}{1 - 34.266\%} \times 100\%) \times 4.20331840 \times 10^8 = 1.10154173 \times 10^8$ MeV/ML, whereas ($H_G - H_{G, +ve\ control}$) induced by 5 μM dose of imatinib mesylate = $(90.1\% - \frac{10\%}{1 - 83.127\%} \times 100\%) \times 4.20331840 \times 10^8 = 1.29601523 \times 10^8$ MeV/mL, which represent the amount of energies yield by 1, 2.5, 5 μM doses of imatinib mesylate/mL derived as established in the pathologic model for cellular staging in case of constancy in number of cells conducted by Moawad [8-10].

TKIs Inhibit Proinflammatory Cytokines Production

Measuring TNF levels by ELISA in culture supernatants from C1.MC/57.1 mast cells of control, +ve control and treated groups shows that imatinib at 1- 5 μ M dramatically reduced mast cell production of TNF- α to levels similar to those in the unstimulated cell populations. Stimulating C1.MC/57.1 mast cells with SCF contribute to the pathogenesis of RA by producing proinflammatory cytokines where TNF level was increased 1000% from control sample (120 pg/mL) to the +ve control one (1200 pg/mL), while the addition of imatinib was able to decrease PDGFbb-induced TNF release by mast cells in treated groups as shown in table 1; Addition of 5 μ M dose of imatinib was able to restore normal level of TNF in control sample (120 pg/mL) diminishing RA symptoms to show that TKIs suppress PDGFbb-induced TNF production.

4. Discussion

The aim of this work is to differentiate normal tissue from that of RA induction and evaluate the ability of TKIs to inhibit autoimmune cells production of proinflammatory cytokines. Autoimmune B cell and mast cell C1.MC/57.1 cell lines were chosen for conducting staging assays and proinflammatory cytokine release measuring to determine the relation between H_G and TNF- α level in RA tissue as human rheumatoid synovium is an autoimmune disease characterized by large number of mast cells correlates with the activity of the disease and contributes to the pathogenesis of experimental arthritis [2]. Tissue differentiation was conducted by pathologic staging system clarified that H_G of RA tissue is in contrast to cancerous tissue less than that of normal counterpart as a result of the excess in cytokines production. New therapies that target specific pathways involved in RA pathogenesis are needed; here imatinib was shown robustly prevents and treats RA by selectively inhibiting a spectrum of signal transduction pathways central to the pathogenesis of RA. Imatinib abrogate PDGFR signaling in a mouse model of RA able to inhibit a narrow spectrum of tyrosine kinases including PDGFR α/β (IC50 = 0.1 μ M), c-Fms (IC50 = 1.4 μ M), and c-Kit (IC50 = 0.1 μ M) as well as macrophage production of proinflammatory cytokines; PDGFR -induced TNF- α production [20-22]. Our in vitro data indicate that imatinib potently inhibits diverse cellular responses that synergize in inducing inflammation and thereby the formation of pannus tissue, which invades and destroys adjacent cartilage and bone in RA. Imatinib decreased C1.MC/57.1 mast cells proliferation as well as TNF production in response to PDGFR stimulation. As the mechanisms underlying the initiation and progression of RA remain undefined, our findings in staging the detected tissues suggest that RA induction in a tissue starts by local cell cycle disruption acts via increasing cell doubling time and consequently cell growth energy[15-18], increasing H_G , initiating and

activating a tumour formation which stimulates the autoimmune adapting cells to produce TNF acts via diminishing tumour formation restoring normal cell doubling time and decreasing H_G once again. Inflammation effect and damage in synovitis and joint destruction in murine arthritis and human RA by TNF- α is produced in cases of either secreting more amounts of TNF- α than required to prevent tumour formation or as a result in time-response difference between the slow rates of increasing H_G as commonly shared in tumours formation and its fast rates of decreasing by autoimmune cells. Thus, RA is an “immune-mediated” disease, in which errors of the immune system function against the PDGF receptor (PDGFR) stimulate the over expression of TNF- α that cause joint destruction. Such proposed mechanism for RA induction is supported by the confirmed findings of high levels of TNF- α in the areas of RA tissue of lowest H_G compared to that of normal counter part of relatively higher H_G . From table 2, the sudden decrease in H_G induced in

$H_{G, +ve\ control}$ to be $\frac{1}{10}$ of $H_{G, control}$ (4.16128522×10^8

MeV $\rightarrow 4.16128522 \times 10^7$ MeV) by stimulating the C1.MC/57.1 mast cells with SCF, increases the level of TNF- α in +ve control sample to be 10 times (120pg/mL $\rightarrow$ 1200pg/mL) of that in the control one as shown in table 1 to express the inverse proportion between H_G and TNF- α level of tissues on inducing RA as their product was constant in each of the control and +ve control samples. Such inverse proportion between H_G and TNF- α level of tissues confirms the proposed mechanism of RA induction. On the contrary, the mechanism by which RA tissue responds to TKIs involves inhibition of monocyte/macrophage, B cells, mast cells, and fibroblast- like synoviocytes (FLSs) proliferation, differentiation, and TNF-production that could reduce disease activity in RA. Staging model by the quantitative response determination of 3H -TDR incorporation to identify H_G is a valuable method, reliable to predict TKIs dose effectiveness and highly sensitive to allow easier and more frequent monitoring to a mouse model of RA response to TKIs therapy. The ability of imatinib to suppress PDGF induced proliferation of rising H_G of RA tissue to bounce back was confirmed as energy yield by TKIs doses was 100% identical to the increase induced in H_G of RA tissue ($p \leq 0.0001$) to provide a clear cut criterion for accepting the hypothesis of staging the induction and the attenuation of RA through 3H -TDR incorporation. The addition of 1 and 5 μ M doses of imatinib mesylate increased $H_{G, +ve\ control}$ by 200% and 300% respectively attenuating RA symptoms, decreasing TNF level by 87.5% and 90% to be $\frac{1}{8}$ and $\frac{1}{10}$ of that in +ve control sample

respectively to restore normal TNF level in control sample. Thus diminishing RA symptoms in a tissue requires rising its histologic grade which may lead to apoptosis at high doses of the inhibitor as shown in table 1 to indicate that the costs of mitigating the symptoms of RA will be at the expense of the number of cells. Resistance of cancer patients towards imatinib therapy develops frequently in patients of advanced-stage Philadelphia-positive leukemia for their much higher H_G than that of normal counterparts. Accordingly, no resistance would be expected towards RA therapy by imatinib for the lower H_G of RA tissue than that of normal counterparts as shown in table 2 ($H_{G, RA} = 0.1 H_{G, control}$). Thus the demonstrated data suggest that the inhibitory effect of TKIs on TNF level of tissues rising its H_G contributes to TKIs efficacy in RA. As TNF- α is a driver of autoimmune tissue injury in a spectrum of autoimmune diseases, including multiple sclerosis, Crohn disease, psoriasis, and RA [23]. Accordingly in addition to potentially providing benefit in the treatment of autoimmune joint destruction disease in RA, it is anticipated that TKIs have potential as novel therapeutics could also provide efficacy in other autoimmune diseases.

5. Conclusion

Incidence of RA is the result of big increase in TNF level perpetuating local inflammation and joint destruction leads to a major decrease in H_G of tissues with the same ratio in which PDGFR, PDGF, macrophages and mast cells are over expressed. TKIs like FDA-approved drugs imatinib represent a promising and powerful strategy for the treatment of RA and other inflammatory diseases that potently treat RA and inhibit multiple signal transduction pathways that drive pathogenic cellular responses in RA. The immunomodulatory mechanism of TKIs therapy mediated monocyte/macrophage, B cells, FLSs and mast cells proliferation and inhibition of PDGFR signaling, increasing H_G of RA tissue, reducing TNF level to normal rate in normal lesion and thereby provide clinical benefit in RA. In RA therapy, alterations induced by TKIs in H_G and TNF level would be strongly inversely correlated attenuating RA effectively but not in an inverse proportion as in incidence of RA.

Conflict of interest

The author declares that there is no conflict of interest concerning this paper.

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