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Exacerbation of ischemic brain injury in hypercholesterolemic mice is associated with pronounced changes in peripheral and cerebral immune responses

Josephine Herz [a,b], Sabine I. Hagen [b], Eileen Bergmüller [b], Pascal Sabellek [b], Joachim R. Göthert [c], Jan Buer [d], Wiebke Hansen [d], Dirk M. Hermann [b], Thorsten R. Doeppner [b,*]

[a] Department of Paediatrics I, Neonatology, University Hospital Essen, University Duisburg-Essen, Essen, Germany
[b] Department of Neurology, University Hospital, Essen, Germany
[c] Department of Hematology, West German Cancer Center, University Hospital of Essen, Essen, Germany
[d] Institute of Medical Microbiology, University Hospital Essen, University Duisburg-Essen, Essen, Germany

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ABSTRACT

Inflammation contributes to ischemic brain injury. However, translation of experimental findings from animal models into clinical trials is still ineffective, since the majority of human stroke studies mainly focus on acute neuroprotection, thereby neglecting inflammatory mechanisms and inflammation-associated co-morbidity factors such as hypercholesterolemia.

Therefore, both wildtype and ApoE$^{-/-}$ mice that exhibit increased serum plasma cholesterol levels fed with normal or high cholesterol diet were exposed to transient middle cerebral artery occlusion. Analysis of peripheral immune responses revealed an ischemia-induced acute leukocytosis in the blood, which was accompanied by enhanced myeloid cell and specifically granulocyte cell counts in the spleen and blood of ApoE$^{-/-}$ mice fed with Western diet. These cellular immune changes were further associated with increased levels of pro-inflammatory cytokines like IL-6 and TNF-α. Moreover, endogenous stroke-induced endothelial activation as well as CXCL-1 and CXCL-2 expression were increased, thus resulting in accelerated leukocyte, particularly granulocyte accumulation, and enhanced ischemic tissue damage. The latter was revealed by larger infarct volumes and increased local DNA fragmentation in ischemic brains of ApoE$^{-/-}$ mice on Western diet. These effects were not observed in wildtype mice on normal or Western diet and in ApoE$^{-/-}$ mice on normal diet. Our data demonstrate that the combination of both ApoE knockout and a high cholesterol diet leads to increased ischemia-induced peripheral and cerebral immune responses, which go along with enhanced cerebral tissue injury. Thus, clinically predisposing conditions related to peripheral inflammation such as hypercholesterolemia should be included in up-coming preclinical stroke research.

Introduction

Inflammation is involved in stroke-induced brain damage (Iadecola and Anrather, 2011). However, difficulties remain with regard to the translation of inflammation-targeting therapeutic approaches from pre-clinical to clinical studies (Endres et al., 2008; Enlimomab Acute Stroke Trial Investigators, 2001; Fisher et al., 2009), which might be due to a neglect of co-morbidities in clinical trial designs.

Stroke induces inflammatory responses in the ischemic brain. Glial and endothelial cell activation lead to immune cell infiltration, exacerbating ischemic tissue damage (Dirnagl et al., 1999). Nevertheless, stroke also provokes peripheral immune responses, which influence secondary lesion growth and thus modulate long-term outcome (Macrez et al., 2011). These systemic immune reactions involve a rapid activation of the immune system with an increased cytokine and chemokine production in the blood and spleen (Emsley et al., 2003; Offner et al., 2006; Smith et al., 2004), followed by secondary immunosuppression and increased susceptibility to bacterial infections (Dirnagl et al., 2007; Meisel et al., 2005; Prass et al., 2003).

There is emerging evidence that inflammatory factors outside the brain markedly influence stroke susceptibility and outcome. Systemic inflammation has been shown to exacerbate brain damage in experimental models of cerebral ischemia (Langdon et al., 2010; McColl et al., 2009). As such, peripherally delivered IL-1β is known to increase tissue damage via mechanisms involving modulation of tight junction proteins and priming of peripheral innate immune cells (McColl et al., 2008). The clinical relevance of the interaction between peripheral inflammation and brain tissue injury is emphasized by epidemiological and clinical studies, demonstrating a close correlation between increased plasma IL-6 levels and stroke severity (Smith et al., 2004).

* Corresponding author at: Department of Neurology, University of Duisburg-Essen Medical School, Hufelandstr. 55, 45147 Essen, Germany. Fax: +49 201 723 1660.
E-mail address: thorsten.doeppner@uk-essen.de (T.R. Doeppner).
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Nevertheless, the majority of pre-clinical studies do not consider co-morbidities such as hypercholesterolemia, which are frequently shown in stroke patients. Hypercholesterolemia does not only promote atherosclerotic plaque development but also induces local inflammation within the vessel wall of peripheral arteries, which is associated with broad systemic immune changes, affecting almost all immune cell subtypes of the innate and adaptive immune system (Drechsler et al., 2010; Smith et al., 2010; Wu et al., 2009).

A few pre-clinical studies emphasize the role of the immune system in the combined setting of hyperlipidemia and stroke, but are hampered due to focusing on single molecules and immune cell subsets (Kim et al., 2008). Until now, there is only limited information about the modulation of peripheral immune responses which cannot be appropriately attributed to effects either of hypercholesterolemia or brain ischemia due to insufficient controls. Finally, most studies do not discriminate how hypercholesterolemia is induced. Thus, dietary and genetic interventions for induction of hypercholesterolemia are often combined without assessing individual effects of each of these interventions.

In a comprehensive approach we investigated ischemia-induced peripheral immune responses and their concomitant reactions in the ischemic brain with special emphasis on immune cell infiltrates, endothelial cell activation and brain tissue injury. Wildtype and ApoE$^{-/-}$ mice fed with a regular or cholesterol-rich food were used in order to mimic different severities of hypercholesterolemia.

Materials and methods

Mice

Experiments were performed in accordance to National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals with local government approval. Male wildtype and ApoE$^{-/-}$ mice which were generated on the same C57BL/6 genetic background were either fed with a normal chow or a cholesterol rich chow, so called Western diet (TD.88137 Adjusted Calories Diet, Harlan Laboratories) for 6 weeks and submitted to left-sided middle cerebral artery occlusion (MCAO) or sham surgery as described below. Animals were randomly attributed to treatment paradigms, and experimenters were blinded to both treatment and data analysis. After 24 and 72 h, mice were sacrificed for quantification of peripheral blood and spleen leukocyte subsets by flow cytometry as well as for analysis of plasma cytokine concentration and splenic mRNA expression ($n = 6–10$ per group and time point). Immunohistochemical analysis of tissue injury, endothelial and microglia activation, and leukocyte infiltration of ischemic brains was performed at 72 h post-ischemia ($n = 6–8$ per group). Quantification of chemokine abundance (CXCL-1 and CXCL-2) in brain tissue protein lysates was performed via enzyme-linked immunosorbent assay (ELISA) measurements at 24 h post-ischemia ($n = 6–8$). For quantification of peripherally derived leukocyte subsets in ischemic brains, an additional set of mice was generated to perform flow cytometry analysis on tissue isolated immune cell suspensions at 72 h post-ischemia ($n = 15–20$ per group). In total 12–16 mice per group were exposed to MCAO and 6–10 to sham operation for analysis at 24 h post-ischemia. For analysis at 72 h post-ischemia a total number of 21–28 mice per group were exposed to MCAO and 6–10 to sham operation. A detailed description of the experimental setup is presented in Suppl. Fig. S1.

Induction of focal cerebral ischemia

Induction of stroke was performed using the intraluminal monofilament occlusion model as described previously (Herz et al., 2012). Briefly, animals were anesthetized with 1% isoflurane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0 °C using a feedback-controlled heating system. Cerebral blood flow was analyzed by laser Doppler flow (LDF) recordings and was monitored during ischemia for up to 15 min after reperfusion onset. For induction of cerebral ischemia, a midline neck incision was made and the left common carotid artery (CCA) and the external carotid artery were isolated and ligated. A nylon monofilament coated with silicon resin was introduced through a small incision into the CCA and advanced to the carotid bifurcation for induction of MCAO. Twenty minutes later, reperfusion was initiated by monofilament removal. In sham-operated animals, all procedures were performed exactly as for MCAO except for occlusion of the MCA. As such, the left CCA and the external carotid artery were isolated and ligated and a small incision was made in the CCA followed by immediate ligation without introducing a filament. After the surgery, wounds were carefully sutured, anesthesia was discontinued and animals were placed back into their cages.

Analysis of post-ischemic tissue injury and immunohistochemistry

For infarct volume measurement and immunohistochemical analysis, mice were transcardially perfused with ice-cold 0.9% NaCl at 72 h post-ischemia. Brains were removed and fresh frozen on dry ice. To determine infarct volumes 20 μm cryostat sections (every 400 μm between +1 mm and −3 mm from bregma) were stained with cresyl violet followed by a computer-based analysis of infarct volumes on scanned sections using Image J software by subtracting the area of the non-lesioned ipsilateral hemisphere from that of the contralateral side. Infarct volume sizes were calculated by integration of the lesioned areas.

For assessment of cell death, endothelial activation and analysis of inflammatory reaction, 20 μm cryostat sections taken at the level of bregma of mice that were transcardially perfused with 0.9% saline were used for immunohistochemistry using published fluorescence and avidin-peroxidase protocols (Bacigaluppi et al., 2009; Herz et al., 2012). Cell death was analyzed via staining of DNA fragmentation using terminal transferase dUTP nick end labeling (TUNEL) according to the manufacturer's protocol (In situ Cell Death Detection Kit, Roche, Switzerland). For the remaining conventional immunohistochemistries, the following primary antibodies were used: rabbit anti-Iba1 (1:100; Wako, Germany), rat anti-CD45 (1:20, BD Biosciences, Germany), biotinylated goat anti-mouse ICAM-1 (1:50, R&D Systems, USA), rat anti-mouse CD31 (1:500, BD Biosciences, Germany), and goat anti-mouse VCAM-1 (1:100, R&D Systems). For endothelial markers, slides were fixed with methanol/acetone followed by incubation with primary antibodies in phosphate buffered saline (PBS) containing 0.2% Tween-100. As secondary antibodies, Cy3-conjugated anti-rat (1:100, Jackson Lab., UK) antibody, streptavidin Alexa Fluor 488 (1:100, Invitrogen, Germany) and Alexa Fluor 488 conjugated anti-goat antibody (Invitrogen, Germany) were used for CD31/ICAM-1 and CD31/VCAM-1 co-stainings, respectively. For Iba-1 stainings, slides were fixed with 2% paraformaldehyde (PFA) followed by incubation with the primary antibody over night at 4 °C in PBS 0.3% Triton-X. Primary antibody binding was visualized by Alexa Fluor 488 anti-rabbit antibody (1:500, Invitrogen, Germany). For avidin-peroxidase staining of CD45, endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 70% methanol in Tris-buffered saline (TBS). Sections were incubated with biotin-conjugated secondary antibodies, immersed with Vectastain® AB kit (Vector Laboratories, USA) and revealed with diamino benzidine tetrahydrochloride (DAB) (Sigma, USA).

Sections were evaluated under a bright light microscope (Axioplan; Zeiss, Germany) connected to a CCD camera (Microfire; AVT Horn, Germany) and a fluorescence microscope (BX 41; Olympus, Germany) connected to a CCD camera (CC12; Olympus).

Cellular injury was analyzed by counting TUNEL$^+$ cells in 6 different regions of interest (ROI, 62.500 μm^2) and mean values were calculated for individual animals. For analysis of CD31/ICAM-1 and CD31/VCAM-1 co-stainings, positively stained CD31 vessels and ICAM-1 or VCAM-1 vessels were counted separately on photographs of the same region of interest (ROI, 125.00 μm^2 each) in 3 defined ROIs within the lesion