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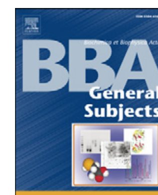

Carbamoyl-PROXYL-enhanced MRI detects very small disruptions in brain vascular permeability induced by dietary cholesterol

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ABSTRACT

Background: Gd-DTPA-enhanced magnetic resonance imaging (MRI) is a conventional method for non-invasive investigation of blood-brain-barrier (BBB) permeability in animal models. It allows the visualization of serious injury to the BBB. We developed a novel approach for detecting very small disruptions in BBB permeability induced by dietary cholesterol by using carbamoyl-PROXYL (CMP) as an MRI contrast probe.
Methods: Mice were separated into two groups: normal diet (ND-mice) and high cholesterol diet (CD-mice). MRI-signal dynamics, plasma cholesterol, matrix metalloproteinase (MMP-9, MMP-2), and the white blood cell profile were analyzed. For the MRI analysis, two regions-of-interest (ROI) were selected: brain (ROI-1) and surrounding area (ROI-2).
Results: In the ROI-2 of ND-mice, CMP- or Gd-enhanced MRI-signal followed typical kinetics with a half-life of signal decay ($\tau_{1/2}$) ~8 or ~15 min, respectively. In CD-mice, the MRI-signal increased continuously without decay.
In the ROI-1 of ND- and CD-mice, MRI-signal enhancement was not detected by Gd-DTPA. In the ROI-1 of ND-mice, CMP-induced MRI-signal enhancement was negligible, while in CD-mice, it was significant ($\tau_{1/2}$>15 min). Hypercholesterolemia increased the plasma levels of MMP-9 and neutrophils.
Conclusions: Hypercholesterolemia increases vascular permeability, which is mediated by MMP-9 and neutrophils.
General significance: Even very small disruptions in brain vascular permeability could be detected by CMP-enhanced MRI but not by Gd-DTPA-enhanced MRI.

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1. Introduction

The blood-brain barrier (BBB) protects the central nervous system from the peripheral circulation. BBB impermeability is maintained by brain microvascular endothelial cells through their tight junctions and the basal lamina, which contain extracellular matrix proteins. BBB disruption occurs in a variety of neurological disorders, including multiple sclerosis, Alzheimer's disease, and AIDS dementia [1–3]. Recent studies have shown that BBB permeability is altered by a high cholesterol diet (CD) in animal models and that peripheral cholesterol metabolism might be affected [4–6]. However, the mechanisms by which dietary cholesterol increases BBB permeability are unknown.

BBB permeability enhancement after ischemia is accompanied by the up-regulation of matrix metalloproteinases (MMPs) [7]. MMP-2 (72-kDa type IV collagenase or gelatinase A) and MMP-9 (92-kDa type IV collagenase or gelatinase B) belong to the subclass of gelatinases. They degrade components of the extracellular matrix in the basal lamina, including collagen, laminin, and zona occludens-1 (ZO-1) in the endothelial tight junctions [8,9]. It has been reported that MMP-2 and MMP-9 are involved in BBB disruption [10–12]. The MMP-9-knockout mice possess reduced BBB permeability after ischemia compared to wild-type mice [13]. Treatment with an MMP inhibitor reduces tissue damage [10,14]. In this context, it can be assumed that MMP-2 and/or MMP-9 could be involved in enhancing the BBB permeability of mice on a CD.

The conventional methods for investigating BBB permeability in animal models are usually invasive or radioactive, such as the detection of serum IgG, exogenous Evans blue dye, or [^{14}C]-sucrose in the brain. The permeability of BBB can be investigated non-invasively by

Abbreviations: BBB, blood-brain barrier; carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; CD, high cholesterol diet; CSF, cerebrospinal fluid; Gd-DTPA, gadolinium-diethylenetriamine pentaacetic acid; MCAO, middle cerebral artery occlusion; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; ND, normal diet; ROI, regions of interest; TIMP, tissue inhibitor of metalloproteinase; ZO-1, zona occludens-1

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magnetic resonance imaging (MRI), using usually gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) as a contrast agent [15]. In addition, Gd-DTPA-enhanced MRI allows for the visualization of severe BBB injury. In this study, we developed a new approach to visualize very small changes in BBB permeability, using 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) as an MRI contrast agent [16]. Gd-DTPA and carbamoyl-PROXYL are water-soluble and non-permeable across the BBB. Gd-DTPA possesses a relatively high molecular weight (742.79), while carbamoyl–PROXYL possesses a relatively low molecular weight (185.24) and low toxicity.

This study aimed to determine whether these probes are appropriate for non-invasive MR imaging of very small disruptions in the BBB induced by dietary cholesterol and to investigate the mechanisms by which BBB permeability is increased by dietary cholesterol.

2. Materials and methods

2.1. Animals

The care, maintenance, and experiments with animals were in accordance with the "Principles of Laboratory Animal Care" (NIH publication number 85–23, revised 1985) and the Guidelines of the Animal Investigation Committee of Chiba University (Chiba, Japan) and National Institute of Radiological Sciences (Chiba, Japan). Our study protocol was approved by the Animal Care and Use Committee of Chiba University.

Male C57Bl/6 mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The mice received a normal diet (ND) (MF; Oriental Yeast Co., Tokyo, Japan) or a CD (ATT6492210; 1.25% [wt/wt] cholesterol, Oriental Yeast Co.) starting at 5 weeks of age. Throughout the experiments, the mice were kept in stainless steel cages with food and water available *ad libitum* and maintained on a 12-h light-dark cycle.

2.2. In vivo MRI measurements

The MRI measurements were performed using a 7.0-Tesla horizontal magnet (Kobelco and Jastec, Kobe, Japan) interfaced to a Bruker Avance-I console (Bruker BioSpin, Rheinstetten, Germany) and controlled with ParaVision 4.0.1 (Bruker BioSpin).

The mice were anesthetized with isoflurane (1.2%, Abbott Japan, Tokyo, Japan) and placed in a body holder (Rapid Biomedical, Rimpar, Germany), stomach side down and head fixed. A polyethylene catheter (PE-10, Becton-Dickinson, NJ, USA) was placed in the tail vein for probe administration. The mice were then placed in the ^{1}H-volume radio-frequency (RF) resonator (Bruker BioSpin) with a surface RF receiver (Rapid Biomedical). Rectal temperature was maintained at 37.0 ± 0.5 °C using an automatic controlled electric heater and monitored using an optical temperature probe (FOT-M and FTI-10, FISO Technology, Quebec, Canada). A respiration sensor (SA Instruments, Edison, NY, USA) was placed on the chest for monitoring.

Before probe administration, five control images of the mouse brain were acquired with the following parameters: T_1-weighted incoherent gradient-echo sequence (fast low-angle shot), repetition time = 75 ms, echo time = 3.2 ms, flip angle = 45 degrees, field of view = 19.2 × 19.2 mm, number of averages = 4, scan time = 19.2 seconds, matrix = 64 × 64, slice thickness = 1.0 mm, and number of slices = 4. We selected the coronal slice orientations with a $300 \times 300 \times 1000\ \mu m^3$ nominal voxel resolution. Ninety-six seconds after starting the MRI scan, with 5 images acquired as pre-administration data, 100 μL of Gd-DTPA (Meglumine Gadopentetate, Bayer HealthCare, Osaka, Japan) (final concentration - 0.125 mmol/kg; Gd-DTPA was dissolved in dimethylsulfoxide) or 100 μL of nitroxide probe (carbamoyl-PROXYL, Sigma-Aldrich, St. Louis, MO, USA) (final concentration - 0.4 mmol/kg; carbamoyl-PROXYL was dissolved in dimethylsulfoxide) was injected into the tail vein. T_1-weighted images were acquired continuously for ~20 min. Mice injected with dimethylsulfoxide only (using the same volume – 100 μL) served as controls. The MRI data were analyzed using the ImageJ (National Institute of Health, Bethesda, MD, USA) software. The regions of interest (ROI) were defined as the entire brain (ROI-1) and the soft tissues surrounding the brain (ROI-2) (Fig. 1). The MRI signal intensity after probe injection was normalized to the average MRI signal intensity before injection (first 5 frames) using the ImageJ software.

2.3. Determination of plasma cholesterol levels

Blood samples were taken from the tail vein in a heparinized microhematocrit tube. The blood samples were centrifuged at 12,000 g for 5 min at room temperature to obtain plasma. Plasma was stored at −80 °C to measure cholesterol and MMP. The total cholesterol levels were determined by a modification of the cholesterol oxidase method by using kit reagents (Wako Pure Chemical Industries, Osaka, Japan). The high-density lipoprotein (HDL) cholesterol levels were measured using the cholesterol oxidase assay on the supernatant from the precipitate of non-HDL lipoproteins with phosphotungstic acid and magnesium chloride (Wako Pure Chemical Industries). The non-HDL cholesterol levels were calculated as the HDL cholesterol levels subtracted from total cholesterol levels.

2.4. Determination of plasma MMP-2 and MMP-9 levels

An enzyme-linked immunosorbent assay (ELISA) was used to determine plasma total MMP-2 and total MMP-9 levels according to the manufacturer's instructions. The MMP-2 ELISA kit (Human/Mouse/Rat MMP-2 [total], Quantikine; R&D Systems, Inc., Minneapolis, MN, USA) detects pro-, active, and tissue inhibitor of metalloproteinase (TIMP)-complexed MMP-2. The MMP-9 ELISA kit (Mouse MMP-9 [total], Quantikine; R&D Systems, Inc.) detects pro-, active, and TIMP-complexed MMP-9. Plasma obtained from mice at 15 weeks of age was analyzed.

2.5. White blood cell differential analysis

Blood samples from mice at 36 weeks of age were taken from the tail vein in an EDTA-treated microhematocrit tube. Blood samples for the time course analysis of the change in white blood cells were taken from the vena cava of mice anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg, Dainippon Sumitomo Pharma Co., Osaka, Japan), and the samples were transferred to an

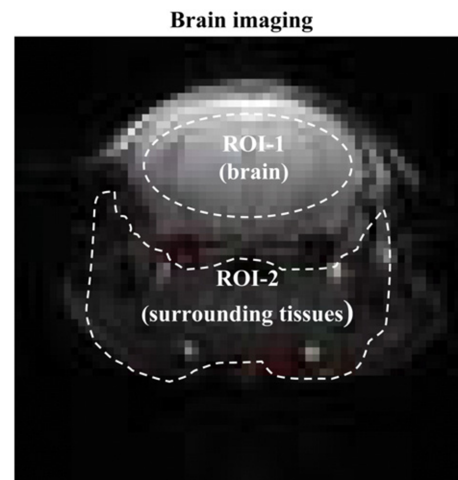

Fig. 1. Definition of the regions of interest (ROI). The ROI-1 and −2 were defined as the brain and the soft tissues surrounding the brain, respectively.

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