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Intravenous Infusion of Nerve Growth Factor–Secreting Monocytes Supports the Survival of Cholinergic Neurons in the Nucleus Basalis of Meynert in Hypercholesterolemia Brown–Norway Rats

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Abstract

The recruitment of monocytes into the brain has been implicated in Alzheimer's disease and recent studies have indicated that monocytes can reduce amyloid plaque burden. Our previous investigations have shown that hypercholesterolemic rats develop cognitive, cholinergic, and blood–brain barrier dysfunction, but do not develop amyloid plaques. This study was designed to evaluate the effects of repeated intravenous (i.v.) infusion (via the dorsal penile vein) of primary monocytes on cognition, the cholinergic system, and cortical cytokine levels in hypercholesterolemia Brown–Norway rats. In addition, we also transduced the monocytes with nerve growth factor (NGF) to evaluate whether these cells could be used to deliver a neuroprotective agent to the brain. Our results indicate that repeated i.v. infused monocytes migrate into the brains of hypercholesterolemic rats; however, this migration does not translate into marked effects on learning. Animals receiving NGF-loaded monocytes demonstrate slightly improved learning and significantly elevated cholinergic neuron staining compared to treatment with monocytes alone. Furthermore, our data indicate that repeated infusion of monocytes does not lead to elevated cytokine secretion, indicating that no inflammatory response is induced. This study provides an experimental attempt to evaluate the effects of blood-derived primary monocytes in hypercholesterolemia rats.

Keywords

monocytes; brain; hypercholesterolemia; NGF

Recent evidence indicates that monocytic cells may have important implications and therapeutic potential in the treatment of Alzheimer's disease (AD; Malm et al., 2010). These studies show that monocytes or monocyte-derived cells can phagocytose β -amyloid (A β) peptides, migrate to amyloid plaques, and reduce plaque burden (Stalder et al., 2005; Simard et al., 2006; El Khoury et al., 2007; Lebson et al., 2010).

Monocytes are bone marrow-derived pluripotent cells that circulate throughout the bloodstream before entering organs and differentiating into tissue-specific macrophages or dendritic cells to aid in host defense and tissue repair. Previous investigations suggest that neurodegenerative or inflammatory stimuli can lead to the transmigration of blood-derived monocytes across the blood–brain barrier (BBB) and ultimately their engraftment into the central nervous system (CNS; D’Mello et al., 2009; Rezai-Zadeh et al., 2011). The migration of these cells into the CNS has been implicated in a number of neurological disorders (Mildner et al., 2011; Rezai-Zadeh et al., 2011); however, their role in disease pathogenesis remains unclear. Indeed, if these cells are to be used for therapeutic purposes, more studies are needed to determine what role their recruitment into the CNS may play in further inflammatory insult and disease exacerbation (Yong and Rivest, 2009). It also remains unknown whether monocyte recruitment can occur during signs of early pathological disruptions, including BBB leakage without extensive amyloid plaque deposition.

Hypercholesterolemia, or elevated cholesterol, is associated with learning deficits and AD-related neuropathological changes (Sparks et al., 2000; Ghribi et al., 2006; Granholm et al., 2008; Thirumangalakudi et al., 2008; Schreurs, 2010). Some epidemiological studies show that elevated cholesterol levels can increase the risk of dementia (Haag et al., 2008; Reiss and Voloshyna, 2012). More convincingly, a recent genome-wide analysis has shown that mutations in genes associated with cholesterol metabolism lead to increased susceptibility to AD (Jones et al., 2010). We have previously demonstrated that hypercholesterolemic Sprague Dawley rats (induced by high-cholesterol diet) exhibit prominent cognitive and cholinergic deficits (Ullrich et al., 2010). Overwhelming evidence has demonstrated that a cholesterol-enriched diet can lead to A β accumulation (for review see Shobab et al., 2005; Maulik et al., 2013). Although our model does not show amyloid plaques, these rats do exhibit elevated levels of A β and tau as well as disruption of the BBB (Ullrich et al., 2010).

The aim of this study was to evaluate the effects of repeated intravenous (i.v.) infusion of primary monocytes in hypercholesterolemia inbred brown-Norway rats. This model provides a unique opportunity to study whether monocytes can serve as delivery vehicles in the presence of a compromised BBB, but in the absence of amyloid plaques. In particular, we are interested in whether monocytes secreting nerve growth factor (NGF), a large protein responsible for the survival and function of cholinergic neurons, could counteract any of the cognitive and cholinergic deficits associated with hypercholesterolemia.

MATERIALS AND METHODS

Animals and Diet

Male Brown-Norway rats (aged 6 months) were housed in the animal department of Innsbruck Medical University with free access to food and water and a 12-hr/12-hr light/dark cycle. All animals were fed with a special diet supplemented with 5% cholesterol for 5 months. The diet contained the following ingredients: 450 g/kg cornstarch, 140 g/kg casein, 155 g/kg maltodextrin, 100 g/kg sucrose, 40 g/kg soybean oil, 50 g/kg fiber, 35 g/kg mineral mix, 1.8 g/kg L-cystine, 1.4 g/kg choline chloride, 0.0008 g/kg butylhydroxytoluol, 10 g/kg vitamin mix (without folic acid), 1 g/kg chocolate aroma, 0.002 g/kg folic acid, and 50 g/kg

cholesterol (Ssniff Special Diet GmbH, Soest, Germany). All animal experiments were approved by the Austrian Ministry of Science and Research (BMWF-66.011/0044-II/3b/2011 and BMWF-66.011/0059-II/3b/2011) and conformed to the Austrian guidelines on animal welfare and experimentation. All possible steps were taken to reduce suffering and the number of animals used in this study.

Isolation of Primary Rat Monocytes

Primary rat monocytes were freshly isolated as we previously described, with some modifications (Humpel, 2008; Böttger et al., 2010; Hohsfield and Humpel, 2010; Hohsfield et al., 2013a,b). Brown-Norway rats (250 g; Himberg, Austria) were anaesthetized by an intraperitoneal injection of 40 mg/kg body weight thiopental (Sandoz, Kundl, Austria) and perfused with 500 ml of 4°C-prechilled 10 mM phosphate-buffer saline (PBS)/2.7 mM EDTA/25 mg/ml heparin, pH 7.3, through the left ventricle. The collected effluent was centrifuged at 550g for 10 min at 4°C. The cell pellet was resuspended in 50 ml of 10 mM PBS/1% bovine serum albumin (BSA; Serva, Heidelberg, Germany)/2.7 mM EDTA, pH 7.3, and carefully overlaid on a Percoll working solution. After centrifugation at 500g for 30 min at 4°C, peripheral blood mononuclear cells (PBMC) were harvested from the interphase. PBMC were then washed once with 50 ml PBS, and $\sim 20 \times 10^6$ PBMC were resuspended in 100 μ l of PBS/BSA/EDTA. Monocytes were purified from PBMC by negative magnetic selection. PBMC were incubated in a cocktail consisting of four different purified anti-rat monoclonal antibodies (20 μ g of each: CD8a [clone OX-8], CD5 [clone OX-19], CD45RA [clone OX-33], PAN T [clone OX-52]; all from Cedarlane Laboratories, Szabo, Austria) for 10 min at 4°C, with shaking. PBMC were washed once with PBS and resuspended in 100 μ l of PBS/BSA/EDTA and 40 μ l of MACS Goat Anti-Mouse IgG Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC were incubated for 15 min at 4°C on a shaker and after incubation were washed once with PBS. The cells were resuspended in 1,000 μ l PBS/BSA/EDTA and then applied to a MS-MACS column fixed to a strong magnet. The purified monocytes were centrifuged and pooled for further experiments. Approximately 10×10^6 cells were isolated from one adult rat. The described isolation procedure yields approximately 90–95% CD68-positive monocytes (Moser and Humpel, 2007; Humpel, 2008). During this preparation, monocytes were counted with a Coulter Z series cell counter (Fischerlehner & Kucera, Innsbruck, Austria) in a range from 5.5 to 10 μ m.

PKH67 Labeling of Monocytes

To visualize injected monocytes, freshly isolated rat monocytic cells were labeled with PKH67 Green Fluorescent Cell Linker (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' instructions as previously described (Böttger et al., 2010). Prior to Biporter protein delivery, monocytes (max. 2×10^7) were pelleted and then subsequently incubated with 4 μ M PKH67 dye in 1 ml diluent C. After 5 min of incubation, cells were washed twice with serum-containing medium and then resuspended in culture medium for further use.

Generation of NGF-Secreting Monocytes

Primary rat monocytes were loaded with recombinant NGF (Sigma) using the Bioporter Protein Transfer Reagent (QuickEase) as described previously (Böttger et al., 2010; Hohsfield et al., 2013b). Briefly, two vials of Bioporter reagent were prepared: 2.5 μ l of Bioporter reagent was mixed with or without (control) 100 ng of recombinant NGF in 100 μ l of sterile PBS (pH 7.4) and then incubated with the reagent for 5 min at 20°C. After incubation, 2.5×10^6 monocytes were resuspended in 400 μ l OptiMEM and added to two vials, each containing diluted Bioporter reagent. The cells were then incubated for 3 hr rotating at 10 rpm (Pluriplex rotor). After incubation, cells were centrifuged and dissolved in 500 μ l of OptiMEM. The cells were then pooled (5×10^6 cells), placed into a new Eppendorf tube, and washed three times with OptiMEM. After washes, the cell pellet ($\sim 5 \times 10^6$ cells) was resuspended in 1.5 ml of prewarmed heparinized (100 U/ml) saline (0.9% NaCl) solution.

Intravenous Infusion of Monocytes

After 3 months of cholesterol diet, male Brown-Norway rats began receiving i.v. injections of primary monocytes (obtained from other male brown-Norway rats), either loaded or not with NGF, weekly for 2 months. The rats were anesthetized by an intraperitoneal injection of thiopental (400 μ l/100 g; 12.5 mg/ml). After this, 100 μ l heparinized saline with or without 5×10^6 monocytic cells was administered via the dorsal penile vein. Animals receiving saline infusion alone served as negative controls. As a control, monocytes were also acutely infused and analyzed 24 hr after a single injection.

Evaluation of Spatial Learning With an Eight-Arm Radial Maze

Five months after the start of the experiment (1 week following the last of i.v. injection), spatial learning and memory were assessed by using a partially baited eight-arm radial maze (PanLab, Spain) as previously described (Pirchl et al., 2010; Ullrich et al., 2010). Prior to behavioral testing, all animals were placed on a restricted diet to increase motivation (2 g food pellets/animal/day for 3 days) and subsequently acclimatized to the maze and experimental setup. Spatial learning and memory performance was tested during five consecutive daily sessions (S) consisting of five trials per day. Four arms were baited with food pellets (chocolate cereal). Once all baits had been found or the 10-min time limit exceeded, the trial was ended. To exclude olfactory interference, crumbs from the baits were placed under each food cup of all arms and after each trial the maze apparatus was cleaned with 70% ethanol. The behavioral test was automatically controlled and monitored by a computer equipped with Mazesoft 8.1.9 software. Quantification of cognitive performance (spatial learning and memory) was performed as previously described (Pirchl et al., 2010, 2012) by calculating the percentage of correct visits (choices) as a measure for enhanced learning.

Tissue Collection and Extracts

Three days after the last spatial learning test (12 days following last i.v. injection) or 24 hr after a single acute injection, animals were anesthetized by subcutaneous injection of sodium thiopental (12.5 mg/ml, 1 ml). Liver, lung, and spleen were removed, placed on a cork, and

immediately frozen in a CO₂ stream. After this, the brain was removed, and the frontal cortex was dissected from the left hemisphere and frozen at –80°C (for inflammatory markers and NGF evaluation). The right brain hemisphere was postfixed overnight in 4% paraformaldehyde (PFA), stored in 20% sucrose/sodium azide solution and then frozen in a CO₂ stream. Frozen cortical tissue was dissolved in 100 µl ice-cold PBS containing a protease inhibitor cocktail (P-8340; Sigma; for NGF and inflammatory markers), homogenized using an ultrasonic device (Hielscher Ultrasonic Processor), and then centrifuged at 16,000g for 10 min at 4°C. The supernatant was collected, and samples were stored at –80°C until further use. Total protein was determined by Bradford protein assay.

NGF ELISA

Cortical NGF levels were measured in cortex extracts using an indirect sandwich enzyme-linked immunosorbent assay (ELISA; Promega, Madison, WI) as previously described (Zassler and Humpel, 2006; Böttger et al., 2010). Briefly, 96-well ELISA plates were coated with a monoclonal anti-NGF antibody diluted in carbonate coating buffer (pH 9.7) and incubated overnight at 4°C. Plates were then blocked using 1× blocking buffer (200 µl/well) for 1 hr at 20°C. After incubation, NGF standards (0–100 pg/well) or tissue extracts were added to plates and incubated for 6 hr at 20°C. After washes, plates were incubated with a monoclonal rat anti-NGF antibody overnight at 4°C. After a second round of washes, the plate was incubated with horseradish peroxidase-conjugated anti-rat antibody (1:4,000) for 2 hr at 20°C. Plates were again washed and incubated with enzyme substrate (TMB One solution; Promega) for 15 min at 20°C. The enzyme reaction was stopped by adding 1 N HCl, and the absorbance was measured at 450 nm with a microplate ELISA reader (Zenyth 3100 ELISA reader or LambdaE, MWG). Sample values were calculated from a standard curve in the linear range.

Inflammatory Markers Measured by ELISA

The detection of inflammatory proteins (monocyte chemotactic protein-1 [MCP-1], macrophage inflammatory protein-2 [MIP-2], tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β]) was performed using the Thermo Scientific SearchLight Protein Array technology (THP Medical Products, Vienna, Austria) according to the manufacturer's recommendations (Bio-Rad, Hercules, CA) and as previously described (Hohsfield and Humpel, 2010). Briefly, cell extracts (diluted 1:2 in diluent) or calibrated standards were added to coated wells of the provided plate and incubated for 3 hr. After washing, the biotinylated antibodies were added and after 30 min of incubation, the wells were washed again and, incubated with streptavidin-horseradish peroxidase conjugate. After the final washing step, the SuperSignal Chemiluminescent Substrate was added. All incubation steps were carried out on a shaker at 20°C. The luminescent signal was detected with a compatible CCD imaging and analysis system, and the absorbance was measured at 450 nm. The concentration of each sample was quantified by comparing the spot intensities with the corresponding standard curves calculated from the standard sample results in SearchLight Array Analyst software.

Evaluation of Fluorescent Cell Deposition in Organs

To evaluate the number of fluorescent cells in the brain and organs, tissues were collected, mounted onto a cork and frozen under a stream of CO₂. With a cryostat, the tissue was cut into 40-μm sections and mounted with PBS. The sections were then rapidly visualized under the fluorescence microscope (Leica DMIRB). Fluorescent microscopic images were obtained with Improvision Openlab 4.0.4 imaging software, captured with a fluorescein isothiocyanate (FITC) filter. To count fluorescently labeled monocytes, three to five sections of each organ or brain (cortex and hippocampus) were evaluated with FITC filter L5 (excitation 480/40, emission 527/30) and with TRITC filter Y3 (excitation 535/50, emission 610/75) under the 20× objective. Sections from animals that did not receive monocyte injections were also evaluated and served as negative controls. Some sections were immunohistochemically counterstained with laminin.

Immunohistochemistry

Immunohistochemistry was performed as previously described under free-floating conditions (Ullrich et al., 2010; Pirchl et al., 2012). After PFA fixation and sucrose immersion, the right brain hemisphere was placed on a cork, frozen in a CO₂ stream, and subsequently sectioned into 40-μm sections with a cryostat (Leica CM 1950). The brain sections were then washed with PBS and incubated in PBS/0.1% Triton (T-PBS) for 30 min at 20°C, with shaking. To quench endogenous peroxidase, sections were treated with PBS/1% H₂O₂/5% methanol. After incubation, the sections were then blocked in T-PBS/20% horse serum (Gibco Invitrogen)/0.2% BSA (Serva) for 30 min at 20°C, with shaking. After blocking, brain sections were incubated with primary antibodies against choline acetyltransferase (ChAT; 1:750; AB144P; Chemicon) or laminin (1:500; Sigma) in T-PBS/0.2% BSA overnight at 20°C. The sections were then washed and incubated with secondary antibody Alexa Fluor 488 anti-rabbit (laminin; 1:200; Molecular Probes Invitrogen, Eugene, OR) or biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) in T-PBS/0.2% BSA for 1 hr at 20°C, with shaking. For fluorescence microscopy, brain sections were then mounted with a glass coverslip and Vectashield mounting medium (Vector Laboratories) and visualized under the microscope. For immunoperoxidase staining, sections were rinsed with PBS and incubated in avidin-biotin complex solution (Elite ABC kit; Vector Laboratories) for 1 hr at 20°C, with shaking. Finally, the sections were washed with 50 mM Tris-buffered saline (TBS) and incubated in 0.5 mg/ml 3,3'-diaminobenzidine (DAB; Sigma)/TBS/0.003% H₂O₂ at 20°C in dark until signal was detected. Once DAB staining was visible, the reaction was stopped by adding TBS to the sections. The brain sections were rinsed with TBS, mounted onto glass slides, coverslipped with Entellan (Merck, Darmstadt, Germany), and then evaluated by microscopy. Images were captured with an Olympus BX61 (ProgRes C14 camera) microscope equipped with Openlab 5.5.0 imaging software. Quantification of ChAT-positive neurons in the nucleus basalis of Meynert (NBM) was performed by a blind observer and evaluated in four sections chosen at -0.8, -1.5, -2.2, and -3.0 mm from bregma according to a rat brain atlas (Paxinos and Watson, 1986).

Statistical Analyses

All data are given as mean $\pm$ SEM (n = independent experiments). Multistatistical analysis was obtained by one-way ANOVA with Fisher LSD post hoc test for comparison within groups or the standard unpaired two-tailed Student's t -test for comparison between two groups. $P < 0.05$ was considered the minimum level of statistical significance.

RESULTS

Effects of NGF-Secreting Monocyte Infusions on Cholinergic Neurons

The majority of cholinergic neurons that innervate the neocortex are located in the NBM (Fig. 1A,B) playing an important role in learning and memory. These neurons have been shown to degenerate during neurodegenerative disease. Repeated monocyte infusion (8 weeks, once per week) alone did not result in any significant changes in ChAT-positive staining compared with vehicle infusions (Fig. 1C). However, we observed a significant elevation in the number of ChAT-positive cholinergic neurons in the NBM (located between 1.5 and 2.2 mm from Bregma) in animals infused with NGF-secreting monocytes compared with animals infused with monocytes alone (Fig. 1D). Furthermore, analysis of NGF in cortical extracts revealed a tendency for an increase in NGF content after repeated infusions of NGF-secreting monocytes (Table I), compared with vehicle or monocytes alone (Table I).

Effects of NGF-Secreting Monocyte Infusions on Cognitive Function

In this study, an eight-arm radial maze was used to evaluate spatial learning after 2 months of weekly infusions. We observed that i.v. infusion of monocytes alone slightly reduced cognitive performance in spatial learning compared with vehicle-infused animals in session 3 (Fig. 2A). On the other hand, animals receiving infusions of NGF-secreting monocytes exhibited slight improvement in spatial learning compared with animals receiving naïve monocytes in session 3 (Fig. 2B). No significant difference in total memory errors between groups was observed (data not shown).

Monocyte Deposition in the Brain and Peripheral Organs

Monocytes appeared to migrate into the cortex of repeatedly infused animals (Fig. 3A). Some of these fluorescent cells were associated with laminin-positive blood vessels (Fig. 3B). Fluorescent (PKH67-positive) cell deposition was most pronounced (approximately 200 cells/mm² of tissue) in the lungs (Fig. 3C) and the liver and spleen (approximately 50 cells/mm² of tissue); however, few or no cells were observed in the brain of acute 24-hr-treated animals (Fig. 3C). Figure 3D shows the deposition of PKH67-positive cells in cortical and hippocampal regions 12 days after the last chronic infusion (2 months, weekly), but does not distinguish between perivascular and parenchymal cells. Interestingly, animals receiving NGF-secreting monocytes exhibited a threefold increase in the number of PKH67-positive cells compared with those receiving naïve monocytes.

Effects of Monocyte Infusions on Cortical Inflammatory Markers

To evaluate possible inflammatory response effects of monocyte infusions, we also extracted cortical tissue from the animals and measured inflammatory markers. The

expression of all inflammatory markers (MCP-1, IL-1 β , TNF- α , MIP-2) did not change significantly between the animal groups (Table I).

DISCUSSION

The aim of the present study was to evaluate whether intravenously infused monocytes could prove beneficial or detrimental to cognition, the cholinergic system, or the inflammatory cytokine status of the rat brain. We were also interested in determining whether NGF-loaded monocytes could improve any behavioral or cholinergic deficits associated with high-cholesterol diet. To examine the effects of i.v. infusion of monocytes, animals were given weekly i.v. injections containing either 5×10^6 monocytes or heparinized saline (vehicle) via the dorsal penile vein for 2 months. We show that monocytes migrate into the brain and that NGF-secreting monocytes support cholinergic neurons of the NBM.

Hypercholesterolemia Rat Model

Increasing evidence indicates that hypercholesterolemia and cholesterol metabolism are associated with the progression of cognitive impairment and neurodegeneration in AD (Puglielli et al., 2003; Maulik et al., 2013). Alongside enhanced A β deposition, high-cholesterol diet also leads to the loss of BBB integrity and enhanced expression of inflammatory markers (Sparks et al., 2000; Ghribi et al., 2006; Schreurs et al., 2012). Our previous investigations have shown that diet-induced hypercholesterolemia in male Sprague Dawley rats results in AD-like deficits including: impaired learning and memory, reduced survival of cholinergic neurons in the NBM, reduced cortical acetylcholine, elevated inflammatory factors, elevated cortical A β , tau, and phospho-tau 181, and increased small cortical bleedings (Ullrich et al., 2010). In the present study, we used Brown-Norway rats to minimize donor–recipient immune responses. The effects of cholesterol and anesthesia as well as repeated infusion in this rat strain have been recently reported in detail (Hohsfield et al., 2013a).

Monocyte Infiltration Into the Brain of Hypercholesterolemia Rats

Several investigations provide evidence that monocytes from the bloodstream migrate into the CNS during neurodegenerative disease (Mildner et al., 2009; D'Mello et al., 2009; Rezai-Zadeh et al., 2011). Recent evidence has indicated that monocytes can infiltrate the brain and home directly to sites of amyloid deposition (Stalder et al., 2005; Simard et al., 2006; Lebson et al., 2010) and are capable of eliminating and preventing the formation of amyloid deposits (Simard et al., 2006; Lebson et al., 2010). In addition, studies have shown that these blood-derived cells produce neuroreparative factors and down-regulate the release of proinflammatory factors (Schwartz and Shechter, 2010). These converging results elucidate a beneficial role of monocyte recruitment into the AD brain (Rezai-Zadeh et al., 2011).

To track cell migration in our rat Brown-Norway strain, monocytes were fluorescently labeled prior to their administration into animals using the PKH67 cell linker dye. For evaluation purposes, all FITC-positive cells were compared against the TRITC channel to

rule out any misidentified debris or nonspecific labeling. This was necessary because no specific monocyte markers that can distinguish monocytes from resident macrophages/microglia were available and all organs displayed some level of autofluorescence. Monocytes have a relatively short half-life (Lebson et al., 2010), so we also evaluated monocyte organ deposition 24 hr after a single monocyte infusion. We worried that monocytes would be undetectable after the 12 days needed for behavioral testing. Our short assay for monocyte migration revealed that monocytes are present in the peripheral tissues (liver, spleen, and lungs), but do not enter the brain 24 hr after monocyte infusion in cholesterol-fed rats. These findings support previous data demonstrating the high level of monocyte infiltration in peripheral organs compared to that seen in the brain of nontransgenic mice (Lebson et al., 2010). After repeated i.v. injection of monocytes for 2 months, on the other hand, we did observe deposition of fluorescent cells in the cortex and hippocampus of hypercholesterolemic rats. We were able to observe some associations of fluorescent-positive cells in the brain with laminin-positive blood vessels, suggesting that the fluorescent cells that we observed were cells entering from the bloodstream. However, we cannot confirm how many cells have entered the parenchymal area vs. how many have remained in the perivascular space. Another possible explanation for a decreased amount of migrated cells could be the long incubation between the last monocyte infusion and the time required for behavioral testing, resulting in a lack of cells or PKH67 dye for adequate visualization. Because the interval between infusions was more than the half-life of monocytes, it is possible that the accumulation of cells was not sufficient enough to result in a significant amount of cell deposition in the brain.

Effects of NGF-Secreting Monocytes in the Brains of Hypercholesterolemic Rats

In AD, patients show significant deficits in cholinergic neurons in the NBM of the basal forebrain, a loss strongly correlated with cognitive impairment (Francis, 2005). Extensive evidence has shown that NGF is the most potent molecule in promoting cholinergic neuron survival and function. Despite demonstrated success in counteracting cholinergic decline (Aloe et al., 2012), NGF-based therapies have remained hampered by the molecule's inability to cross the BBB and by the adverse side effects elicited from its nonspecific distribution (e.g., weight loss, severe back pain). Thus, recent clinical trials have turned to delivery methods that seek to target specific brain regions, avoiding widespread NGF exposure. Thus, we and others have proposed the use of noninvasive measures to deliver NGF to the brain effectively (Covaceuszach et al., 2009; Böttger et al., 2010; Hohsfield et al., 2013b).

We have previously demonstrated that primary rat monocytes can be successfully loaded with NGF using Bioporter protein delivery technology. These cells secrete approximately 0.8 ng/ml NGF per 10^6 cells per 24 hr, with no apparent changes to phenotype or function (Hohsfield et al., 2013b). In the present study, we used the same method to generate NGF-secreting monocytes for i.v. infusions in vivo. We were interested in determining whether NGF-loaded monocytes could exert a beneficial effect on cognition and neuropathology in cognitively impaired hypercholesterolemic rats. Here, we show that NGF-secreting monocytes counteract the decline of cholinergic neurons without marked effects on learning. We demonstrate that injection of these NGF-secreting cells results in significantly improved

survival of cholinergic neurons in the NBM compared with animals receiving monocytes alone, however, this treatment does not have a dramatic effect on cortical NGF. It is possible that longer treatment is needed in order to observe a more pronounced effect on cognitive function and neuroprotection.

Monocytes exhibit very short life spans, thus it is also possible that treatment is required more often than once per week. This is a limitation of the current study, and future studies should consider a shorter interval of injection than the cell life span in order to achieve a cumulative and significant effect. On the other hand, it is also possible that at the time of cortical tissue collection NGF had been taken up, metabolized or bound to other molecules and cells. Such a high metabolic turnover may be caused by cellular uptake and subsequent retrograde transport to cell bodies. Thus, these unchanged NGF levels may simply reflect the technical limitations of our tissue collection. However, we believe that these results are encouraging. Repeated i.v. injection of monocytes did not exacerbate cholesterol-induced cognitive impairment or enhance proinflammatory marker levels. These results suggest that the repeated infusion of monocytes does not lead to further inflammation or disease aggravation in the brain. However, further studies are needed to better characterize this neuroinflammation; our data offers only a small glimpse of some of the mediators involved in the inflammatory response. Specifically, it would be interesting to evaluate the effects of monocyte infusion on microglia phenotype and function as well as the infiltration of other leukocyte subtypes into the brain.

Overall, the present study outlines the limitations and difficulties of infusing monocytes into hypercholesterolemic rats. However, it also provides evidence that i.v. infusion of NGF-secreting monocytes can result in improved cholinergic neuron survival without causing significant changes in cognitive impairment or proinflammatory cytokines/chemokines. These findings agree with previous reports indicating minor benefits from CNS infiltration of blood-derived monocytic cells alone, specifically in slowing A β deposition in AD (Lebson et al., 2010) and exhibiting anti-inflammatory properties in spinal cord injury (Shechter et al., 2009).

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Fig. 1.

Effects of weekly monocyte infusions on choline acetyltransferase-positive neurons in the nucleus basalis of Meynert. Male brown-Norway rats were fed a 5% cholesterol diet for 5 months; during the last 2 months these animals received either weekly i.v. injections of saline (vehicle, circles, $n = 10$; C), primary monocytes alone (pM-(-), solid triangles, $n = 7$; C,D), or monocytes loaded with NGF (pM-NGF, open triangles, $n = 7$; D). Twelve days after the last injection, brains were collected, sectioned ($40\ \mu\text{m}$), and stained for choline acetyltransferase (ChAT), a marker for cholinergic neurons (A,B). The number of ChAT-positive neurons was quantified in the nucleus basalis of Meynert located between 0.8 and 3.0 mm from bregma under $10\times$ or $20\times$ objective by a blind observer. Three to six brain sections were evaluated per animal. Values are mean $\pm$ SEM ChAT-positive neurons/brain section. Statistical analysis was performed using an unpaired Student's t -test (* $P < 0.05$, ** $P < 0.01$). Scale bar = $200\ \mu\text{m}$ in A; $30\ \mu\text{m}$ for B.

Fig. 2.

Effects of weekly monocyte i.v. infusions on the cognitive performance of cholesterol-fed brown-Norway rats. Male brown-Norway rats were fed a 5% cholesterol diet for 5 months; during the last 2 months, these animals received either weekly i.v. injections of saline (vehicle, circles, $n = 10$; A), primary monocytes alone (pM-(-), solid triangles, $n = 7$; A,B), or monocytes loaded with NGF (pM-NGF, open triangles, $n = 7$; B). After injections, animals were evaluated for cognitive performance in an eight-arm radial maze. Cognitive learning performance was quantified as the percentage of correct visits made to baited arms. Values are mean $\pm$ SEM% correct visits. Statistical analysis was performed by unpaired Student's t -test ($*P < 0.05$).

Fig. 3.

Migration of fluorescently labeled monocytes into peripheral and brain tissue. To evaluate whether monocytes migrated into the brain after infusions, brains and organs (lung, liver, spleen) were collected 12 days following chronic (once per week for 2 months) injection (A,B,D) or following a single injection after 24 hr (C). By immunohistochemical evaluation, PKH67⁺ monocytes were observed in the cortex (A) closely associated with laminin-positive vessels (Alexa Fluor 546; B). C,D: The number of PKH67-labeled monocytes was analyzed using a fluorescence photometer and evaluating cells in organs (C), cortex (open bars, D) and hippocampus (solid bars, D). The number of monocytes is given as mean $\pm$ SEM PKH67⁺ cells/mm². Animals receiving only saline injections served as negative controls (vehicle). The values in parenthesis indicate the number of analyzed animals. Three to six sections of each tissue were evaluated per animal. Scale bar = 30 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Evaluation of Nerve Growth Factor (NGF) and Inflammatory Markers in Cortical Extracts *

Animal group	NGF	MCP-1	MIP-2	TNF- α	IL-1 β
Vehicle	91 $\pm$ 14 (7)	39 $\pm$ 1	0.21 $\pm$ 0.01	BDL	1.5 $\pm$ 0.1
pM(-)	120 $\pm$ 17 (7)	46 $\pm$ 6	0.24 $\pm$ 0.03	BDL	1.7 $\pm$ 0.3
pM-NGF	135 $\pm$ 22 (10)	41 $\pm$ 7	0.24 $\pm$ 0.04	BDL	1.8 $\pm$ 0.3

* Cortical tissue was collected from animals 1 week following chronic i.v. injection (2 months, weekly). Tissue extracts were evaluated by ELISA for NGF and inflammatory markers (monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein-2 [MIP-2], tumor necrosis factor- α [TNF- α], interleukin-1 β [IL-1 β]). Values represent mean $\pm$ SEM pg/mg tissue (n = 5 unless otherwise indicated in parentheses); BDL, below detection limit. Statistical analysis was performed using a one-way ANOVA with a subsequent Fisher LSD post hoc test. Note that none of the treatments affected inflammatory markers, although there was a tendency towards increased cortical NGF following pM and pM-NGF treatment.