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NILOTINIB AND BOSUTINIB MODULATE PRE-PLAQUE ALTERATIONS OF BLOOD IMMUNE MARKERS AND NEURO-INFLAMMATION IN ALZHEIMER'S DISEASE MODELS

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Abstract—Alzheimer's disease (AD) brains exhibit plaques and tangles in association with inflammation. The non-receptor tyrosine kinase Abl is linked to neuro-inflammation in AD. Abl inhibition by nilotinib or bosutinib facilitates amyloid clearance and may decrease inflammation. Transgenic mice that express Dutch, Iowa and Swedish APP mutations (TgAPP) and display progressive Aβ plaque deposition were treated with tyrosine kinase inhibitors (TKIs) to determine pre-plaque effects on systemic and CNS inflammation using milliplex® ELISA. Plaque Aβ was detected at 4 months in TgAPP and pre-plaque intracellular Aβ accumulation (2.5 months) was associated with changes of cytokines and chemokines prior to detection of glial changes. Plaque formation correlated with increased levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1α, IL-1β) and markers of immunosuppressive and adaptive immunity, including, IL-4, IL-10, IL-2, IL-3, Vascular Endothelial Growth Factor (VEGF) and IFN-γ. An inverse relationship of chemokines was observed as CCL2 and CCL5 were lower than WT mice at 2 months and significantly increased after plaque appearance, while soluble CX3CL1 decreased. A change in glial profile was only robustly detected at 6 months in Tg-APP mice and TKIs reduced astrocyte and dendritic cell number with no effects on microglia, suggesting alteration of brain immunity. Nilotinib decreased blood and brain cytokines and chemokines and increased CX3CL1. Bosutinib increased brain and blood IL-10 and CX3CL1, suggesting a protective role for soluble CX3CL1. Taken together these data suggest that TKIs regulate systemic and CNS immunity and may be useful treatments in early AD through dual effects on amyloid clearance and immune modulation.

Key words: plaque, CX3CL1, inflammation, nilotinib, bosutinib.

INTRODUCTION

Alzheimer's disease (AD) is characterized by extracellular plaque deposits derived from β-amyloid precursor protein (βAPP) and accumulation of Aβ peptides (Jarrett et al., 1993; Li et al., 1999; Allinson et al., 2003). Changes of microglia morphology and astrogliosis surrounding plaques suggest inflammation in advanced stages of AD (Akiyama et al., 2000). However, intracellular accumulation of Aβ in transgenic animals and human brains with AD and Down's syndrome (von Kienlin et al., 2005; Lonskaya et al., 2013e) suggests that intraneuronal Aβ may precede plaque deposition in early AD. We previously demonstrated that intraneuronal Aβ triggers AD-like pathology, including inflammation, without detection of extracellular plaques (Burns et al., 2009; Rebeck et al., 2010). Furthermore, pre-plaque Aβ activates microglia and astrocytes and increases some inflammatory markers (Rebeck et al., 2010), suggesting that inflammation may not only be a response to CNS plaque accumulation but also involve communication between Aβ-expressing neurons and the immune environment. Although microglia and astrocytes detect extracellular Aβ through a number of sensors (Landreth and Reed-Geaghan, 2009), intraneuronal Aβ may induce apoptosis, thus triggering neuronal signals to activate microglia and astrocytes (Pereira et al., 2005) independent of extracellular plaques. However, reduction in parenchymal Aβ, independent of intraneuronal Aβ, correlates with a decrease in microglial activation and a reduction in pro-inflammatory molecules (Malm et al., 2015). In AD post-mortem brain, the non-receptor tyrosine kinase Abelson (Abl) is activated in the hippocampus and entorhinal cortex (Derkinderen et al., 2005; Jing et al., 2009; Tremblay et al., 2010; Schlatterer et al., 2011a). Src, a tyrosine kinase that is structurally homologous to Abl, is also associated with AD pathology (Lee, 2005; Reynolds et al., 2008; Ittner et al., 2010). The tyrosine kinase inhibitor (TKI) nilotinib (AMN107) is a multi-target compound that preferentially inhibits Bcr-Abl with an IC_{50} less than 30 nM in murine myeloid progenitor

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Abbreviations: Abl, Abelson; AD, Alzheimer's disease; DAB, 3,3′-diaminobenzidine; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GFAP, Glial Fibrillary Acid Protein; IBA, ionized calcium-binding adapter; IFN, interferon; STEN, Sodium Tris EDTA NP40; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor; VEGF, Vascular Endothelial Growth Factor; WT, wild type.

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cells (Amagai et al., 2015; Medico et al., 2015). Bosutinib (SKI-606) is a dual Sc/Abl inhibitor with an IC_{50} of 1.2 nM and 1 nM in cell-free assays, respectively (Trimmer et al., 2010; Gleixner et al., 2011; Sarkar et al., 2012). Nilotinib and bosutinib are second generation TKIs that are therapeutically used in adult chronic myeloid leukemia (CML) (Kantarjian et al., 2007; Musumeci et al., 2012). We previously demonstrated that a small fraction of nilotinib (1–10 mg/kg) and bosutinib (1–5 mg/kg) crosses the blood–brain-barrier (BBB), inhibits Abl and facilitates autophagic Aβ clearance, leading to neuroprotection and improved cognition (Lonskaya et al., 2012; Hebron et al., 2013a,b; Lonskaya et al., 2013a,c,d).

Pre-plaque interaction between systemic and CNS immunity may be critical to understanding a role for the immune system in early stages of AD – providing insight into the therapeutic potential of anti-inflammatory drugs in early or mild to moderate AD. We previously demonstrated that nilotinib and bosutinib reduce intracellular and plaque Aβ in 4-month-old TgAPP mice that express neuronally derived human APP, 770 isoform, containing the Swedish K670N/M671L, Dutch E693Q and Iowa D694N mutations (Tg-APP) under the control of the mouse thymus cell antigen 1, theta, Thy1, promoter (Davis et al., 2004) and in lentivirus gene transfer mouse models (Lonskaya et al., 2013c, 2014). Plaque accumulates at 4–5 months of age in organotypic cultures from TgAPP mice and microglia accumulation is detected at 9 months around plaques (Humpel, 2015). Here, we aim to determine the immune response in pre-plaque Tg-APP and examine the effects of nilotinib and bosutinib on changes of peripheral and CNS innate and adaptive immunity. This approach may provide evidence that inflammatory modulation may regulate Aβ clearance. These studies demonstrate pre-plaque changes of systemic inflammatory molecules, reflecting possible early communication between central and peripheral immunity to modulate innate and adaptive immune responses. Changes in systemic immune profile were altered by nilotinib and bosutinib treatment in parallel with modulation of inflammatory brain responses to pre-plaque Aβ.

EXPERIMENTAL PROCEDURES

Nilotinib and bosutinib treatment

Transgenic mice (male and female) expressing neuronally derived human APP, 770 isoform, containing the Swedish K670N/M671L, Dutch E693Q and Iowa D694N mutations (Tg-APP) under the control of the mouse thymus cell antigen 1, theta, Thy1, promoter (Davis et al., 2004) and age-matched C57BL/6 mice wild type (WT) were treated with intraperitoneal (I.P.) injection (30 μL) of either 10 mg/kg nilotinib or 5 mg/kg bosutinib or dimethyl sulfoxide (DMSO) every other day for 6 weeks. Nilotinib (Cat# S1033) and Bosutinib (Cat# S1014) were commercially obtained (Selleckchem Inc., Houston, Texas, USA) in 10-mg vials and dissolved in 1 ml DMSO (50 mg/ml), aliquoted to avoid freeze and thaw cycles and stored at −20 °C. A total volume of 30 μL solution with a final concentration of 10 mg/kg nilotinib or 5 mg/kg bosutinib was injected. Mice were obtained from Jackson's Laboratories as breeding pairs and the colonies were propagated at Georgetown University Department of Comparative Medicine. All animal experiments were conducted with the approval and oversight of the Georgetown University Animal Care and Use Committee (GUACUC).

Tissue collection and milliplex enzyme-linked immunosorbent assay (ELISA)

To isolate blood and brain tissues, animals were deeply anesthetized with a mixture of Xylazine and Ketamine (1:8) and 50–150 μl of whole blood was collected via cardiac puncture. To wash out the remaining blood from vessels and reduce contamination, animals were perfused with 10 ml of 1× saline for 4 min and the brain was collected and immediately homogenized in 0.5 ml ELISA buffer and centrifuged at 5000×*g* for 15 min at 4 °C to precipitate the pellet. We customized a highly sensitive milliplex® MAP Kit (Cat # MPXMCYTO-70K, Millipore) with color-coded microspheres (beads) and fluorescent dyes; through precise concentrations, the beads simultaneously and specifically capture mouse cytokines, including IL6, IL-1α, IL-1β, tumor necrosis factor (TNF)-α, IL-2, IL-3, IL-4, IL-10, VEGF, Interferon (IFN)-γ, CCL2, and CCL5. A total of 25 μL of sample was introduced into a plate containing the microspheres and the reaction mixture was incubated with Streptavidin-PE conjugate and reporter molecule as described in the manufacturer's protocol. Using a Luminex® machine, microspheres are first passed through a laser which excites the internal dyes in the microspheres; a second laser excites the PE, which is the fluorescent dye on the reporter molecule, and a high-speed digital-signal processor identifies each individual microsphere and quantifies the bioassay.

Immunohistochemistry of brain sections

Animals were deeply anesthetized with a mixture of Xylazine and Ketamine (1:8), washed with 1× saline for 1 min and perfused with 4% paraformaldehyde (PFA) for 15–20 min. Brains were quickly dissected, immediately stored in 4% PFA for 24 h at 4 °C, and transferred to 30% sucrose at 4 °C for 48 h. Brains were cut using a cryostat at 4 °C into 20-micron thick coronal sections and stored at −20 °C. Immunohistochemistry was performed on 20-μm-thick sections. Astrocytes were probed (1:200) with monoclonal anti-Glial Fibrillary Acid Protein (GFAP) antibody (Millipore Corporation, Reading, Massachussets, USA) and microglia were probed (1:200) with ionized calcium-binding adapter (IBA)-1 polyclonal antibody (Wako, Richmond, Virginia, USA). Dendritic cells were probed (1:200) with CD11b polyclonal antibodies (Thermo Fisher, Weston , Florida, USA). Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) was performed according to the manufacturer's protocol (Life Technologies, Miami, Florida, USA). Human $Aβ_{1–42}$ was probed (1:200) with rabbit polyclonal specific anti-$Aβ_{1–42}$ antibody (Zymed) that recognizes amino acids 1–42. Mouse monoclonal (6E10) antibody (Covance) (1:100) with 3,3′-diaminobenzidine (DAB) was also used (Covance).