Induction of Multiple Sclerosis and Response to Tyrosine Kinase Inhibitors

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Received: 22 March 2013 / Accepted: 17 September 2013 / Published online: 28 September 2013
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Abstract The goal of this work is to determine the role of the autoimmune cells in multiple sclerosis (MS) induction and the immunomodulatory mechanism of therapy with tyrosine kinase inhibitors (TKIs) in MS attenuation. Samples (5×10^5 cells per well) of C6 and primary rat astrocytes were stimulated with 10 ng/mL of platelet-derived growth factor (PDGFbb) as a positive control forming a mouse model of MS. PDGFbb was added to the astrocytes in the absence or presence of 0.1 and 1 μ M of imatinib. Proliferation of C6 and primary rat astrocytes samples were assessed for samples staging by the addition of 1 μ Ci of 3 H-thymidine per well. Samples of RAW 264.7 cells were stimulated for 48 h with 10 ng/mL of PDGFbb in the absence or presence of 0.1 and 1 μ M of sorafenib. Tumour necrotic factor (TNF) levels in culture supernatants from RAW 264.7 cells were measured by ELISA. The histologic grade (H_G) and the level of TNF of the mouse model of MS was 1/5 and 5 times respectively of those in the control one to clarify that MS induction is due to a major decrease in H_G inversely proportional to the accompanied increase in TNF level perpetuating local inflammation and demyelination in MS lesion. The addition of 0.1 and 1 μ M doses of imatinib increased H_G of the mouse model of MS by 6 and 11 times respectively while 0.1 and 1 μ M doses of sorafenib decreased TNF level to be 1/2 and 1/5 of that in the

mouse model of MS respectively restoring normal rate of TNF level of normal lesion to show that H_G and TNF level would be strongly inversely correlated ($r = -0.99$) in attenuating MS effectively by TKIs therapy but not in an inverse proportion as in MS induction.

Keywords Platelet-derived growth factor · Tumour necrotic factor · Histologic grade

Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disease attacks myelinated axons in the central nervous system (CNS), characterized by destroying the myelin and the axon in variable degrees. The exact mechanisms by which innate immune cells contribute to MS progression are not known yet [1]. There is compelling evidence that T cell-mediated inflammatory events in the CNS of patients with MS play a role in the pathogenesis of this disease which characteristically associated with the over-production and activity of proinflammatory cytokines transforming growth factor β (TGF β) and platelet derived growth factor (PDGF) [2]. A recent study showed that MS patients have macrophages which is a type of innate immune cells phagocytose myelin in brain lesions in MS patients and promote immune cell infiltration and inflammation in the CNS inducing demyelination [3, 4]. Thus, PDGF and TNF pathways are genetic biomarkers exert neurotoxic and chemoattractant effects that cause gradual damage in CNS and complications in MS patients [5]. 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 [6]. Therefore new therapies that target specific pathways involved in MS

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pathogenesis are needed. As tyrosine kinase inhibitors (TKIs) inhibit a select set of tyrosine kinases that are directly implicated in the pathogenesis of MS. Thus, TKIs is a one of those therapies that can treat autoimmune diseases inhibiting signaling pathways implicated in MS, including those mediated by the tyrosine kinases c-Fms and platelet-derived growth factor receptor (PDGFR) [7, 8]. 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) [9–11]. For instance, imatinib and sorafenib inhibited astrocyte proliferation mediated by PDGFR, whereas GW2580 and sorafenib inhibited macrophage TNF production mediated by c-Fms and PDGFR, respectively [12]. 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 [9–11]. In an attempt to understand the immunological basis of MS current approach tests the ability of TKIs to attenuate MS symptoms in a mouse model of MS.

Materials and Methods

For in vitro studies, as conducted and described by Crespo et al. [12]; imatinib and sorafenib were purchased from LC laboratories in powder form and resuspended in sterile vehicle (0.5 % hydroxypropyl methylcellulose ±0.05 % Tween-80). Concentrations equivalent to those reached by Crespo et al. dosing regimen were used for all in vitro experiments. Macrophage cell line RAW 264.7 cells were grown in RPMI media supplemented with 10 % fetal calf serum (FCS), L-glutamine, penicillin/streptomycin (P/S), and non-essential amino acids (NEAA). Primary rat astrocytes from the cerebral cortex of P2 mouse pups were grown in DMEM media supplemented with 10 % FCS, 2 mM L-glutamine, P/S NEAA, sodium pyruvate, insulin (5 µg/mL), NAC (5 µg/mL), and 10 µM hydrocortisone. The C6 rat astrocyte cell line was cultured in DMEM supplemented with 10 % FCS, L-glutamine, P/S, and NEAA. C6 and primary astrocyte proliferation was assessed by ³H-thymidine incorporation. Briefly, 5 × 10⁵ cells per well were plated in a 96-well plate in 200 µL of media and left incubating at 37 °C overnight to allow the cells to adhere. Astrocytes were then stimulated with 10 ng/mL of PDGFbb or 10 ng/mL of TNF as a positive control forming a mouse model of MS [13, 14]. PDGFbb and TNF were added to the astrocytes in the absence or presence of 0.1 and 1 µM of imatinib. Cells were cultured for 24 h before

Table 1 Labeled index of ³H-TDR incorporation in C6 and primary astrocyte groups treated by 0.1 and 1 µM doses of imatinib, and TNF level in RAW 264.7 cells treated by 0.1 and 1 µM doses of sorafenib

Imatinib dose (µM)	Labeled index (%Li)	Sorafenib dose (µM)	TNF level (pg/mL)
0 (Control)	55 $\frac{5}{9}$ %	0 (Control)	12
0 (+ve Control)	91 $\frac{1}{9}$ %	0 (+ve Control)	60
0.1	46 $\frac{2}{3}$ %	0.1	30
1	2 $\frac{2}{9}$ %	1	12

the addition of 1 µCi of ³H-thymidine per well of total volume of 460 µL. After 18 h, the cells were frozen or harvested and corrected counts per minute were counted using a beta plate reader. The effect of TKIs in activating the macrophage cell line RAW 264.7 cells was tested by measuring TNF levels in supernatants from RAW 264.7 cells by ELISA in culture stimulated for 48 h with 10 ng/mL of PDGFbb in the absence or presence of 0.1 and 1 µM of sorafenib. Results are representative of three independent experiments. The mean ± SEM of triplicates is shown in Table 1 at statistical significance of **P* < 0.05 by Student's *t* test, compared with cells stimulated in the absence of inhibitor.

Results and Analysis

TKIs Inhibit PDGF-Induced Astrocyte Proliferation

Data and results as shown by Crespo et al. [12]; imatinib was able to inhibit PDGF-induced astrocyte proliferation. Labeled index (Li) of ³H-TDR incorporation in C6 and primary astrocyte treated groups was decreased in dose dependent manner due to proliferation inhibition induced by imatinib mesylate doses (Table 1). On the other hand, the number of cells was constant in treated groups where no apoptosis induced up to 1 µM dose of imatinib mesylate.

Thus, H_G of the control, +ve control and treated samples of C6 and primary astrocyte groups were identified according to Moawad pathologic staging system as follows [9–11]:

The released energy of 1 µCi/well (0.460 mL) of tritiated thymidine (half-life time = 12.32 years, decay energy = 0.01859 MeV [15]) per mL during the incubation of 18 h is equivalent to:

$$E_{3\text{H-TDR}} = \frac{1\text{mL} \times 10^{-6} \times 3.7 \times 10^{10}}{0.460\text{ mL}} \times \frac{12.32 \times 3.16 \times 10^7}{\ln 2} \times \left(1 - e^{-\frac{\ln 2 \times 18 \times 60 \times 60}{12.32 \times 3.16 \times 10^7}}\right) \times 0.01859 \Rightarrow$$

Table 2 The histologic grade (H_G in MeV) of C6 and primary astrocyte 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)	$H_G - H_{G,+ve \text{ control}}$ (in MeV)	Energy of imatinib dose ($E_{\text{Imatinib Dose}}$ in MeV)
0 (Control)	4.30840136×10^7		0
0 (+ve Control)	8.61680272×10^6		0
0.1	5.17008163×10^7	4.30840136×10^7	4.30840136×10^7
1	9.47848299×10^7	8.61680272×10^7	8.61680272×10^7

$$E_{3H-TDR} = 9.69390306 \times 10^7 \text{ MeV/mL.}$$

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

$$H_G = U\% \times E_{3H-TDR} \text{ MeV} \quad (1)$$

where $U\%$ is the unlabeled fraction of the detected cells by $^3\text{H-TDR}$ and E_{3H-TDR} is the energy of the used tritiated thymidine, (H_G) of the control, +ve control and treated samples of C6 and primary astrocyte groups were identified as shown in Table 2 [9–11].

While the percentage of decrease in Li by $^3\text{H-TDR}$ incorporation of E_{3H-TDR} expresses the increase in H_G than $H_{G,+ve \text{ control}}$ as a result of the induced cell cycle arrest (CCA) by the studied drug in case of cell number constancy as shown in Eq. 2 [9–11].

$H_G - H_{G,+ve \text{ control}} = \%$ decrease in Li by $^3\text{H-TDR}$ incorporation $\times E_{3H-TDR}$ (Eq. 2). Thus from Eq. 2, alterations in the histologic grades ($H_G - H_{G,+ve \text{ control}}$) induced by 0.1 and 1 μM doses of imatinib mesylate were as follows:

($H_G - H_{G,+ve \text{ control}}$) induced by 0.1 μM dose of imatinib mesylate = $(91\frac{1}{9}\% - 46\frac{2}{3}\%) \times 9.69390306 \times 10^7 = 4.30840136 \times 10^7 \text{ MeV/mL}$, while ($H_G - H_{G,+ve \text{ control}}$) induced by 1 μM dose of imatinib mesylate = $(91\frac{1}{9}\% - 2\frac{2}{9}) \times 9.69390306 \times 10^7 = 8.61680272 \times 10^7 \text{ MeV/mL}$, which represent the amount of energies yield by 0.1 and 1 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 [9–11]. The H_G of C6 and primary astrocyte control, +ve control and treated groups, the corresponding alterations in H_G induced by imatinib doses that equivalent to their energy are shown in Table 2.

Table 2 shows a major decrease in H_G (80 %) from the control group to the +ve control one forming the mouse MS model to confirm that in MS induction H_G and TNF level would be in an inverse proportion as will be clarified in the discussion.

TKIs Inhibit Proinflammatory Cytokines Production

Data and results as shown by Crespo et al. [12]; measuring TNF levels by ELISA in culture supernatants from

macrophage cell line RAW 264.7 cells of control, +ve control and treated groups by sorafenib as shown in Table 1 clarifies that macrophages contribute to the pathogenesis of MS by producing proinflammatory cytokines as TNF level was increased 500 % from control sample (12 pg/mL) to the +ve control one (60 pg/mL), while the addition of sorafenib was able to decrease PDGFbb-induced TNF release by macrophages in treated groups of RAW264.7 cells. Addition of 1 μM dose of sorafenib was able to restore normal level of TNF in control sample (12 pg/mL) diminishing MS symptoms to show that TKIs suppress PDGFbb-induced TNF production by macrophages. Tables 1 and 2 show a major increase induced in H_G (1,000 %) of the mouse MS model (+ve control group) due to TKIs therapy to restore normal level of TNF in the control group to confirm that in attenuating MS symptoms effectively by TKIs therapy, H_G and TNF level would be strongly inversely correlated as will be clarified in the discussion.

Discussion

The aim of this work is to differentiate normal tissue from that of MS induction and evaluate the ability of TKIs to inhibit macrophage production of proinflammatory cytokines. Tissue differentiation was conducted by pathologic staging system clarified that H_G of MS tissue is in contrast to cancerous tissue less than that of normal counterpart as a result of the excess in cytokines production. Here, imatinib was shown robustly prevents and treats MS by selectively inhibiting a spectrum of signal transduction pathways central to the pathogenesis of MS. Imatinib and sorafenib abrogate PDGFR signaling in a mouse model of MS; able to inhibit PDGF-induced astrocyte proliferation as well as macrophage production of proinflammatory cytokines; PDGFR-induced TNF- α production. Our in vitro data indicate that imatinib potently inhibits diverse cellular responses that synergize in inducing inflammation and thereby demyelination in MS. Imatinib decreased C6 and primary astrocyte proliferation as well as TNF production in response to PDGFR stimulation. As the mechanisms underlying the initiation and progression of MS remain undefined, our findings in staging the detected tissues

suggest that MS induction in a tissue starts by local cell cycle disruption acts via increasing cell doubling time and consequently cell growth energy [16–18] 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 CNS is produced in cases of either secreting more amounts of TNF 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, MS is an “immune-mediated” disease, in which errors of the immune system function against the PDGF receptor (PDGFR) stimulate the over expression of TNF that cause damage in CNS. Such proposed mechanism for MS induction is supported by the confirmed findings of high levels of TNF in the areas of MS lesions of lowest H_G compared to that of normal lesions of relatively higher H_G . From Table 2, the sudden decrease in H_G induced in $H_{G,+ve\text{control}}$ to be $\frac{1}{5}$ of $H_{G,\text{control}}$ by stimulating the rat astrocytes with PDGFbb, increases the level of TNF in +ve control sample to be 5 times of that in the control one as shown in Table 1 to express the inverse proportion between H_G and TNF level of tissues on inducing MS as their product was constant in each of the control and +ve control samples. Such inverse proportion between H_G and TNF level of tissues confirms the proposed mechanism of MS induction. On the contrary, the mechanism by which MS tissue responds to TKIs involves inhibition of monocyte/macrophage proliferation, differentiation, and TNF-production that could reduce disease activity in MS. 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 MS response to TKIs therapy [9–11]. The ability of imatinib and sorafenib to suppress PDGF induced proliferation of astrocytes rising H_G of MS tissue once again was confirmed. The considerable increases in the percentages of cells labelled by thymidine of MS model before and after TKIs therapy rising H_G reflecting the response to therapy were inversely proportional to the induced decreases in TNF levels of MS model ($P \leq 0.0001$) to provide a clear cut criterion for accepting the suggestion of staging the induction and the attenuation of MS through ^3H -TDR incorporation. From Table 2, the addition of 0.1 and 1 μM doses of imatinib mesylate increased $H_{G,+ve\text{control}}$ by 6 and 11 times respectively attenuating MS symptoms, decreasing TNF level to be $\frac{1}{2}$ and $\frac{1}{3}$ of that in +ve control sample respectively to restore normal TNF level in the control sample as shown in

Table 1. These therapeutic measurements clarify also the strong inverse correlation ($r = -0.99$) between H_G and TNF level of tissues on attenuating MS symptoms. Thus diminishing MS symptoms in a tissue requires to rise its H_G from $H_{G,+ve\text{control}}$ to be more than twice $H_{G,\text{control}}$ to indicate that MS induction needs much less alterations in H_G than that required for MS attenuation. Resistance towards cancer therapy by imatinib develops frequently in patients of advanced-stage Philadelphia-positive leukemia for their much higher H_G than that of normal counterparts [19]. In addition, administering high imatinib dose in cancer therapy of advanced malignancy is capable to induce apoptosis irrespective of high H_G of the malignant cells. Accordingly, no resistance is expected towards MS therapy by imatinib for the much lower H_G of MS lesion than that of normal counterparts besides the much lower administered dose of imatinib in MS therapy not capable to induce apoptosis in contrast to the administered one in cancer therapy of advanced malignancy. Thus the demonstrated data suggest that the inhibitory effect of TKIs on TNF level of tissues raising its H_G contributes to TKIs efficacy in MS. As TNF- α is a driver of autoimmune tissue injury in a spectrum of autoimmune diseases, including rheumatoid arthritis, Crohn disease, psoriasis, and MS [20]. Accordingly in addition to potentially providing benefit in the treatment of autoimmune demyelination disease in MS, it is anticipated that TKIs have potential as novel therapeutics could also provide efficacy in other autoimmune diseases.

Conclusion

MS induction is due to a major decrease in H_G as a result of disruption in autoimmune anti-tumour response, inversely proportional to the accompanied increase in TNF level perpetuating local inflammation and demyelination in MS lesion in which PDGFR and PDGF are over expressed. TKIs like FDA-approved drugs imatinib and sorafenib represent a promising and powerful strategy for the treatment of MS and other inflammatory diseases that potently treat MS and inhibit multiple signal transduction pathways that drive pathogenic cellular responses in MS. The immunomodulatory mechanism of TKIs therapy mediated monocyte/macrophage proliferation and inhibition of PDGFR signaling, increasing H_G of MS lesion, reducing TNF level to normal rate in normal lesion and thereby provide clinical benefit in MS. In MS therapy alterations induced by TKIs in H_G and TNF level would be strongly inversely correlated ($r = -0.99$) attenuating MS effectively but not in an inverse proportion as in MS induction.

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

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