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Comparison of MR images and histochemical localization of intra-arterially administered microglia surrounding ß-amyloid deposits in the rat brain

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Summary. The therapeutic use of microglial cells has recently received some attention for the treatment of Alzheimer disease (AD), but few non-invasive techniques exist for monitoring the cells after administration. Here we present a magnetic resonance imaging (MRI) technique for tracking microglia injected intra-arterially *in vivo*. We micro-injected Aß42 into the left hippocampus and saline into the right hippocampus of rats. We then administered microglia, which were labeled with enhanced green fluorescent protein (EGFP) gene and Resovist, into the carotid artery. After monitoring exogenously administered microglia using MRI, we compared the MR images and the histochemical localization of administered microglia. MRI revealed clear signal changes attributable to Resovist-containing microglia in Aß-injected areas. Histochemistry demonstrated that EGFP-positive microglia accumulated around Aß deposits and internalized the peptide. This study demonstrates the usefulness of MRI for non-invasive monitoring of exogenous microglia, and suggests a promising future for microglia/macrophages as therapeutic tools for AD.

Key words: Alzheimer's disease, ß-amyloid, Microglia, MRI, Senile plaque

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Introduction

Senile plaques and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD). According to the amyloid cascade hypothesis, aggregation of ß-amyloid (Aß) in senile plaques is an early and necessary event in AD (Hardy and Higgins, 1992; Hardy and Selkoe, 2002). Inhibition of Aß deposition, and the removal of Aß from the brain, are therefore important goals for AD prevention and treatment (Hardy and Selkoe, 2002). Several potential methods have been proposed for removal of Aß deposits, including vaccination (Schenk et al., 1999), sequestration (Matsuoka et al., 2005), gene therapy (Marr et al., 2004), and cell therapy (Bard et al., 2000; Wyss-Coray et al, 2003). One of the suggested forms of cell therapy involves the use of microglia for their strong ability to remove Aß deposits (Bard et al., 2000; DiCarlo et al., 2001; Wyss-Coray et al, 2001; Wilcock et al., 2003), although microglia have characteristics that are potentially disadvantageous as well as beneficial (McGeer and McGeer 2001; Schenk and Yednock, 2002).

Exogenously administered microglia can phagocytose Aß and degrade it, as shown using unfixed brain sections from both AD cases and amyloid precursor protein transgenic mice (Bard et al., 2000). Recently, Akiyama and McGeer demonstrated that the reduction of senile plaques occurred in cortical areas affected by incomplete ischemia in a typical AD (Akiyama and McGeer, 2004). In those areas, Aß fragments were detected in activated microglia. These findings suggested that exogenous microglia might be of therapeutic use in AD if they could be delivered into the brain through an area where there was breakdown of the blood-brain barrier (BBB).

For such cell therapeutics, it is very important to monitor administered cells non-invasively. However, few non-invasive techniques exist for monitoring the cells after administration. In this study, we used a rat model of AD, and examined the localization of exogenously administered microglia using magnetic resonance imaging (MRI) and histochemistry. The imaging employed magnetic resonance (MR) microscopy as a non-invasive method for monitoring exogenously injected cells in living animals, a technique that may have future application for human studies (for reviews, Allport and Weissleder 2001; Bulte et al., 2002; Heckl et al., 2004).

Materials and methods

Cell culture and gene transformation

A mouse microglial cell line (EOC 13.31, purchased from ATCC, CRL-2468, Manassas, Va, USA) expressing EGFP was used in this study, because these cells were easily distinguished from *in situ* brain microglia. Microglia were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20% Ladmac supernatant, and 1% penicillin/streptomycin mixture (Nacalai Inc., Osaka, Japan). The cell lines remained immortalized only while in the medium containing colony stimulating factor (Ladmac supernatant). For labeling microglial cells, we transfected a construct consisting of chicken ß-actin promoter (CAP) and the reporter gene, enhanced green fluorescent protein (EGFP), into cultured microglia. The chicken ß-actin promoter was kindly donated by Dr Miyazaki (Niwa et al., 1991). As a transfection reagent, we used the hemagglutinating virus of Japan envelope (HVJ-E, GenomONE, Ishihara Sangyo Kaisha. Ltd, Osaka, Japan) (Kaneda et al., 2002; Shimamura et al, 2003; Toyoda et al., 2004). After the transfection, we screened for microglia consistently expressing EGFP by adding Genetecin (500 µg/ml) to the medium.

Aß injection into the rat hippocampus

All experimental procedures were approved by the Committee on Animal Care of the Shiga University of Medical Science. Male Wistar rats (260-300 g) were anesthetized with sodium pentobarbital (50 mg/kg, IP) and immobilized in a stereotaxic apparatus. For Aß administration, we dissolved the lyophilized human Aß42 (synthesized in the Central Research Laboratory at Shiga University of Medical Science) in 10 mM phosphate-buffered saline (PBS, pH 7.4). We preincubated Aß to make aggregations as seen in senile plaques of human AD, and Aß was injected into the rat hippocampus as described previously (Takata et al., 2003). In brief, Aß42 was incubated at 37°C overnight, then Aß42 (4.5 µg in a final volume of 2 µL) was injected at 3.5 mm caudal, 2.0 mm lateral to the bregma (left side). The depth of the syringe was 3.5 mm. The same volume of saline was injected into the contralateral (right) side. Injection rate was 1.0 µL/min and the steel pipette was left in place for an additional 5 minutes before being withdrawn.

Injection of microglia into the carotid artery

For MRI, microglia were labeled with super-paramagnetic iron particles (Resovist) using the HVJ-E vector, as previously described (Toyoda et al, 2004). Microglia with EGFP gene and/or Resovist were washed with new medium, and harvested into a 12-mL tube. After centrifugation for 2 minutes at 200 g, cells were resuspended in 10 mM PBS to a final concentration of approximately $6x10^6$/mL. We selected the internal carotid artery as the injection route, because many microglia were trapped in the lung when we injected labeled cells into the tail veins of mice. One day (n=5) or 3 days (n=5) after injection of Aß42, rats were anesthetized and the skin of their necks was incised to open the carotid artery sheath. A bolus of $3x10^6$ cells in 0.5 ml PBS was injected into the internal carotid artery over 30 seconds.

MRI tracing of microglial cells in vivo

One day after microglial injection, MR images were acquired with a 7T Unity Inova MR scanner (Varian, Palo Alto, Calif). A gradient echo sequence was used with acquisition parameters of 180-ms repetition time, 25-ms echo time, 40° flip angle, 60x60-mm^2 field of view, 1-mm slice thickness and 256x256 matrices. During the MRI sessions, the spontaneously breathing animals were anesthetized with 1.5% isoflurane in 50% O_2 and 50% N_2.

Tissue preparation

After obtaining MR images, rats were deeply anesthetized with pentobarbital (70 mg/kg) and perfused through the aorta with approximately 300 ml of 10 mM PBS followed by 300 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After the perfusion fixation, the animals were kept in ice for 30 minutes, and then perfused with 1000 mL of ice-cold 0.1 M phosphate buffer containing 15% sucrose. The brain was cut into 20-µm-thickness sections in a cryostat.

Quantification of fluorescent cells

Three hippocampal sections (20 µm) from each rat were visualized using a fluorescent microscope (Olympus IX50/IX70, Japan). The hippocampus was divided into the square area (113,400 μm^2) and the photographic data were saved on computer via a CCD camera. Fluorescent cells in the whole hippocampal region were counted in both sides, and the data were converted into the number of labeled cells per square

mm. All data are shown as mean ± SEM. Statistical significance was assessed using the Student t test. Significance was set at $P<0.05$.

Fluorescent immunohistochemistry

Sections were incubated overnight at 4°C with mouse monoclonal antibody against Aß (4G8, Signet Laboratory Inc., Mass, USA, 1:1000). After washing with 0.1 M PBS containing 0.3% Triton-X 100 (PBST), sections were incubated for 3 hours at room temperature with Cy5-labeled secondary antibody (Chemicon, Temecula, Calif, USA, 1:100). Photographs were taken under a laser confocal microscope (LSM510 META, Germany). For controls, sections were stained with the omission of the primary antibody or using the Aß antibody pre-absorbed with 20 µg/ml of Aß peptides.

Prussian Blue and 4G8 Double Staining

After incubating with mouse monoclonal antibody against Aß (4G8, 1:1000), some sections were stained with peroxidase-labeled anti mouse IgG complex (Histofine, 1:20, Nichirei, Osaka, Japan). A brown color was developed with 0.02% 3, 3'-diaminobenzidine in 50 mM Tris-HCl buffer (pH 7.6). The sections were then incubated for 30 minutes with 1% potassium ferrocyanide in 1% HCl at room temperature, to stain iron particles with a blue color (Prussian blue method).

Results

MR images detected accumulation of administered cells in the Aß-injected sites within 24 hours. Figure 1 shows horizontal MR images of rat brain scanned 24 hours after intra-arterial injection of Resovist-labeled microglial cells. A dark region was detected in the left hippocampus (Fig. 1A, arrow). Little MR signal change was detected in the saline-injected right side. After cutting the brain in the same plane as the MRI, sections were stained with mouse monoclonal antibody against Aß and by the Prussian blue method (Fig. 1B,C). At low magnification, the Aß injection site was seen in the same region as detected by MRI (Fig. 1B, arrow). The MR signal area was larger than that of the histochemically detected region. At high magnification (Fig. 1C), an accumulation of blue-colored microglia with Resovist (Fig. 1C, arrow) was apparent at brown-colored Aß deposits (Fig. 1C, arrowhead).

Figure 2A shows a coronal MR image of rat brain scanned 24 hours after intra-arterial injection of Resovist-labeled microglia. For a comparison, figure 2B demonstrates a coronal MR image of a control rat injected with Aß peptide and saline but not administered with Resovist-labeled microglia. In the control rat, needle routs were weakly visualized in both sides of the brain. In the rat injected with Resovist-labeled microglia, needle routes were clearly visualized in both hippocampi (Fig. 2A), compared with MR images of rats before injecting Resovist-labeled microglia (Fig. 2B). In addition, a dark area was detected in the CA2 region of the left hippocampus (Fig. 2A, arrow). Histochemical examination demonstrated that blue-colored microglia with Resovist accumulated around the brown-colored Aß deposits (Fig. 2C,D). Few exogenous microglia were observed in the right hippocampus (Fig. 2E). Under confocal microscopy, clusters of EGFP-positive microglia were seen at the diffuse red-colored Aß deposits (Figs. 2F-H).

Cell counts were performed on Aß-injected and saline-injected sides of the hippocampus. At 1 day and 3 days following Aß42 or saline injection, the density of

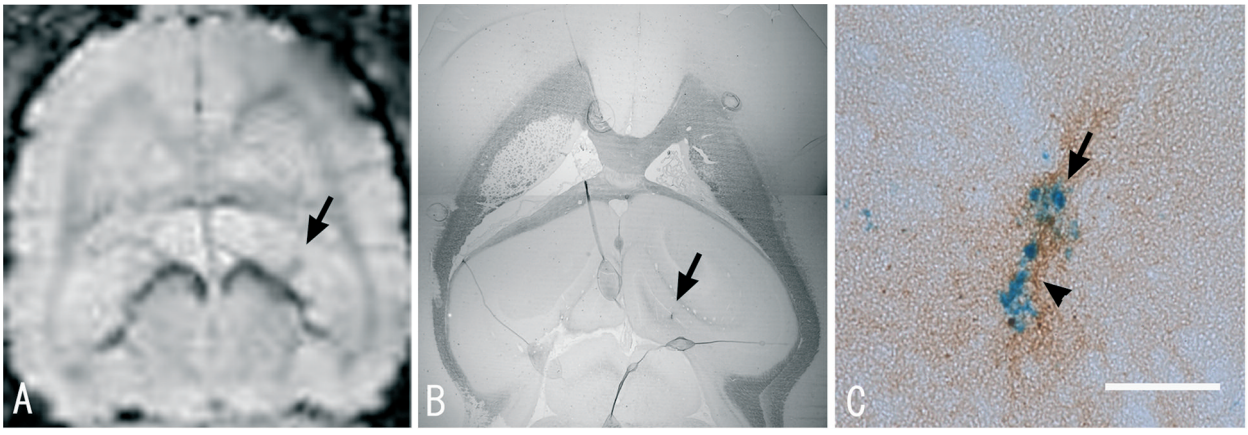

Fig. 1. Magnetic resonance image **(A)** and histochemical staining **(B and C)** in rat brain injected with Aß and saline. Microglia injected one day after Aß injection. **A.** A decrease in MR signal is apparent in the Aß-injected region (arrow). **B.** High contrast image revealing the white matter, for comparison with the MR image. An Aß-injected area is visible (arrow) in a section corresponding to that imaged by MR microscopy. The section was immunostained using the Aß antibody after Prussian blue staining. **C.** At high magnification, the accumulation of blue-colored microglia containing Resovist (arrow) is evident around brown-colored Aß-deposits (arrowhead). Scale bar: A, B, 2 mm; C, 50 µm.

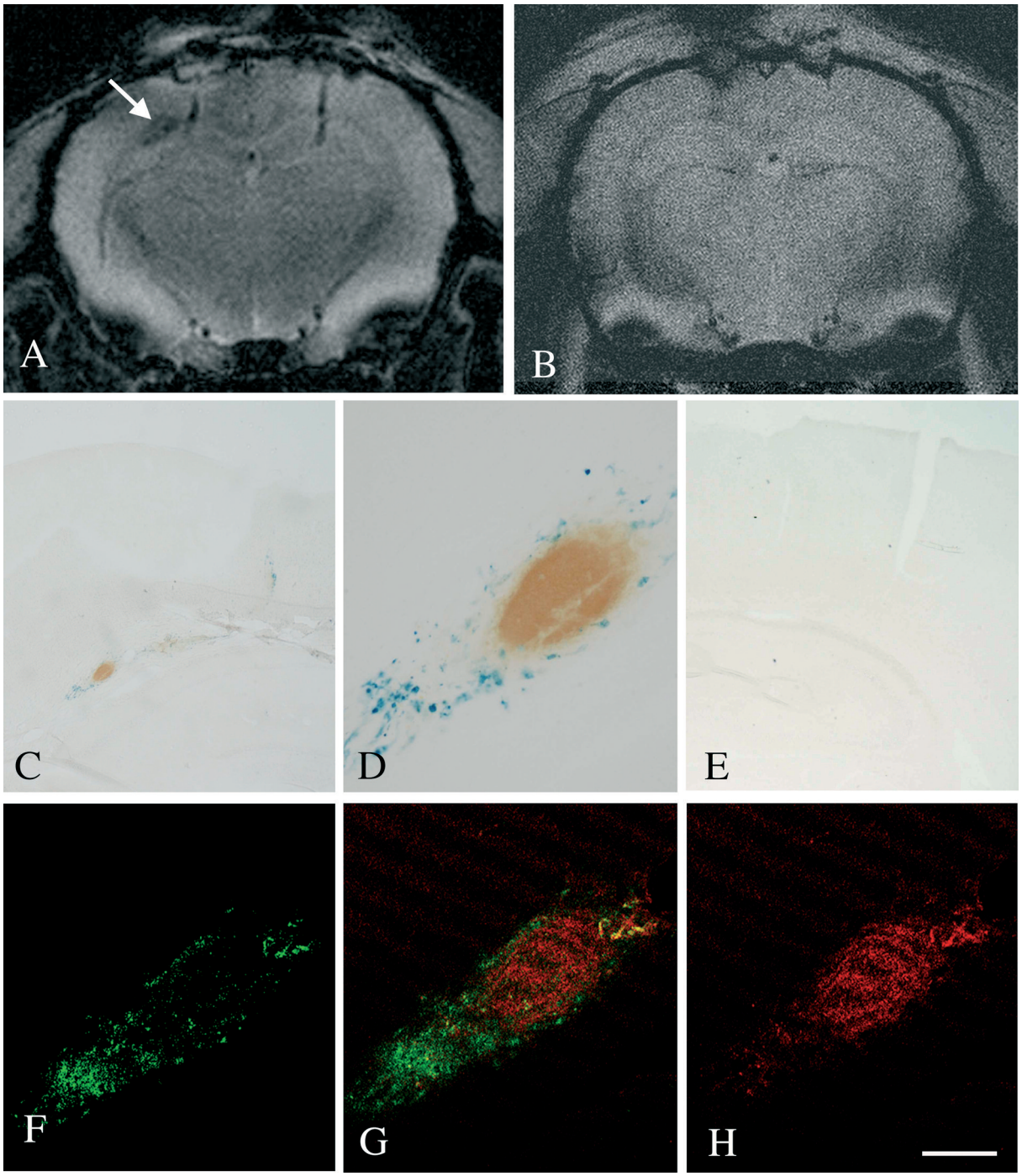

Fig. 2. Coronal sections of rat hippocampus injected with Aß peptides (left side) and saline (right side). **A.** MR image from a living rat. Microglia injected three days after Aß injection. Resovist reduces proton signals and is indicated by dark regions. Note that a dark area attributable to Resovist is apparent in the CA2 region of the left hippocampus (arrow). **B.** MR image from a control rat before injecting Resovist-labeled microglia. **C and D.** Double staining for Resovist (Blue) and Aß (brown) in the left hippocampus of the same rat as shown in A. E; Double staining for Resovist (Blue) and Aß (brown) in the right hippocampus. F, G and H; Confocal microscopy of the section adjacent to that shown in D. F; Many EGFP-positive green cells are seen. G; Merged picture showing EGFP and Aß signals. H; Red Aß deposits labeled with Cy5. Scale bar: C, E, 700 µm; D, F-H, 100 µm.

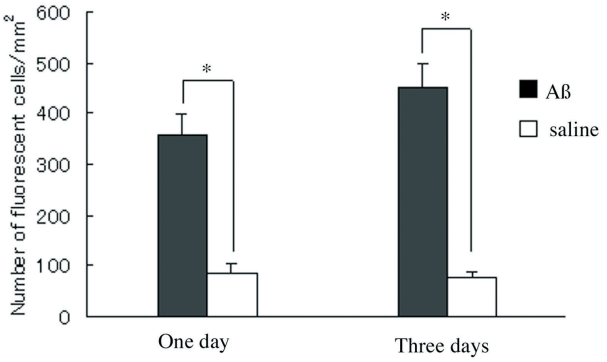

Fig. 3. Quantitative analysis of EGFP-positive microglia detected in the Aß- and saline-injected sides. Data represent the mean ± SEM (n=5). * P<0.05.

EGFP-positive microglia was significantly higher on the Aß-injected side than on the saline-injected side (Fig. 3).

In addition to the Aß mass in the hippocampus, small red-colored Aß deposits were scattered at the peripheries of the large Aß mass and throughout the needle route (Fig. 4A,D). Some microglia expressing green EGFP signals were seen in these areas (Figs. 4B and E). Merged images revealed some yellow cells containing both EGFP signal and Aß42 signal (Figs. 4C and F, arrows), suggesting that some of the exogenous microglia took up Aß peptide. No staining for Aß was observed in the control sections (Fig. 4G).

Discussion

In the present study, MRI clearly detected microglia accumulating at Aß deposits. Activated microglia have previously been detected in the brain of AD patients by positron emission tomography using a ligand against benzodiazepine receptors (Cagnin et al., 2001; Versijpt et al., 2003). However, this is the first study to use MRI

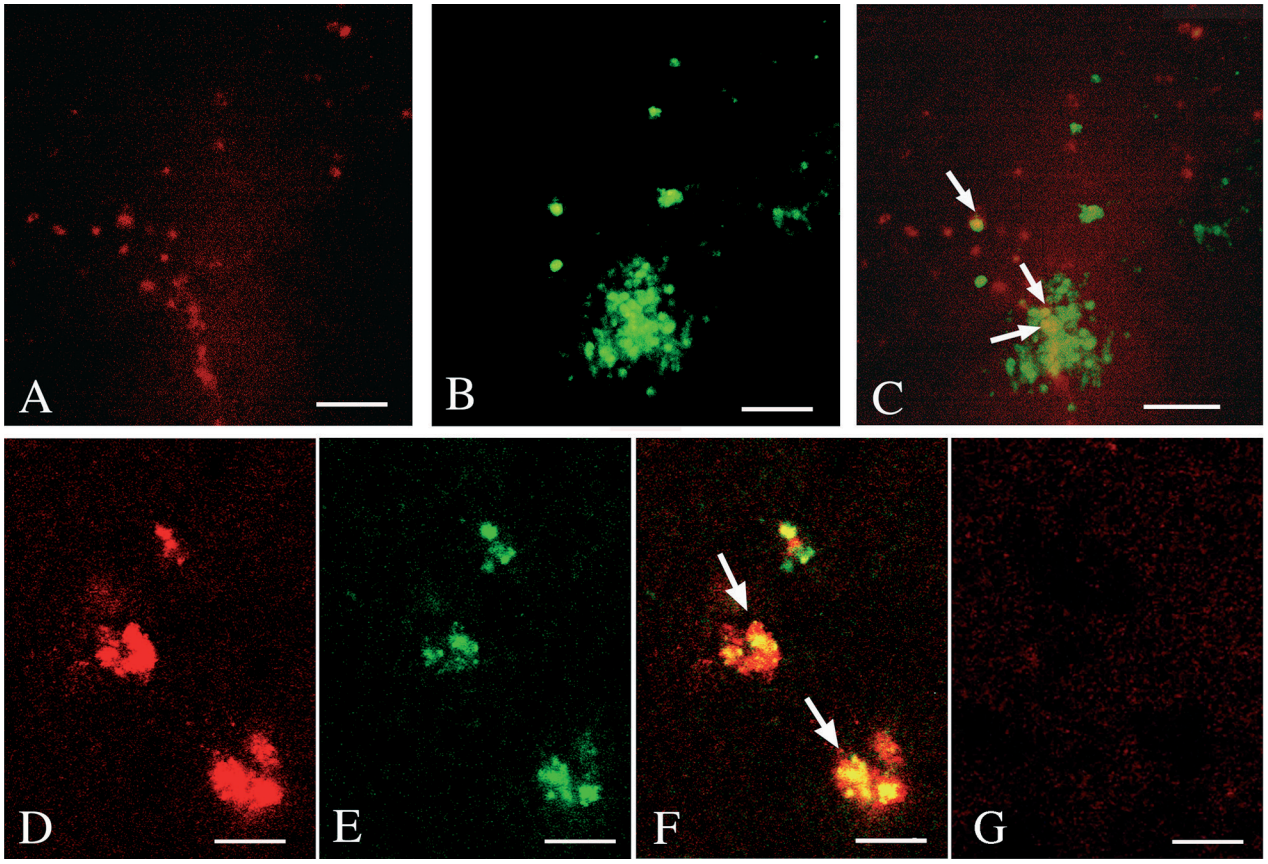

Fig. 4. Double staining for EGFP-positive microglia and Aß deposits. Microglia injected three days after Aß injection. The sections were observed by fluorescent microscopy (**A, B, C**) or laser confocal microscopy (**D, E, F, G**) for Aß (**A, D, G)**, EGFP (**B, E**), and for both EGFP and Aß (**C, F**). Some yellow cells containing both EGFP signal and Aß42 signal are observed (arrows in **C and F**). **G.** A section was stained using Aß antibody pre-absorbed with 20 µg/ml of Aß peptides. Scale bar: A-C, 30 µm; D-G, 10 µm.

to demonstrate exogenously administered microglia accumulating around Aß deposits in living animals. We used HVJ-E as a novel system of effective magnetic labeling for MRI. Several transfection agents have previously been employed to incorporate paramagnetic Fe^{3+} particles into cells. These have included carboxy-terminated dendrimer, FuGene, Superfect, Polyfect, PLUS/lipofectamine, Effectene, and poly-L-lysine (Allport and Weissleder 2001; Bulte et al., 2002; Heckl et al., 2004; Toyoda et al., 2004). Since most such agents have the same cationic charge as Fe^{3+}, their efficiency is reduced to some extent. Because the HVJ-E vector has no charge and uses membrane fusion activity to transfer the gene into cells, effective magnetic labeling of transplanted cells is obtained (Toyoda et al., 2004). In the present study, the areas of the MRI signals were larger than the areas of histochemical labeling. This occurred because iron particles reduce MR signals not only of iron-containing cells but also of the surrounding areas.

Histochemical examination demonstrated that intra-arterially injected microglia accumulated around Aß deposits in good agreement with the MR images. Interestingly, some microglial cells showing both EGFP and Aß-Cy5 signals were scattered at the peripheries of the Aß injected sites and at the needle routes. These findings suggest that some intra-arterially injected microglia phagocytose Aß. A previous study using frozen sections showed that in the presence of antibodies against Aß, microglia gather around senile plaques and phagocytose Aß peptides (Bard et al, 2000). Recently, Akiyama and McGeer suggested that activated microglia also had the ability to phagocytose Aß non-specifically (Akiyama and McGeer, 2004). It is likely that both specific and non-specific mechanisms contribute to microglial phagocytosis of Aß.

Because amyloid deposition is thought to be the key step in the pathogenesis of AD, removal of Aß from the brain is an important aim in the treatment of AD. Our results suggest that exogenously administered microglia have therapeutic potential. Microglia injected into the rat carotid artery can migrate through the broken blood-brain barrier (BBB) to the hippocampus, where they accumulate around Aß deposits and phagocytose Aß peptides. Previous studies have reported that some types of microglia can pass through an intact BBB (Imai et al., 1997; Sawada et al., 1998), raising the possibility that selective use of these microglia could find application in cell therapy for AD. In addition to microglia, macrophages may also have therapeutic potential, because the distinction between microglia and macrophages is not clear.

In the brain of AD patients, many reactive microglia surround senile plaques (McGeer and McGeer 2001). While such reactive microglia may be beneficial, they could alternatively cause harm (McGeer and McGeer 2001; Schenk and Yednock 2002). Further studies will be needed to confirm the effectiveness of microglia/macrophages for the treatment of AD. If microglia/macrophages are to be used therapeutically, they will need to be observed non-invasively after transplantation. Recent advances in MR microscopy, as applied in this study, may offer a means to achieve this.

Conclusion

We compared MR images and histochemical localization of intra-arterially administered microglia surrounding ß-amyloid deposits in the rat brain. MRI revealed clear signal changes attributable to Resovist-containing microglia in Aß-injected areas of living rats. In good agreement with MR images, histochemical examination demonstrated that intra-arterially injected microglia positive for EGFP and Resovist had accumulated at sites of Aß deposition in the rat hippocampus. Our findings indicate the usefulness of MRI as a non-invasive means of detecting transplanted microglia, with the potential for future clinical application.

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References

Akiyama H. and McGeer P.L. (2004). Specificity of mechanisms for plaque removal after A beta immunotherapy for Alzheimer disease. Nat. Med. 10, 117-118.

Allport J.R. and Weissleder R. (2001). In vivo imaging of gene and cell therapies. Exp. Hematol. 29, 1237-1246.

Bard F., Cannon C., Barbour R., Burke R.L., Games D., Grajeda H., Guido T., Hu K., Huang J., Johnson-Wood K., Khan K., Kholodenko D., Lee M., Lieberberg I., Motter R., Nguyen M., Soriano F., Vasquez N., Weiss K., Welch B., Seubert P., Schenk E. and Yednock T. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer' s disease. Nat. Med. 6, 916–919.

Bulte J.W., Duncan I.D. and Frank J.A. (2002). In vivo magnetic resonance tracking of magnetically labeled cells after transplantation. J. Cereb. Blood Flow Metab. 22, 899-907.

Cagnin A., Brooks D.J., Kennedy A.M., Gunn R.N., Myers R., Turkheimer F.E., Jones T., Banati R.B. (2001). In-vivo measurement of activated microglia in dementia. Lancet 358, 461-467.

DiCarlo G., Wilcock D., Henderson D., Gordon M. and Morgan D. (2001). Intra-hippocampal LPS injections reduce Aß load in APP + PS1 transgenic mice. Neurobiol. Aging 22, 1007-1012.

Hardy J.A. and Higgins G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. Science 256, 184-185.

Hardy J. and Selkoe D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353-356.

Heckl S., Pipkorn R., Nagele T., Vogel U., Kuker W. and Voight K. (2004). Molecular imaging: Bridging the gap between neuoradiology

and neurohistology. Histol. Histopathol. 19, 651-668.

Imai F., Sawada M., Suzuki H., Kiya N., Hayakawa M., Nagatsu T., Marunouchi T. and Kanno T. (1997). Migration activity of microglia and macrophages into rat brain. Neurosci. Lett. 237, 49-52.

Kaneda Y., Nakajima T., Nishikawa T., Yamamoto S., Ikegami H., Suzuki N., Nakamura H., Morishita R. and Kontani H. (2002). Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. Mol. Ther. 6, 219-226.

Marr R.A., Guan H., Rockenstein E., Kindy M., Gage F.H., Verma I. Masliah E. and Hersh L. B. (2004). Neprilysin regulates amyloid Beta peptide levels. J. Mol. Neurosci. 22, 5-11.

Matsuoka Y., Shao L., Debnath M., Lafrancois J., Becker A., Gray A., Aisen P., Mathis C., Klunk W. and Duff K. (2005). An Abeta sequestration approach using non-antibody Abeta binding agents. Curr. Alzheimer Res. 2, 265-268.

McGeer P.L. and McGeer E.G. (2001). Inflammation, autotoxicity and Alzheimer disease. Neurobiol. Aging 22, 799–809.

Niwa H., Yamamura K. and Miyazaki J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193-199.

Sawada M., Imai F., Suzuki H., Hayakawa M., Kanno T. and Nagatsu T. (1998). Brain-specific gene expression by immortalized microglial cell-mediated gene transfer in the mammalian brain. FEBS Lett. 433, 37-40.

Schenk D.B. and Yednock T. (2002). The role of microglia in Alzheimer's disease: friend or foe? Neurobiol. Aging 23, 677-679.

Schenk D., Barbour R., Dunn W., Gordon G., Grajeda H., Guido T., Hu K., Huang J., Johnson-Wood K., Khan K., Kholodenko D., Lee M., Liao Z., Lieberburg I., Motter R., Mutter L., Soriano F., Shopp G., Vasquez N., Vandevert C., Walker S., Wogulis M., Yednock T., Games D. and Seubert P. (1999). Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature 400, 173-177.

Shimamura M., Morishita R., Endoh M., Oshima K., Aoki M., Waguri S., Uchiyama Y. and Kaneda Y. (2003). HVJ-E envelope vector for gene transfer into central nervous system. Biochem. Biophys. Res. Commun. 300, 464-471.

Takata K., Kitamura Y., Tsuchiya D., Kawasaki T., Taniguchi T. and Shimohama S. (2003). Heat shock protein-90-induced microglial clearance of exogenous amyloid-beta1-42 in rat hippocampus in vivo. Neurosci. Lett. 344, 87-90.

Toyoda K., Tooyama I., Kato M., Sato H., Morikawa S., Hisa Y. and Inubushi T. (2004). Effective magnetic labelling of transplanted cells with HVJ-E for magnetic resonance imaging. Neuroreport 15, 589-593.

Versijpt J.J., Dumont F., Van Laere K.J., Decoo D., Santens P., Audenaert K., Achten E., Slegers G., Dierckx R.A., Korf J. (2003). Assessment of neuroinflammation and microglial activation in Alzheimer's disease with radiolabelled PK11195 and single photon emission computed tomography. A pilot study. Eur. Neurol. 50, 39-47.

Wilcock D.M., DiCarlo G., Henderson D., Jackson J., Clarke K., Ugen K.E., Gordon M.N. and Morgan D. (2003). Intracranially administered anti-Abeta antibodies reduce beta-amyloid deposition by mechanisms both independent of and associated with microglial activation. J. Neurosci. 23, 3745-3751.

Wyss-Coray T., Lin C., Yan F., Yu G.Q., Rohde M., McConlogue L., Masliah E. and Mucke L. (2001). TGF-beta 1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. Nat. Med. 7, 612-618.

Wyss-Coray T., Loike J.D., Brionne T.C., Lu E., Anankov R., Yan F., Silverstein S.C. and Husemann J. (2003). Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. Nat. Med. 9, 453-457.

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