Chronic Mild Hypoxia Delays MOG-Specific Th17 Response and Induces CXCL12 and CXCL13 in EAE.

Esen, N., Serkin, Z., Dore-Duffy, P.

Dept. of Neurology, Wayne State University School of Medicine, Detroit- MI

ABSTRACT

Background: In experimental autoimmune encephalomyelitis (EAE), and animal model of multiple sclerosis (MS), it is well documented that chronic inflammatory activity can lead to compromised delivery of oxygen and glucose at lesion sites. Therapeutic approaches geared to restoring metabolic balance may be useful.

Objectives: Previously, we have reported that in vivo exposure to chronic mild hypoxia (10% oxygen) leads to vascular remodeling that results in amelioration of myelin oligodendrocyte glycoprotein peptide (35-55) (MOG)-mediated EAE. In the present study we further define mechanisms.

Methods: C57BL/6 mice were immunized with MOG and some of them were kept in the hypoxia chambers (day 0) and exposed to 10 % oxygen fr 3 weeks, while the others were kept at normoxic environment. Sham immunized controls were included in both hypoxic and normoxic groups. Animals were sacrificed at pre-clinical and peak disease periods for tissue collection and analysis.

Results: Delayed disease onset, which was significant following exposure to 10% oxygen correlated with the decreased evidence of inflammation in the spinal cord of immunized animals. Decreased evidence of infiltration correlated with decreased numbers of CD4+ T cells in the hypoxic spinal cords and delayed Th17 specific cytokine responses. These observations did not appear to be due to hypoxia-induced changes in the ability of MOG peptide to induce a proliferative response of T-cells in this model. In addition, for the first time, here we presented evidence that exposure to chronic mild hypoxia induced a significant increase in the number of CD11b^{+}CD45^{low} microglia in the hypoxic EAE spinal cords. Chemokines CXCL12 and CXCL13 were significantly induced in the spinal cords of hypoxic EAE mice.

Conclusion: Results suggest that acclimatization to mild hypoxia incites a number of endogenous adaptations that modulate the inflammatory response so that this system can be used to pinpoint possible new therapeutic targets in neurodegenerative diseases.

INTRODUCTION

In Multiple sclerosis , it has been suggested that loss of bioenergetics balance described as “virtual hypoxia” [1, 2] may play a major role in the initiation of axonal degeneration [2, 3], and in turn may induce immune system [4]. Therapeutic intervention aimed at restoration of vascular function and metabolic homeostasis should have a profound effect on tissue plasticity and disease outcome in both MS and EAE. In the brain, maintenance of metabolic homeostasis is the result of a coordinated effort between the cellular constituents of the neurovascular unit [pericytes, endothelial cells, astrocytes and neurons] [5, 6, 7]. These cells make fine tuned regulatory adjustments that promote survival and maintain the balance between oxygen and glucose availability and neuronal metabolic demand. In the adult animal adaptive mechanisms reflect a continuous matching of tissue oxygen with capillary density and are important in tissue repair. We have reported that *in vivo* acclimatization to chronic mild hypoxia comparable to that encountered at moderate high altitude induced endogenous adaptation to the stress stimuli and amelioration of the signs and symptoms of MOG-peptide induced EAE [8]. Exposure of animals to chronic mild hypoxia on the same day of immunization significantly postponed the time required for development of clinical symptoms. When animals were subjected to chronic mild hypoxia following the appearance of clinical symptoms chronic inflammation was suppressed. Evidence of vascular remodeling and decreased infiltration of leukocytes was observed in both protocols. In the present study, we have further investigated the potential mechanisms involved in amelioration of EAE and have focused and questioned whether exposure to chronic mild hypoxia altered immune mechanisms responsible for induction of disease activity in MOG peptide induced EAE.

MATERIALS and METHODS

Immunization: Female C57BL/6 mice (6-8wk) (Jackson laboratories, Bar Harbor, MN) were immunized with (MOG) peptide 35-55, (final concentration=200 µg) emulsified with complete Freund's adjuvant (CFA) containing dead 5 mg/ml Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI) sc. in two injection sites at hind flanks of each mouse. All mice were immunized by intraperitoneal (ip) injection with 150 ml of pertussis toxin (300 ng) (List Biological Laboratories, Campbell, CA) immediately after immunization and 2 days later. Mice were evaluated on a daily basis for overt signs of illness, and clinical signs of EAE using a 5-point scoring system: 0 - no symptoms; 1 – Limp tail; 2 –Limp tail and hind limb weakness; 3 - partial hind limb paralysis; 4 - full hind limb paralysis; 5 – moribund state, or death by EAE.

Exposure to normobaric hypoxia - Animals are housed in the Biospherix Hypoxia chambers calibrated to administer 10% oxygen for varying periods of time. In this study mice were exposed to hypoxia at day 0 (day of immunization). Control normoxic animals were house on the bench top next to the hypoxia chambers under normal oxygen conditions. Following exposure to moderate low oxygen, animals were sacrificed for tissue acquisition at varying times per our approved protocol.

IFNγ and IL-17A ELISPOT Assay: MOG specific response of T cells isolated from immunized mice either exposed to hypoxia or normoxia was also analyzed by ELISpot assay. Briefly splenocytes (2x10^5 cells/ well) were plated into IL-17A or IFN-γ coated 96-well filtration plates (Millipore) and stimulated with MOG peptide overnight at 37˚C in humidified 5% CO2/air incubators. After 24 hours the plates were incubated with the appropriate biotinylated antibodies (all antibodies from ebiosciences) and the cytokine producing cells were visualized with streptavidin-alkaline phosphotase system (Vector Laboratories) and the plates were analyzed using the CTL ImmunoSpot Analyzer (Cellular Technology Limited, Shaker Heights, OH) with ImmunoSpot software. The frequency of cytokine-producing cells was expressed as the difference between the mean number of spots and the mean background for each experiment. The data were presented as the mean ± SEM of each group, n= 3 mice, and performed in triplicate.

Flow cytometry: To determine whether exposing immunized mice to hypoxia influenced the influx of immune cells, T cells, PMNs, macrophages, microglia were quantitated by FACS analysis, as previously described [9] . Following extensive washes and incubation in Fc block (BD Biosciences) to minimize nonspecific antibody binding to FcRs, cells were stained with directly conjugated antibodies for four-color FACS to detect PMNs and macrophages (CD11b$^+$, CD45^{high}), and microglia (CD11b$^+$, CD45$^{low-intermediate}$). To enumerate EAE-associated T cell infiltrates, cells were analyzed using antibodies to CD3 and CD4. Cells were analyzed using a BD FACSAria with compensation set based on the staining of each individual fluorochrome alone and correction for autofluorescence with unstained cells.

ELISA : Spinal cords were homogenized and the protein levels of CXCL12 and CXCL13 in the supernatants were quantified using ELISA kits (both from R&D Systems) following the manufacturer’s instructions (level of sensitivity = 15.6 pg/ml). The results were normalized to the total protein levels.

Immunohistochemistry: At various time points animals were utinized and following tissue perfusion with PBS, spinal cords were flash frozen and dissected with cryostat. Sections following air-drying and fixation were incubated with 1.5 % methanolic H_2O_2 for 10 min and blocked with 5 % normal goat serum in 1 % BSA for 30 min. CXCL13 immunostaining was visualized with Vectostatin ABC and diaminobenzidine substrate kit (both from Vector Labs) according to the manufacturer’s instructions.

RESULTS

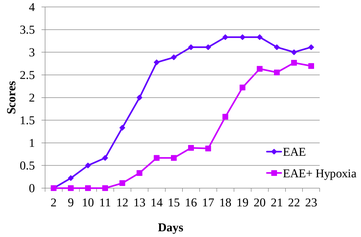

Figure 1: Chronic mild hypoxia delayed onset of EAE. Mice were immunized with MOG35-55 peptide+ CFA. Immediately following immunization, one group of animals was placed in the normobaric hypoxia chambers. EAE only group was left at the normoxic environment all the time. The graph presents the Mean ± SDEV of scores (n=12 mice/ group) including the scores 0 from two independent experiments. The two-way Anova analysis revealed that the difference was statistically significant (p< 0.001).

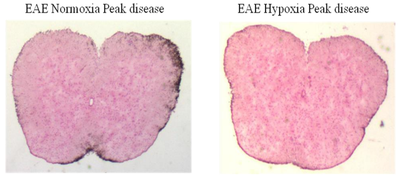

Figure 2: Chronic mild hypoxia decreased the number of cells infiltrated into the spinal cord. Spinal cords from either normoxic or hypoxic EAE mice were dissected and were stained with H&E for the evidence of inflammatory activity.

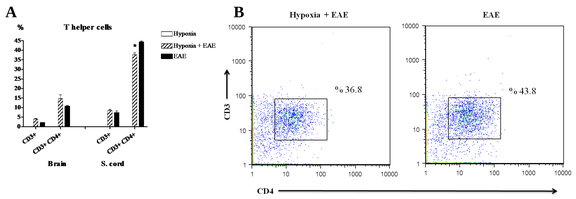

Figure 3: CD4+ T cells were less in the hypoxic EAE spinal cords at Day 14. Immune cells were recovered from mice (2 animals/group/time point) at days 7, and 14 following MOG immunization as described in the Materials and Methods and analyzed by FACS. Since T cells were undetectable at day 7, the graph shows the results from the day 14 samples. Results are presented as the percentage of positive cells for each population (Mean ±SD) (**A**), and as the representative dot plot (**B**). Significant differences between hypoxia exposed and normoxic EAE mice are denoted with asterisks (*, p<0.05).

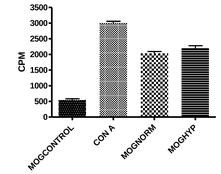

Figure 4: Mild hypoxia did not affect the MOG-induced proliferative capacity of splenocytes. Total splenocytes from EAE induced normoxic and hypoxic mice were stimulated with MOG and Con A for 72 h whereupon [3H]-thymidine incorporation was counted by using a scintillation beta-counter. The results are presented as the Mean ±SD of cpm values from triplicate cultures.

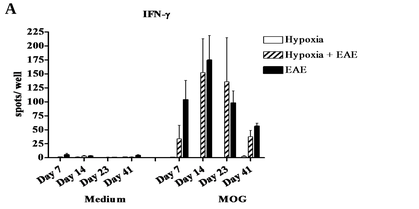

Figure 5: Hypoxia delayed the sensitization of IL-17A producing T cells. Spleens were removed from either hypoxic or norrmoxic MOG-immunized or control mice at days 7, 14, 23, and 41 post-immunization, and single cell suspensions (at 2x10^5 cells/well) were plated into either IFN-γ (**A**) or IL-17A (**B**) coated 96-well filtration plates in the presence of MOG or medium alone. Following the overnight incubation, spots in the wells were analyzed using CTL ImmunoSpot Analyzer and the software. The data were presented as the mean ± SEM of each group, n= 3 mice, and performed in triplicate. Significant differences between hypoxia exposed and normoxic EAE splenocytes are denoted with asterisks (*, p<0.05).

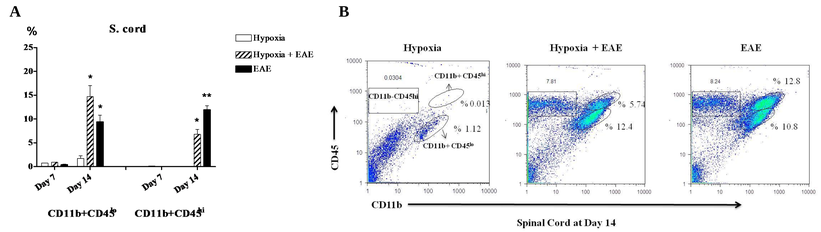

Figure 6: Hypoxia increased the CD11b+CD45lo microglia population in EAE. Immune cells were recovered from mice (2 animal/group/time point) at days 7, and 14 following MOG immunization as described in the Materials and Methods ,and analyzed by FACS. Microglia were differentiated from myeloid cells based on their low level of CD45 expression. The percentage of cells (**A**) and the dot plots (**B**) showed that there were more CD11b+ CD45lo microglia and less CD11b+CD45^{hi} myeloid cell population in EAE mice under hypoxic conditions. The data were presented as the mean ± SEM of each group, n= 2 mice, and performed in duplicate. Significant differences between hypoxia exposed and normoxic EAE cells are denoted with asterisks (*, p<0.05, ** p< 0.001).

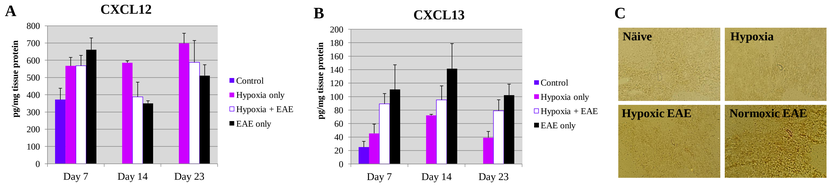

Figure 7: Hypoxia modulated CXCL12 and CXCL13 expression. Spinal cords were homogenized and analyzed for the levels of CXCL12 (**A**) and CXCL13 (**B**) proteins by ELISA as described in the Materials and Methods . The data were presented as the mean ± SEM of each group, n= 3 mice, and performed in duplicate. Significant differences between hypoxia exposed and normoxic EAE splenocytes are denoted with asterisks (*, p<0.05). (**C**) Suppression of CXCL13 expression under hypoxic conditions was also shown by immunohistochemically in the spinal cord sections.

CONCLUSION

Results from this study confirmed that acclimatization to hypoxia has delayed the onset of EAE and lead to less severe clinical symptoms. Exposure to hypoxia along with the angioplastic changes has modulated some immunological changes so that the disease onset has delayed. We think that acclimatization to hypoxia leads to less immune cell infiltration, and delayed IL-17 production of activated T cell in EAE. In addition, the chemokine CXCL13 which has been shown to be deleterious in EAE has found to be attenuated during hypoxic acclimatization. Further understanding of the mechanisms governing angioplasticity and immunomodulation would potentially pinpoint new targets for therapeutic intervention.

ACKNOWLEDGEMENT

This study is supported by the National Multiple Sclerosis Collaborative Research Center Award to Dr. Dore-Duffy.

REFERENCES

[1] Lassmann H. Hypoxia-like tissue injury as a component of multiple sclerosis lesions. J Neurol Sci 2003:206(2):187-91.
[2] Trapp BD, Stys PK. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. Lancet Neurol 2009:8(3):280-91.
[3] Lehnardt S, Schott E, Trimbuch T, Laubisch D, Krueger C, Wulczyn G, et al. A vicious cycle involving release of heat shock protein 60 from injured cells and activation of toll-like receptor 4 mediates neurodegeneration in the CNS. J Neurosci 2008:28(10):2320-31.
[4] Bechtold DA, Smith KJ. Sodium-mediated axonal degeneration in inflammatory demyelinating disease. J Neurol Sci 2005:233(1-2):27-35.
[5] Dore-Duffy P, LaManna JC. Physiologic angiodynamics in the brain. Antioxid Redox Signal 2007:9(9):1363-71.
[6] Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. Pharmacol Rev 2005:57(2):173-85.
[7] Ward NL, LaManna JC. The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol Res 2004:26(8):870-83.
[8] Dore-Duffy P, Wenzel M, Katyshev V, Cleary K. Chronic mild hypoxia ameliorates chronic inflammatory activity in myelin oligodendrocyte glycoprotein (MOG) peptide induced experimental autoimmune encephalomyelitis (EAE). Adv Exp Med Biol 2011:701:165-73.
[9] Esen N, Blakely PK, Rainey-Barger EK, Irani DN. Complexity of the microglial activation pathways that drive innate host responses during lethal alphavirus encephalitis in mice. ASN Neuro 2012:4(4):207-21.