Therapeutic effect of IL-12/23 and their signaling pathway blockade on brain ischemia model

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Abstract

Recently, T cell cytokines such as IL-17 and IFN-γ have been shown to play important roles in the progression of brain injury induced by ischemia. We have shown that IL-23 from infiltrated macrophages activates γδT cells, thereby inducing IL-17 from these cells. However, deletion of the IL-23 gene in mice showed a more dramatic protective effect against brain ischemia reperfusion (I/R) model than γδT cell depletion did, suggesting that IL-23 plays some other pivotal role in brain injury in addition to its role in I/R induction. To develop therapeutic methods based on these findings, we examined the effect of the JAK kinase inhibitor CP-690550 and an anti-IL12/23 monoclonal antibody on an I/R model. CP-690550 efficiently inhibited IL-17 production from memory T cells in vitro and partly suppressed infarct volume increase after I/R. Anti-p40 antibody, which blocks both IL-12 and IL-23, efficiently suppressed I/R injury and improved recovery of neurological deficits. The number of IL-17-producing cells was decreased by anti-p40 antibody treatment. Thus the JAK inhibitor and anti-p40 antibody, both of which have already been under trial for the treatment of several human inflammatory diseases, appear to be promising therapeutic agents for the amelioration of stroke.

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1. Introduction

Stroke is a leading cause of death and disability worldwide, but no successful therapy has been established that can be initiated at the subacute phase of brain infarction [1,2]. As thrombolysis is currently the only approved therapy, there is a dramatic need for additional therapeutic targets. Recombinant tissue-type plasminogen activator (rt-PA) may be used within a period which has recently been lengthened to 4.5 h after the onset of symptoms, but it is not indicated after that point because the risk of secondary intracranial hemorrhage after treatment begins to outweigh the potential benefits [3], and many stroke patients are not eligible for rt-PA treatment at any time. Therefore a novel approach is needed, and must be effective even in late phase of ischemia.

Previous studies have suggested that T lymphocytes appear to be localized to the infarction boundary zones 24 h after reperfusion [4,5], and that they play essential roles in brain ischemic injury [6,7]. Antibodies directed against lymphocyte adhesion receptors blocked T-cell transmigration and reduced stroke volumes at day 1 after transient middle cerebral artery occlusion (tMCAO) in rats. Recombination activating gene 1 (RAG1) – deficient mice, which lack functional T and B cells, are protected from cerebral ischemia after 24 h, and this protection is lost upon reconstitution of RAG1 + mice with T cells from wild-type littermates. Detailed analysis of lymphocyte subsets has revealed that T cells, but not B cells, are detrimental during stroke, though the molecular mechanism underlying this phenomenon has not been clarified.

We have shown that, among the effector T cell cytokines, IL-17 plays a more important role in I/R injury than IFN-γ does [8], and that IL-17 and γδT cells are good therapeutic targets for I/R injury. The major producer of IL-17 in the ischemic brain is infiltrated γδT cells, while IFN-γ is produced by Th1 and CTLs. IL-17 is strongly induced from γδT cells as well as from Th17 cells by IL-23 [9–11], which is mostly produced by infiltrated macrophages. IL-23-KO mice consistently exhibit no IL-17 production, and are resistant against I/R injury, much more so than IL-17-KO mice. This suggests that IL-23 has an additional target other than IL-17. The nature of this target and the action of IL-23 remain to be investigated.

From a therapeutic point of view, IL-23 could be a good target along with IL-17, given that anti-p40 antibody has been proven or suggested to be effective for the treatment of psoriasis, psoriasis arthritis, IBD and MS [12–15]. Anti-p40 antibody blocks both IL-12 and IL-23 and may thereby effect the suppression of Th1 and Th17
simultaneously. Accordingly, we investigated the effect of p40 antibody on mouse brain I/R stroke model.

Given that IL-23 activates STAT3 through the receptor-associated JAK tyrosine kinases [16], we hypothesized that a JAK kinase inhibitor might also suppress the activity of IL-23. The JAK kinase inhibitor CP-690550 (tasocitinib, developed by Pfizer Inc., New York, NY, USA) is now in phase II/III clinical trials for RA treatment, and has been shown to be very effective. Therefore we also examined the effect of CP-690550 on I/R injury. In the present study, we show that both anti-p40 antibody and CP-690550 administered at the onset of cerebral ischemia can reduce lesion size and improve neurological outcome after experimental stroke.

2. Materials and methods

2.1. Mice

Eight- to ten-week-old male C57BL/6 mice weighing approximately 25 g (Nihon Jikken Doubutsu, Tokyo, Japan) were housed in a cage with ad libitum access to food and water. All experiments were approved by the Institutional Animal Research Committee of Keio University (approval number: 08004).

2.2. Chemicals and antibodies

 CP-690550, a small molecule inhibitor of JAK-3 [17], was purchased from Selleck Chemicals (Houston, TX, USA). mAbs (clone C17.8) against p40 (a common subunit for IL-12 and IL-23) [18] were purified through Protein G-column chromatography from ascites of nude mice transplanted with hybridoma.

2.3. Murine focal brain ischemia model

We induced focal brain ischemia as described previously [8]. Briefly, the mice were anesthetized with halothane in a mixture of 70% nitrous oxide and 30% oxygen. We maintained head temperature at 36 °C with a warming lamp and measured cerebral blood flow before and during ischemia by means of laser Doppler flowmetry at the ipsilateral parietal bone (approximately 1–2 mm posterior to the bregma). Each mouse’s resting CBF value was regarded as the baseline for that mouse, and changes in it after the induction of brain ischemia were expressed as percentages of the resting value. After ligation of the right common carotid artery (CCA), the right middle cerebral artery (MCA) was occluded with a 7-0 nylon monofilament with a rounded tip. We then inserted an 11-mm-long filament into the right CCA. The distance from the suture tip to the right CCA bifurcation was approximately 9 mm. During right MCA occlusion, a reduction in CBF of more than 60% was confirmed by laser Doppler flowmetry. Sixty minutes after brain ischemia, we withdrew the filament to allow reperfusion of the right MCA territory. CP-690550 (25 μg/g body weight) dissolved in 70% natural saline, 15% ethanol and 15% dimethyl sulfoxide (DMSO) or anti-p40 antibody (25 μg/g body weight) in PBS were administered i.p. just before the filament evulsion.

On days 3 and 7 after reperfusion, the mice were sacrificed by means of deep anesthesia. One-millimeter-thick serial coronal slices from the brains were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in order to measure the infarct volume. For MAP2 immunostaining, the mice were fixed through transcardial perfusion with cold PBS followed by 4% paraformaldehyde/PBS. One-millimeter-thick serial coronal slices from the brains were embedded in paraffin sections and immunostained with MAP2-specific antibody. We identified the infarct area through TTC staining and MAP2 immunostaining and calculated infarct volume.

2.4. Neurological assessment

Neurological deficit was evaluated in a blinded fashion using a 4-point-scale neurological score as follows: 0, no observable neurological deficits (normal); 1, failure to extend left forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe) [19].

2.5. Measurement of IL-17 production from T cells

CD3+ cells were purified from spleens and lymph nodes by means of negative selection using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), and CD3+ cells were further purified through FACS sorting as CD3+CD4+CD44+ cells or CD3+TCRγδ+ cells. Cells were cultured at 37 °C (5% CO2) in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME, and stimulated with 2 μg/ml plate-bound anti-TCR mAb (clone 145-2C11) and 1 μg/ml soluble anti-CD28 mAb (clone 57.31) in the presence or absence of IL-23 (25 ng/ml) with the indicated concentrations of CP-690550. IL-17 production was determined through ELISA and quantitative RT-PCR [20].

2.6. Quantitative real-time PCR

The ischemic brain tissues were lysed in RNAiso (Takara Bio, Shiga, Japan). Real-time PCR was performed on cDNA samples using Power SYBR Green (Applied Biosystems, Carlsbad, CA, USA). The relative quantitation value is expressed as 2-ΔΔCt, where ΔCt is the difference between the mean Ct value of duplicate measurements of the sample and the endogenous hypoxanthine phosphoribosyltransferase 1 (hprt1) control.

2.7. Intracellular cytokine staining

For surface and intracellular cytokine staining, infiltrating inflammatory cells prepared using Percoll as previously described [8] were restimulated for 4.5 h with 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich, St. Louis, MO, USA), 1 μg/ml ionomycin (Sigma–Aldrich), and 1 μM brefeldin A (eBioscience, San Diego, CA, USA). Surface staining was performed for 15 min with the corresponding mixture of fluorescently-labeled antibodies. The infiltrating inflammatory cells from ischemic brain tissue were sorted by surface markers: macrophages – CD45 high, CD11b high; microglia – CD45 intermediate, CD11b intermediate; T lymphocytes – CD45 high, CD3+; γδT lymphocytes – CD45 high, CD3+, TCRγδ+. After surface staining, the cells were suspended in Fixation Buffer (eBioscience), and intracellular cytokine staining was performed according to the manufacturer’s instructions using anti-IL-17-APC and anti-IFNγ-APC [21]. FACS analysis was performed on a FACS Cantoll instrument (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo Software (TreeStar, Ashland, OR, USA) [21].

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). We performed one-way analysis of variance (ANOVA) followed by post hoc multiple comparison tests (Dunnett’s correction) to analyze differences among three or more groups of mice. Between two groups of mice, an unpaired Student’s t-test was performed to determine statistical significance. P < 0.05 was considered to indicate a significant difference.
3. Results

3.1. CP-690550 suppresses IL-17 production from T cells

We have shown that IL-17 plays a progressive role in brain injury due to I/R, and that the blockade of γδT cells, which produce IL-17, is effective to protect mice from infarct volume growth at a late phase. Since the JAK-STAT pathway is involved in IL-23 receptor signal transduction, blockade of JAK tyrosine kinase activity may also reduce IL-17 production. Therefore we examined the effect of CP-690550 on IL-17 production from γδT cells as well as activated memory CD4+ T cells in response to IL-23. Since the number of γδT cells isolated by FACS was very low, we also examined CD44+ memory type CD4+ T cells for ELISA. As shown in Fig. 1A, CP-690550 efficiently suppressed IL-17 production by γδT cells as well as memory CD4+ T cells. We also observed suppression of STAT3 activation, but not of ERK activation, by CP-690550 under these conditions (data not shown).

3.2. Reduction of ischemic infarction by CP-690550

We investigated the effect of CP-690550 in ischemic brain damage in mice. The dose of CP-690550, 25 mg/kg body weight, was chosen because we had observed that this dose was effective in a collagen-induced arthritis model in mice (data not shown). We used an experimental stroke model consisting of reversible filament-induced MCAO with reperfusion after 60 min. CP-690550 or vehicle was injected intra-peritoneally after I/R. The degree of CBF reduction was similar in the two groups (Fig. 1B). As shown in Fig. 1C, the functional neurological deficit induced by I/R surgery was significantly improved in mice receiving CP-690550 7 days after MCAO compared to control mice. Infarct size was smaller in...
mice treated with CP-690550 3 days after MCAO than in untreated mice, as shown in Fig. 1D, although we have not examined sufficient number of mice for statistical analysis. These data suggest that inhibition of the JAK kinase effectively suppressed brain injury induced through I/R.

3.3. Anti-p40Ab reduces ischemic brain damage

We examined the effect of anti-p40 antibody in ischemic brain damage in mice (Fig. 2). Importantly, animals treated with anti-p40 antibody exhibited a striking improvement in neurological outcome (Fig. 2A). One to two days after MCAO, most of the vehicle-treated animals exhibited a severe deficit, with a neuroscore of 1 or more, whereas basically all of the anti-p40 antibody-treated animals showed only a mild deficit on day 1 (Fig. 2A). Anti-p40 antibody did not influence the reduction of cerebral blood flow during MCAO (Fig. 2B), and did not significantly alter blood pH or the levels of the blood gases pO₂ and pCO₂ (data not shown). In addition, infarct size was consistently and significantly (n = 9, p = 0.002) smaller in anti-IL-12p40 antibody-treated mice than in vehicle-treated controls 7 days after MCAO (Fig. 2C and D). In anti-IL-12p40 antibody-treated mice, IL-1β levels were slightly lower, although this was not statistically significant (Fig. 3A). Levels of other cytokines, including TNF-α and IL-6, and levels of other factors that have been shown to promote brain damage, including MMP3/9 and ICAM-1, were almost identical (Fig. 3B, C and D). Therefore, treatment with anti-p40 antibody did not seem to have a strong impact on the expression of inflammatory factors produced by macrophages.

We then examined the number of T cells that had infiltrated into the brain 3 days after MCAO. IL-17- and IFNγ-positive cell counts were estimated through intracellular cytokine staining and FACS. As shown in Fig. 4A, CD3+ T cell counts were drastically reduced by anti-p40 antibody treatment. The numbers of CD4+ helper T cells and IFNγ-producing cells, in contrast, were slightly but not significantly reduced by the same treatment (Fig. 4B and C). In contrast, IL-17-producing γδT cells were reduced by anti-p40 antibody treatment (Fig. 4D). A representative FACS data for IL-17 production are shown in Fig. 4E. These data suggest that anti-p40 antibody works primarily on γδT cells rather than on helper T cells or Th1 cells.

3.4. Infiltration of IL-17-positive γδT cells was suppressed by anti-p40Ab

We next investigated the mechanism underlying the protective effect of anti-p40 antibody against I/R injury. Expression levels of inflammatory cytokines and other inflammatory factors, most of which were derived from macrophages, were examined in the whole brain on day 3 after MCAO by real time RT-PCR in PBS-treated mice (n = 9), anti-IL-12p40 antibody-treated mice (n = 8), and sham-operated mice (n = 2) (Fig. 3). In anti-IL-12p40 antibody-treated mice, IL-1β levels were slightly lower, although this was not statistically significant (Fig. 3A). Levels of other cytokines, including TNF-α and IL-6, and levels of other factors that have been shown to promote brain damage, including MMP3/9 and ICAM-1, were almost identical (Fig. 3B, C and D). Therefore, treatment with anti-p40 antibody did not seem to have a strong impact on the expression of inflammatory factors produced by macrophages.

4. Discussion

Our results showing that CP-690550 and anti-p40 antibody cause a significant reduction in infarct size along with a striking improvement in neurological outcome seem extremely promising. The fact that these compounds are already in clinical phase III trials
for psoriasis makes the possibility of translating our results into the setting of a clinical trial more feasible [13].

The mechanism by which anti-p40 antibody effects these improvements remains to be clarified. We have previously observed a very strong protective effect on infarct size to result from IL-23 gene disruption [8]. In that study, we also observed reduced inflammatory cytokines and inflammatory factors in IL-23-KO mice. We did not observe the same drastic effect to result from anti-p40 antibody treatment, although anti-p40 antibody clearly reduced the infiltration of IL-17-producing cd T cells. The discrepancy between the results of IL-23 gene deletion and those of IL-23 protein depletion through treatment with an antibody remains to be explained. One possibility is that macrophages and microglias were already altered in IL-23-KO mice, while such abnormalities were not generated through antibody treatment. Of course we could not rule out the possibility of incomplete depletion of IL-23 by anti-p40 antibody.

We have previously shown that IL-23-KO mice are more resistant to brain I/R injury than IL-17-KO mice [8]. IL-23-KO mice exhibited smaller infarct areas from day one after I/R, while IL-17-KO mice exhibited infarct volumes similar to those in WT mice on day 1, though after day 3 and 4 their infarct volumes increased much more slowly than those of WT mice did. This suggests that IL-23 has an unknown function in I/R injury in addition to its role in the induction of IL-17. Our current experiments with anti-p40 antibody resulted in greatly improved neurological outcome, suggesting that IL-23 may directly affect neural cell death/survival or function. Incubating neural cells with IL-23 did not result in apoptosis, however. Other cytokines produced by T cells that have been activated through stimulation with IL-23 may also play a role in brain injury: IL-23 has been shown to induce not only IL-17 but also IL-22 in Th17 cells, LTi-like cells, and innate lymphoid cells [22]. IL-22 has been shown to be responsible for acanthosis in keratinocytes and colitis in several models [23,24]. Thus, IL-22 from γδT cells may be an additional factor that promotes brain damage after I/R. Further study is necessary to define the additional unknown role or roles of IL-23 in brain injury.

In this study, we demonstrated that treatment with JAK inhibitor is also protective against I/R injury. This is probably due to the JAK inhibitors’ ability to suppress cytokine production and/or

<Figure 3. Relative changes in the levels of mRNAs encoding inflammatory cytokines and factors in ischemic brain tissue on day 3 as measured by quantitative RT-PCR. Relative mRNA levels normalized with hpert1 are shown. The numbers of mice in each group were as follows: sham (n = 2), PBS (n = 9), anti-p40 Ab (n = 8).>
function. Although CP-690550 suppresses IL-17 production by T cells, other mechanisms may also be responsible for its effect. CP-690550 was originally developed as an immunosuppressive drug [25], since it inhibits IL-2 signaling. Other immunosuppressive drugs, including FK506, cyclosporine and FTY720, have been shown to reduce the severity of brain injury due to I/R [26,27]. Thus it was not completely unexpected that the JAK inhibitor would have a similar effect on I/R injury. CP-690550 may target not only T cells but also other immune cells, given that JAK inhibitors inhibit various cytokine signaling pathways including IL-12, IL-23, and IL-2. Therefore, JAK suppression may be a more potent therapy than standard immunosuppressive drugs. A precise comparison between the effects of JAK inhibitors on I/R injury and those of classical immunosuppressive agents needs to be performed.

In conclusion, we have shown that cytokine suppression by a JAK inhibitor or by an antibody has a protective effect against brain damage induced by I/R injury. This finding will facilitate the development of new therapies for the treatment of stroke.

Disclosures

The authors have no conflicting financial interests.

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