SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TITOLO DELLA RELAZIONE:

Investigating the role of neuroinflammation on loss of cognitive function - effects of CSF-1R blockade by PLX5622 on irradiation-induced memory deficits

RELAZIONE:

Thanks to this SIF-grant, I've spent 6 months at the University of California, San Francisco under the supervision of professor Susanna Rosi. Rosi's lab research is focused on the impact of brain injuries and the subsequent neuroinflammation on cognitive function and during those 6 months I had the chance to be involved in several projects, including the study of the effects of CSF-1R blockade by PLX5622 on irradiation-induced memory deficits (Feng et al., 2016).

Background

Whole-brain irradiation (WBI) is commonly used for the treatment of primary brain tumors and brain metastases. Following fractionated whole-brain irradiation (fWBI), 50–90 % of long-term survivors (>6 months) have irreversible cognitive decline. The underlying molecular mechanisms that result in the loss of cognitive function after radiotherapy are not completely understood, and consequently, there is no treatment to prevent these adverse effects. Improving the quality of life of the growing population of patients who have received radiation treatment is an important objective.

WBI causes a number of deleterious cellular responses including neuronal dysfunction, blood–brain-barrier dam- age, astrocyte and microglia activation, and infiltration of peripherally derived monocytes. Neuronal in- jury and loss is not the only pathway contributing to cognitive deficits, since activation of non-neuronal cell types also affects overall brain function. We and others have shown that radiation induces infiltration of peripheral myeloid cells that depend on CCR2 signaling and that loss of the cytokine receptor CCR2 prevented the development of radiation-induced long-term cognitive deficits with no influence on neurogenesis. WBI induces up-regulation of pro-inflammatory cytokines and chemokines, including CCL2, which facilitates the recruitment of CCR2+ monocytes into the CNS. Following a single dose of 10 Gy WBI, deletion of CCL2 ameliorates deficits in hippocampal neurogenesis. We demonstrated an increase of monocyte accumulation in the brain, as well as a de- crease of microglia, 7 days following a single dose of 10 Gy WBI [5]. The recruitment of circulating monocytes into the CNS is regulated by the production of a number of soluble chemokines that interact with their cell surface receptors. One of these, colony-stimulating factor 1 receptor (CSF-1R), is a transmembrane tyrosine kinase receptor encoded by the c-fms proto-oncogene. CSF-1/CSF-1R signaling regulates the survival, proliferation, chemotaxis, and differentiation of monocytes and macrophages. Loss of CSF-1R results in complete elimination of microglia and severe monocyte deficits, and mice lacking CSF-1 have markedly reduced numbers of microglia.

Our group, and others, has used a single dose of WBI to model radiation-induced brain injury. However, in clin-ical treatment, virtually all patients receive fractionated brain irradiation with the goal of reducing toxicity to normal tissue. Here, we model the effects of fWBI in young adult mice by using a fractionated treatment paradigm (3 × 3.3 Gy) and explore the outcomes of CSF-1R blockade by PLX5622, analog of another CSF-1R inhibitor PLX3397. In other preclinical studies, PLX5622 has been used to diminish peripheral monocytes/macrophages. Similar to PLX3397, treatment with higher dose of PLX5622 (1200 ppm) depletes microglia in the CNS. Recently, Dagher et al. showed that PLX5622 treatment (300 ppm) ameliorated cognitive deficits in aged Alzheimer's mice. In addition, our preliminary results suggest that lower (300 ppm) and higher (1200 ppm) doses of PLX5622 treatment achieved similar effect in reducing circulating monocytes in the periphery. In light of these results, we treated young adult mice with lower

dose of PLX5622 (300 ppm) and evaluated cognitive outcomes at 1 month after fWBI, the earliest time point we see cognitive deficits in our hands. Our data show that fractionated brain irradiation, similar to single-dose irradiation, results in hippocampal-dependent memory deficits and loss of dendritic spine density in hippocampal granule neurons. Strikingly, CSF-1R blockade by PLX5622 can prevent memory deficits and dendritic spine density loss in mice treated with fWBI. Flow cytometry analyses of myeloid populations following treatment with PLX5622 demonstrate a strong correlation between improved cognitive performance and both decreased microglia numbers and monocyte accumulation in the brain. Using a clinically relevant model and pharmacologic approach, our data show that CSF-1R blockade by PLX5622 can prevent fWBI-induced cognitive deficits in mice by preventing loss of synaptic dendritic spines. These data implicate a new and therapeutically tractable role for infiltrating monocytes and microglia after brain irradiation in loss of synaptic function.

Methods

Compound

Control and PLX5622 (300 ppm formulated in AIN-76A standard chow, Research Diets, Inc.) chows were provided by Plexxikon Inc (Berkeley, CA). Approximately 1.2 mg of PLX5622 was ingested by each mouse per day (calculation based on 4 g/mouse chow daily).

Animal procedures

All animal experiments were conducted in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco (UCSF), following the National Institutes of Health Guidelines for animal care. C57BL/6J male mice were purchased from the Jackson Laboratory. CX3CR1+/GFP/CCR2+/RFP animals were generated by crossing CX3CR1GFP/GFP/CCR2RFP/RFP with C57BL/6J mice as previously described. Starting at 8 weeks old, C57BL/6J mice were treated with PLX5622 or control chow for 21 days. Cranial irradiation started 7 days after drug treatment was initiated. Mice used for Golgi staining were euthanized at the end of the first NOR test (33 days after fWBI) were euthanized and perfused with ice-cold PBS, and the right hemispheres were used for Golgi staining. Mice used for flow cytometry analyses were euthanized at indicated time relative to the last day of fWBI.

Radiation treatment

All the mice were anesthetized with intraperitoneal injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mix and placed 16.3 cm from a cesium-137 source (JL Shepherd & Associates). The eyes and body were shielded by a lead collimator that limited the beam to a width of 1 cm. An extra lead plate was used to block exposure of the trachea. Irradiated groups received 1.65 Gy of irradiation on both side of the head to accumulate 3.3 Gy for each fractionated irradiation. Three fractions were delivered every other day over 5 days to accumulate a total dose of 10 Gy. Sham animals underwent the same procedures without radiation.

Novel object recognition test

All the mice used for the NOR test were housed in a room with reversed light cycle (12 light/12 dark) for at least 2 weeks before tests. Tests were conducted during the dark cycle. The mice were handled 5 min each day for 5 days before habituation. An open arena (30 cm × 30 cm×30 cm; L×W×H) was placed in a dimly lit behavior test room with an overhead camera. The mice were allowed to explore the open arena for 10 min for two consecutive days. On day 3, two identical objects were placed in the arena and mice were allowed to explore for 5 min. On day 4, one of the objects was replaced by a novel object and mice were allowed to explore for 5 min. Trials were recorded by the overhead camera and analyzed by an automatic video tracking system (EthoVision, Noldus) for movement tracking or by manual scoring for exploratory behavior. Exploratory behavior was defined as the animal directing its nose toward an object at a distance less than 2 cm. Objects were secured in the arena with magnets. Arena and objects were wiped with 70 % ethanol between trials to eliminate odor cues.

Delayed matching-to-place dry maze test

Delayed matching-to-place (DMP) dry maze test was used to measure special working memory. Briefly, we used a modified Barnes maze with 40 escape holes (D=5 cm, 16 holes on the outer ring with 50-cm distance to the center, 16 holes on the middle ring with 35-cm distance to the center, and 8 holes on the inner ring with 20-cm distance to the center). All holes were uncovered with the exception of the escape hole, which is covered with a dark escape tube (a black PVC tube). The light was set to approximately 1200 lux, and a

noise (2 kHz, 85 dB) was used during the test. Visual cues were placed on three sides of the maze. Mice were giving four trials each day with interval of 10 min. Mice were placed at the center of the maze under a dark box for 30 s. The trial started when the box was removed and ended when the mice found the escape hole within 90 s. Mice were guided to the escape hole by the experimenter if they could not find it within 90 s. Noise was turned off, and the escape hole was covered immediately after the mice entered. The mice were returned to their home cage after a 10-s delay. The maze surface and the escape tube were cleaned with 70 % ethanol (v/v) after each trial to minimize odor cues. The escape tube was kept at the same location and changed on each test days. Trials were recorded by an overhead camera and analyzed by Ethovision (Ethovision, Noldus).

Metric distance test

Metric distance test was used to measure hippocampal function as previously described by Goodrich-Hunsaker et al. with alterations to suit test in mice. Briefly, the test was performed on two consecutive days during the dark cycle with one habituation phase and 3 trials each day. On day 1, the mice were put into an open arena (30 cm×30 cm×30 cm, L×W×H) for 5 min. On trial 1, the mice were put into the arena with two identical objects placed at a distance of 28 cm to each other for 5 min. On trial 2, the mice were put into the arena with the same setting as trial 1 for 5 min. On trial 3, the distance between the objects was shortened to 14 cm and the mice were allowed to explore for 5 min. There was a 3-min interval between each trial, and the mice were put back into their home cage after each trial. One day 2, all trials were performed with the same setting as day 1 except that the distance between objects on trial 3 was changed to 21 cm. The objects and the arena were cleaned with 70 % (v/V) ethanol after each trial to minimize odor cues. Trials were recorded, and total time exploring the object pair was scored. Total time exploring during trial 1 on each test day was used as baseline of exploration.

Flow cytometry

The mice used for flow cytometry analysis were euthanized and perfused with ice-cold PBS. The brains were removed and immediately placed into ice-cold HBSS. Brain samples were then dissociated using a Neural Tissue Dissociation Kit (P) (Miltenyi Biotec). Dissociated cells were resuspended in 10 ml of 30 %

Percoll solution (Sigma) in an RPMI medium and laid over a 1 ml 70 % Percoll solution layer. After centrifugation at 800g for 30 min at 4 °C, interphase cells were transferred to a new 15-ml Falcon tube and washed with RPMI. Cell pellets were resuspended with FACS buffer (DPBS with 0.5 % BSA fraction V) and blocked with one volume of blocking solution (5 % normal mouse serum, 5 % normal rat serum, 5 % normal rabbit serum, 2 % FBS, and 1 % BSA fraction V in ×1 DPBS) for 30 min and stained for 30 min with fluorophore-conjugated antibodies on ice (CD45-BV711, CD11b-AF700, Ly6C-Pacific Blue, and Ly6G-PE were purchased from BD Pharmingen); 7AAD was used to exclude dead cells. Data were collected on an Aria III sorter (BD) and analyzed with FlowJo v10 software (Tree Star Inc.). At least 20,000 and 200,000 vi- able events were collected from each brain and blood sample, respectively.

Golgi staining

Brain hemispheres stayed in Golgi staining solution (A Modified Golgi-Cox Stain for Neural Cells, Docket No. D4433, Cornell University) for 14 days and were transferred into 30 % sucrose in ×1 PBS overnight at 4 °C. The next day, tissues were transferred into fresh 30 % sucrose solution, protected from light and stored at 4 °C for at least 2 days. Brains were cut into 100 µm sections with a vibratome (VT1000 S, Leica, Wetzlar, Germany), mounted on gelatin pre-treated slides and dried for 2 days. Samples were then developed with the developing solution (A Modified Golgi-Cox Stain for Neural Cells, Docket No. D4433, Cornell University), covered, and dried. Images were taken on a Keyence 7000 system under a ×100 objective lens with immersion oil for hippocampal granule neurons. For each sample, 18–30 images were taken (2–3 images per section, 8–12 sections per mouse, N = 5–6 per treatment group) and used for dendritic spine density analysis. All protrusions from the dendrites were manually counted as spines regardless of morphology. A total length of at least 3000 µm of dendrites was analyzed from each animal using ImageJ (National Institutes of Health).

Statistical analysis

NOR test results are shown as mean percentage of time spent on exploring each object (time exploring familiar or novel object/total exploring time) or mean discrimination index ((time exploring novel object − time exploring familiar object)/total exploring time) ± SEM. Metric distance test results are shown as

percentage of time spent on exploring both objects in trial 1 on each test day. Results for DMP and metric distance tests were analyzed with ordinary two-way ANOVA with Bonferroni's test for post hoc comparisons using day and experimental group as independent factors. Results for NOR test, dendritic spine density analysis, and flow cytometry with PLX5622 and fWBI treatments were analyzed with ordinary two-way ANOVA with Tukey's test for post hoc comparisons using PLX5622 and fWBI as independent factors. Results for temporal analysis of monocyte accumulation were analyzed with one-way ANOVA with Bonferroni's test for post hoc comparisons. All other comparisons between two sets of data were determined using t test. Error bars are shown as mean ± SEM. De- tails of each statistical analysis were described in figure legends. Graphs were analyzed and plotted with Graph- Pad Prism 6 software (GraphPad Software, Inc).

Results

To determine the effects of therapeutically relevant fractionated doses of irradiation on hippocampal-dependent memory, adult male C57BL/ 6J mice were treated with three fractions of 3.3 Gy irradiation every other day to a total dose of 10 Gy and tested with a series of cognition tests at different time points. All mice tolerated fWBI and gained weight normally through the duration of study.

Hippocampal-dependent recognition memory was measured by the NOR test 4 and 13 weeks after the last fraction of radiation. In the 4 weeks' NOR test, the mice in the non-irradiated group showed significantly higher preference toward the novel object while mice in the irradiated group had no preference toward either object. Consistent with these observations, the discrimination index was reduced in the irradiated group ($p < 0.01$) (Fig. 1c). There was no difference in distance traveled or total exploring time between the sham and irradiated groups. We observed similar results in the 13 weeks' NOR test.

The matrix distance test was also used to evaluate hippocampal-dependent memory deficits 8 weeks after fWB. Sham animals could detect both large and small changes in object locations and spent more time exploring the object pair in novel locations while irradiated animals failed to recognize these changes. There was no significant difference in total exploring time during both test days. Working memory was measured by DMP test at 5 and 12 weeks after irradiation. We observed no significant difference in latency

to the first escape hole between sham and irradiated animals at both time points. There was no significant difference in velocity between sham and irradiated groups during the tests. Taken together, these results suggest that fractionated whole- brain radiation impairs hippocampal-dependent memory without affecting working memory in mice.

To investigate the effects of CSF-1R blockade on cognition after fWBI, we treated mice with PLX5622 (300 ppm) in chow for a total of 21 days, starting from 7 days before irradiation. The NOR test was performed 4 weeks after the last fraction of WBI. The bioanalysis of PLX5622 revealed that drug concentrations remained comparable throughout the treatment. As expected, animals on a control diet showed impaired memory after fractionated WBI while PLX5622-treated mice showed no deficits. The discrimination index analyses revealed a significant difference between the sham and irradiated groups on a control diet. Following PLX5622 treatment, there was no significant difference in the discrimination index between the sham and irradiated group. There was no significant difference in total traveled distance or total exploring time among all experiment groups on the test day. These results demonstrate that treatment with PLX5622 for a brief period that precedes and follows irradiation can fully prevent fWBI-induced hippocampal-dependent memory deficits.

CSF-1R blockade protects against dendritic spine loss after fractionated irradiation Hippocampal neurogenesis is important for special and object recognition memory. Given the significant decrement in hippocampal-dependent memory induced by fWBI, we next sought to determine if the cognitive deficits were related to changes at the synaptic level in the hippocampus. We used Golgi staining to quantify dendritic spine density of granule cells in the dentate gyrus (DG). We did not observe significant changes in dendritic spine densities at the end of PLX5622 treatment. However, at 33 days post fWBI, there is a significant reduction of dendritic spine density in irradiated animals. Strikingly, CSF-1R blockade with PLX5622 prevented dendritic spine density loss and resulted in increased dendritic spine density after fWBI. There was no significant difference between the control sham and PLX5622 sham groups.

Fractionated WBI induces acute delayed monocyte accumulation in the brain To determine the temporal dynamics of monocyte accumulation after fWBI, we used CX3CR1+/GFPCCR2+/RFP reporter mice and flow

cytometry analyses of the microglial and mononuclear cell populations. We first measured the numbers of Cd11b+GFP+RFP+ cells, which represent blood-derived monocytes. There was no significant change in this population after each radiation fraction until 3 days after the last fraction, which recovered after day 7. We observed a significant increase in the inflammatory monocyte population (CD45+GFP+RFP+Ly6Chigh cells) at day 3 and day 7 after the last radiation fraction. There was no significant change in the CD45+GFP+RFP+Ly6Clow population. The population expressing only RFP (GFP−RFP+) remained unchanged throughout the experiment. The microglial population, represented by cells expressing only GFP (GFP+RFP−), remained unchanged until 7 days after the last radiation fraction, then declined by 35 %, followed by recovery after day 14. Taken together, these results demonstrate that fractionated cranial irradiation induces CSF-1R blockade results in reduced blood monocytes and prevents monocyte accumulation in the brain after fWBI CSF-1R is expressed in blood monocytes and is important for the survival, maturation, and differentiation of these cells. We analyzed blood samples from WT mice subjected to fWBI, and we observed a 35 % reduction of CD11b+Ly6G−Ly6Chigh monocytes after 7 and 14 days of PLX5622 treatment. Importantly, there were no significant changes in Ly6Clow monocytes or neutrophils. These results suggest that the Ly6Chigh-expressing monocytes are susceptible to CSF-1R blockade while the Ly6Clow- expressing monocytes are not affected. Further analysis of the brain samples revealed that PLX5622 treatment alone did not cause change in the numbers of Ly6Chigh monocytes. Similar to results seen in CX3CR1+/GFPCCR2+/RFP reporter mice, fractionated brain irradiation significantly increased Ly6Chigh monocytes. However, when treated with PLX5622 no significant difference was detected. There was no change in the numbers of CD45+CD11b+Ly6ClowLy6Gneg monocytes or CD45 +CD11b+Ly6C+Ly6G+ neutrophils. These results suggest that the inhibition of monocyte accumulation in the CNS by CSF-1R blockade is possibly due to reduced numbers of circulating monocytes in the blood.

To determine the effects of CSF-1R blockade on micro- glia, we used flow cytometry analyses to compare numbers of microglia between animals treated with PLX5622 and control chows. We observed a significant reduction of microglia during PLX5622 treatment. Compared to the control groups, the PLX5622-treated groups had a 35 % reduction of microglial population at day −4 and 50 % reduction at day 3. Consistent with

these observations, myeloid markers expressed in microglia were also reduced. However, microglia numbers fully recovered 4.5 weeks after PLX5622 withdrawal. These results demonstrate that CSF-1R blockade by PLX5622 causes temporarily loss of microglia in the brain.

Conclusions

In summary, we demonstrate that transient CSF-1R block- ade by PLX5622 prevents fWBI-induced memory loss, which is associated with preservation of dendritic spine density of hippocampal neurons in the mouse model stud- ied. Therefore, targeting CSF1R signaling could provide a possible approach to prevent incidence and severity of irradiation-induced brain injury.