そして臨床応用を行うことはできなかったことも事実である．ゼラチンハイドロゲル，ポリグリコール乳酸マイクロパーティクル作製では，京都大学再生医学研究所の田畑泰彦教授のグループにお世話になり，東京慈恵会医大DDS研究所の故・水島　裕教授，檜垣　恵教授には，ナノパーティクルに関連する研究でご協力頂いた．リドカイン濃度測定では武庫川女子大学薬学部岡村　昇教授のお世話になった．臨床試験は，京都大学医学部附属病院薬剤部および探索医療センターの協力のもとに現在施行中である．関係各位にこの場をお借りして深謝いたします．

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Valacyclovir for the treatment of Bell's palsy

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Despite recent evidence suggesting that Bell's palsy is associated with reactivation of alfa-herpes viruses, the disease has been treated empirically, and the use of valacyclovir has not been definitively established. In 2007, two prospective, randomised, placebo-controlled trials evaluating valacyclovir were reported in patients with Bell's palsy. One demonstrated that valacyclovir/prednisolone therapy was statistically more effective than placebo/prednisolone therapy in improving the recovery of patients with Bell's palsy, excluding zoster sine herpete. However, considering the cost–benefit ratio of this treatment and the limitations of virological diagnoses, we recommend that valacyclovir should be used in cases of severe palsy within 3 days after the onset of Bell's palsy.

Keywords: Bell's palsy, herpes simplex virus, valacyclovir, zoster sine herpete

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1. Introduction

Bell's palsy is considered the most common cause of facial nerve paralysis, with an annual incidence of around 20 – 30 per 100,000 people [1]. It was defined as an idiopathic peripheral facial nerve paralysis of sudden onset until viral evidences were revealed. Various hypotheses such as ischemic damage, vascular congestion, autoimmune reaction and microbial infection have been proposed for the aetiology of this disease. In 1972, McCormick first suggested that the inflammatory neuropathy of Bell's palsy may be caused by reactivation of herpes simplex virus (HSV) [2]. Since then, much evidence regarding HSV infection has been reported, taking advantage of recent advances in molecular biology. An autopsy study of the facial nerve, using polymerase chain reaction (PCR), revealed that HSV genomes had latently infected the geniculate ganglion in patients without a history of facial paralysis [3]. HSV was also detected in the saliva of normal healthy volunteers, although the incidence was low (3 of 16 patients, 19%) compared with that in Bell's palsy patients (21 of 42 patients, 50%) [4]. Murakami *et al.* investigated the endoneurial fluid of the facial nerve during facial nerve decompression surgery and demonstrated active HSV sequences in 11 of 14 patients (79%) with Bell's palsy [5].

In addition to these human studies, Sugita *et al.* [6] succeeded in inducing facial paralysis in mice by inoculating HSV in the auricular skin; the experiments demonstrated the ability of HSV to induce facial paralysis with a course similar to that of Bell's palsy [7,8]. Later, using this animal model, Takahashi *et al.* [9] induced transient facial paralysis, with features similar to Bell's palsy, by reactivating latent HSV. These findings suggest that reactivated HSV in the geniculate ganglion can be a causative pathogen of Bell's palsy, at least in 20 – 80% of patients. It is considered that primary HSV infection may have occurred during childhood, often without signs or symptoms, and that the virus is incorporated in the neuronal cells of the geniculate ganglion. Various kinds of stresses (physical and/or

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emotional) or decreased cellular immunity may trigger reactivation of latent viruses, inducing facial palsy.

Another important issue in the diagnosis and treatment of Bell's palsy is the difficulty in differentiating it from so-called 'zoster sine herpete' (ZSH), which is caused by reactivation of the varicella zoster virus (VZV), without demonstrating pathognomonic otic vesicles (zoster oticus). As the clinical findings of ZSH are very similar to those of Bell's palsy, it is often misdiagnosed as Bell's palsy. According to serological or PCR studies, ZSH constitutes 8 – 29% of Bell's palsy cases [10,11]. Additionally, Ramsay Hunt syndrome is misdiagnosed as Bell's palsy at the early stage, when it lacks zoster oticus or cochleovestibular dysfunction. In our study, zoster oticus appeared late after the onset of paralysis in 34% of patients with Ramsay Hunt syndrome [12]. As VZV has a higher virulence and causes more severe facial paralysis than HSV, differential diagnosis is important because the treatment modalities are different between the two diseases. VZV needs higher doses of antiviral agents than HSV. Some investigators have attempted to detect viral DNA in the saliva [13] or auricular skin [14] in real time. It is also important to note that there are still unknown causes of acute facial paralysis other than HSV or VZV, although their incidence is believed to be low. Thus, one focus of the treatment of Bell's palsy is whether antiviral agents should be used to improve the prognosis of the paralysis.

2. Valacyclovir

2.1 Overview

The proposed link between Bell's palsy and HSV and VZV has led to the use of an antiviral agent, acyclovir, in the treatment of Bell's palsy. Acyclovir is a nucleotide analogue that interferes with herpes virus DNA polymerases and inhibits DNA replication. Trials of acyclovir therapy with and without steroids have been attempted in patients with Bell's palsy [15-18], but the therapeutic effects remain unclear. Recently, an analogue of acyclovir, valacyclovir, was introduced. Valacyclovir is a prodrug of acyclovir. The bioavailability of valacyclovir is three- to fivefold that of acyclovir [19], suggesting a higher antiviral activity against HSV and VZV. Furthermore, because valacyclovir requires oral administration only twice daily, as opposed to the administration of acyclovir five times per day, better compliance is expected with valacyclovir. To date, only a few reports have addressed the effects of valacyclovir in Bell's palsy patients.

2.2 Pharmacodynamics

Valacyclovir hydrochloride (L-valine,2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl) methoxy]ethyl ester, monohydrochloride) is rapidly and almost completely converted to acyclovir and L-valine. Acyclovir is a specific inhibitor of the herpes viruses, with *in vitro* activity against HSV type 1 and type 2, VZV, cytomegalovirus, Epstein–Barr virus (EBV), and human herpes virus 6. After it has been phosphorylated to the active triphosphate form, acyclovir inhibits herpes virus DNA synthesis. The first stage of phosphorylation requires the activity of a virus-specific enzyme. In the case of HSV, VZV and EBV, this enzyme is the viral thymidine kinase, which is only present in virally infected cells [20]. The requirement for activation of acyclovir by a virus-specific enzyme explains its selectivity. The greater antiviral activity of acyclovir against HSV compared with VZV is the result of its more efficient phosphorylation by the HSV thymidine kinase [21]. Acyclovir triphosphate competitively inhibits the viral DNA polymerase, and the incorporation of the nucleoside analogue results in obligate chain termination, halting viral DNA synthesis and thus blocking virus replication [22]. Resistance is normally due to a thymidine kinase-deficient phenotype. Infrequently, reduced sensitivity to acyclovir has been described as a result of subtle alterations in either the virus thymidine kinase or DNA polymerase [23]. The virulence of these variants resembles that of the wild-type virus.

2.3 Pharmacokinetics and metabolism

After oral administration, valacyclovir hydrochloride is rapidly absorbed from the gastrointestinal tract. Valacyclovir is converted to acyclovir and L-valine by first-pass intestinal and/or hepatic metabolism. Neither valacyclovir nor acyclovir is metabolised by cytochrome P450 enzymes. Plasma concentrations of unconverted valacyclovir are low and transient. Peak plasma valacyclovir concentrations are generally less than 0.3 μg/ml at all doses, occur at a median time of 30 – 100 min post-dose, and are at or below the limit of quantification 3 h after administration [24]. The valacyclovir and acyclovir pharmacokinetic profiles are similar after single and repeat dosing. The binding of valacyclovir to plasma proteins is only 15% [25]. The plasma elimination half-life of acyclovir typically averaged 2.5 – 3.3 h in all studies of valacyclovir in volunteers with normal renal function. Valacyclovir is eliminated principally as acyclovir and the known acyclovir metabolite, 9-carboxymethoxymethylguanine (CMMG), in the urine. Less than 0.5% of the administered dose of valacyclovir is recovered as unchanged drug in the urine. Renal clearance of acyclovir following the administration of a single 1000-mg dose of valacyclovir to 12 healthy volunteers was approximately 255 ± 86 ml/min, representing 41.9% of total acyclovir apparent plasma clearance [23].

2.4 Safety, tolerability and adverse events

Dosage reduction is recommended when administering valacyclovir to patients with renal impairment. Acute renal failure has been reported in patients with underlying renal disease who have received inappropriately high doses of valacyclovir. Precipitation of acyclovir in renal tubules may occur when the solubility is exceeded in the intratubular fluid [26]. Adequate hydration should be maintained. Similar caution should be exercised when administering

Table 1. Clinical trials of valacyclovir for the treatment of Bell's palsy.

Study	Year	No. of participants	Valacyclovir treatment	With steroid	Vs treatment	Effectiveness
Antunes *et al.*	2000	46	1500 mg/day for 7 days	Yes	Steroid and placebo	No
Axelsson *et al.*	2003	56	3000 mg/day for 7 days	Yes	No treatment	Yes
Kawaguchi *et al.*	2007	100	1000 mg/day for 5 days	Yes	Steroid and placebo	No
Hato *et al.*	2007	221	1000 mg/day for 5 days	Yes	Steroid and placebo	Yes

165 valacyclovir to geriatric patients. The $t_{1/2}$ of acyclovir at the 1000 mg dose level was approximately 20% longer in the elderly population, a statistically significant but not clinically significant difference [27]. Elderly patients are also more likely to have CNS symptoms as adverse 170 events. With regard to CNS adverse events observed in clinical practice, agitation, hallucinations, confusion, delirium and encephalopathy have been reported more frequently in elderly patients. Thrombotic thrombocytopenic purpura/hemolytic uremic syndrome, in some cases resulting 175 in death, has occurred in patients with advanced HIV disease and in allogeneic bone marrow transplant and renal transplant recipients participating in clinical trials of valacyclovir, at doses of 8000 mg/day [28].

Adverse events were reported in clinical trials of valacyclovir 180 in healthy patients. Adverse events with high incidences in the herpes zoster study (n = 967) at doses of 3000 mg per day were nausea, headache and vomiting (15, 14 and 6%, respectively) [29]. In a genital herpes study (n = 1159), at a dose of 1000 mg per day, adverse events with high incidences 185 were headache and nausea (15 and 5%, respectively) [30]. Other adverse events such as abdominal pain, diarrhoea and dizziness were rare (< 3%).

3. Clinical efficacy

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According to Peitersen [31], Bell's palsy resolves spontaneously in 71% of patients. Treatment strategies, therefore, should address how to accelerate recovery of the facial palsy and prevent sequelae such as facial contracture, synkinesis and 195 muscular atrophy caused by denervation. Unfortunately, more than 10% of Bell's palsy patients do not recover normal facial movement after conventional, conservative corticosteroid treatment. Various adjunctive treatments, including antiviral agents, have been advocated to improve 200 the prognosis in Bell's palsy. Some trials have evaluated acyclovir therapy in patients with Bell's palsy [15-18]. However, only a few trials have addressed the effects of valacyclovir in the treatment of Bell's palsy.

In a retrospective study, Axelsson *et al.* [32] showed 205 a significantly better outcome in patients with Bell's palsy (n = 56) who were treated with valacyclovir (3000 mg/day) for 7 days and prednisone, compared with patients (n = 56) receiving no medical treatment (Table 1). Forty-nine patients 209 (88%) in the treatment group recovered completely, as compared with 38 patients (68%) in the control group 210 ($p < 0.05$). Antunes *et al.* [33] studied the effects of valacyclovir (1500 mg/day) in 46 Bell's palsy patients in a prospective study and found no statistical differences in the recovery rate between the experimental groups. However, these studies did not include an examination to detect the 215 virological aetiology, and the number of subjects in these studies was too small to allow a conclusion to be drawn about the effects of valacyclovir in Bell's palsy.

In 2007, two prospective, randomised, placebo-controlled trials to evaluate valacyclovir were reported, both involving a 220 large number of patients. Kawaguchi *et al.* [34] reported that the recovery rate in patients with Bell's palsy who were treated with valacyclovir (1000 mg/day for 5 days) and prednisolone (60 mg for the initial 5 days, tapered thereafter) was not significantly higher than that in patients treated 225 with placebo and prednisolone (n = 100). However, recovery in patients with HSV reactivation tended to be higher in the valacyclovir and prednisolone group than in the placebo and prednisolone group.

In our study [35] with a larger number of patients (n = 221), 230 combined treatment with valacyclovir (1000 mg/day for 5 days) plus prednisolone (60 mg for the initial 3 days, tapered thereafter) was compared with placebo plus prednisolone. The complete recovery rate in the former was significantly higher than that in the latter: 96.5% of 235 114 and 89.7% of 107 patients, respectively. Recovery rates were also analysed by classifying the initial severity of facial palsy into three grades. In cases of moderate palsy, all patients with both treatment protocols recovered completely, without sequelae. In cases of complete or severe palsy, the 240 recovery rates with valacyclovir plus prednisolone and placebo plus prednisolone were 95.7% (n = 92) and 86.6% (n = 82), respectively; the former was significantly higher than the latter ($p < 0.05$).

The recovery rates in patients who were treated with 245 valacyclovir plus prednisolone and placebo plus prednisolone within 3 days of symptom onset were 96.6% (84/87) and 88.6% (78/88), respectively. The difference between the groups was significant, indicating that valacyclovir was especially beneficial when administered at an early stage of 250 the disease. The two therapies resulted in similar recovery rates, 96.3% (26/27) in the valacyclovir plus prednisolone group and 94.7% (18/19) in the placebo plus prednisolone group, when treatment was started 4 or more days after the 254

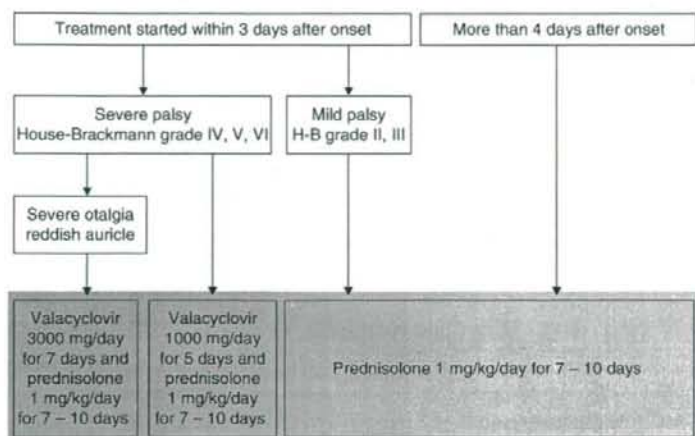

Figure 1. Summary of recommendation for initial treatment of Bell's palsy.

onset of palsy. The study concluded that the administration of the antiviral agent, in addition to the steroid, was advantageous in the treatment of early-stage patients with severe or complete Bell's palsy.

Valacyclovir is a nucleotide analogue that interferes with DNA polymerase, specific to HSV and VZV, and inhibits viral replication. Suggested optimum doses are different between the viruses: 1000 mg of valacyclovir per day for 5 days for HSV and a higher dose of 3000 mg/day for 7 days to treat VZV infection. In our study, ZSH was excluded from the analysis of the recovery rate of Bell's palsy. The successful treatment of ZSH requires valacyclovir at three times the dose used for Bell's palsy [36]. In contrast to Ramsay Hunt syndrome, ZSH does not produce the characteristic aural vesicles, and the differential diagnosis from Bell's palsy is often difficult; ZSH is usually diagnosed retrospectively by serological or PCR analyses. In our study, 18 of 132 patients in the valacyclovir plus prednisolone therapy group were diagnosed with ZSH by serological and PCR studies, and only 13 of those patients (72.2%) recovered completely. Thus, combined therapy with valacyclovir at 1000 mg/day and prednisolone was not suitable for the treatment of ZSH.

In the present study, the quantity of valacyclovir used, 1000 mg/day for 5 days, was sufficient to control HSV reactivation but not to overcome VZV reactivation, which normally requires 3000 mg per day for 7 days. Thus, to improve the recovery rate in facial paralysis due to both HSV and VZV reactivation, 3000 mg/day of valacyclovir is required. However, to simply increase the dose of antiviral agents for all patients seems unwise because higher doses may increase the risk of side effects such as acute renal failure and neurological disorders. Higher doses also increase the cost of medication. Some acyclovir trials showed enhanced recovery in Bell's palsy patients, including those with ZSH, using acyclovir at 2000 mg/day [15,17]. This dose would be sufficient to control HSV reactivation but might not be sufficient to control VZV reactivation, which normally requires 4000 mg/day of acyclovir. Nevertheless, a dose of 2000 mg/day was effective in decreasing pain and inducing early remission of vesicles with zoster [37-38]. Therefore, a dose of 1500 or 2000 mg/day of valacyclovir may be beneficial in the treatment of Bell's palsy patients, including those with ZSH. To improve the recovery rate in Bell's palsy with ZSH, further controlled, randomised studies with such dosages of valacyclovir are needed.

4. Conclusions

Combined therapy with oral valacyclovir plus prednisolone was statistically more effective than therapy with prednisolone plus placebo in improving the recovery rate of patients with Bell's palsy. Although valacyclovir with prednisolone was clearly effective in treating Bell's palsy, the administration of prednisolone with 1000 mg/day of valacyclovir would likely be ineffective in patients with ZSH. The diagnosis of the virological aetiology of Bell's palsy as HSV, VZV, or another aetiology is important to enable antiviral treatment at the optimum dose. However, in a study by Kawaguchi *et al.* [34], out of 150 Bell's palsy patients, it was possible to detect only 12 (8.0%) patients who had reactivation of HSV and only 11 (7.3%) patients who had reactivation of VZV before the initial treatment. Considering the costs and benefits of valacyclovir and the limitations of virological diagnoses, it is recommended that valacyclovir be used in cases of complete or severe palsy within 3 days after the onset of Bell's palsy.

321 ## 5. Expert opinion

Fortunately, Bell's palsy is largely benign, especially when compared with Ramsay Hunt syndrome, and only 325 a few patients with severe or complete facial paralysis require intensive treatment. We believe that the key point in improving the prognosis of Bell's palsy is to diagnose VZV infection earlier and to treat it promptly with an adequate dose of antiviral agent, in combination with 330 steroids [39]. Various attempts are made for prompt detection of the VZV genome using a real-time PCR; for example, from the saliva and the auricular scratch exudate, however at present the test in not sensitive enough. Therefore, we treat Bell's palsy following a protocol that was developed 335 based on the severity of the facial paralysis and the timing of the initial treatment (**Figure 1**). Careful evaluation of the paralysis, together with history-taking and physical examination at the first consultation, is essential for the correct diagnosis of this disease. In mild paralysis, 340 oral administration of prednisolone (1 mg/kg/day for 7 – 10 days) is sufficient because such cases typically show 341 excellent prognoses. In severe or complete paralysis, the treatment differs depending on the timing of the initial treatment. When the treatment is started within 3 days of onset, we prescribe valacyclovir in addition to prednisolone, 345 because VZV and HSV involvement is very common in such patients. In most cases, valacyclovir (1000 mg/day) is administered for 5 days, whereas a higher dose of valacyclovir (3000 mg/day for 7 days) is needed in cases with a suspicion of VZV reactivation. Preceding symptoms 350 such as severe auricular pain, stiff neck and reddish auricle may be signs of VZV reactivation. When the treatment starts more than 4 days after onset, we prescribe prednisolone at the same dose as in moderate paralysis, but with no antiviral agent. 355

Declaration of interest

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Steroid and antiviral treatment for Bell's palsy

Bell's palsy is an acute peripheral unilateral facial paralysis of unknown cause. It is the most common acute facial paralysis, with an incidence of 20–30 per 100 000 people annually. In our series of 3385 patients with acute facial paralysis over the past 30 years, Bell's palsy accounted for 2097 cases (62%). PCR usually identifies fragments of DNA of herpes simplex virus in clinical samples from patients with Bell's palsy. These fragments were detected more often in saliva from patients with Bell's palsy than in saliva from healthy volunteers.[1] Such DNA fragments were also identified in the endoneural fluid of the facial nerve during facial-nerve decompression surgery in 11 of 14 patients (79%) with Bell's palsy.[2] Thus we speculate that reactivation of herpes simplex virus might lead to Bell's palsy: herpes simplex virus is involved in about 31–79% of cases of Bell's palsy.[1–3]

Ramsay-Hunt syndrome is the second most common acute facial paralysis, and is caused by reactivation of latent varicella zoster virus. It is associated with zoster oticus and is often complicated by vestibulocochlear dysfunction. The diagnosis of acute facial paralysis is sometimes complicated by the presence of so-called zoster without vesicles (zoster sine herpete). In the absence of vestibulocochlear dysfunction or zoster in the auricle, such cases are clinically diagnosed as Bell's palsy. Serological and PCR studies show that the prevalence of zoster sine herpete in Bell's palsy ranges from 8% to 28%.[4,5] Therefore although herpes simplex virus is a major cause of Bell's palsy, varicella zoster virus and other unknown causes might also be important (figure).

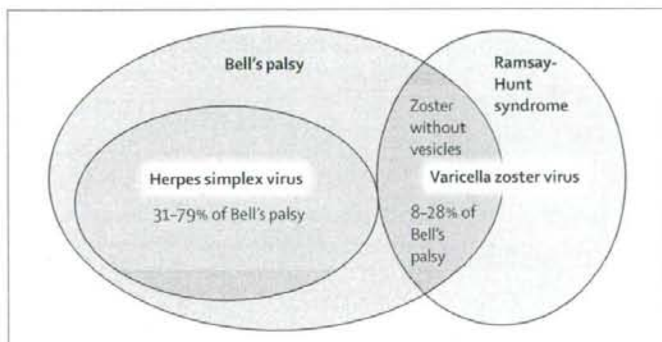

Figure: Involvement of herpes simplex and varicella zoster viruses in acute facial palsy

Steroids and adjunctive antiviral drugs might improve outcome for patients with Bell's palsy. In 2007, two randomised placebo-controlled trials assessed the role of antiviral drugs but gave conflicting findings.[6,7] In a single-blind study in 296 patients, we compared valaciclovir (1000 mg a day for 5 days) plus prednisolone (60 mg for the first 3 days, tapered thereafter) with placebo plus prednisolone.[6] Complete recovery was significantly higher (p=0·045, 95% CI 0·47–0·60) in the valaciclovir group than in the placebo group (110 of 114 [96·5%] patients *vs* 96 of 107 [89·7%] patients, respectively. By contrast, Sullivan and colleagues[7] noted that 92·7% of 124 patients treated with aciclovir (2000 mg a day) plus prednisolone (50 mg a day) for 10 days had complete recovery compared with 96·1% of 127 patients treated with placebo plus prednisolone for 10 days (p=0·28, 95% CI 0·42–0·55). The researchers concluded that an antiviral was not beneficial in improving the prognosis of facial paralysis.

Systematic reviews[8,9] suggest that oral steroids are safe and effective in the treatment of Bell's palsy. Steroids have many side-effects, including anti-inflammatory effects and the suppression of immune reactions. Regardless of the cause, the pathological condition underlying Bell's

palsy involves inflammatory oedema and entrapment neuropathy in the narrow bony facial canal. Therefore treatment with steroids can be advocated regardless of the cause of acute facial paralysis. Antiviral drugs, such as aciclovir, valaciclovir, and famciclovir (the last is only available in some countries), are nucleotide analogues that interfere with DNA polymerase, specific to herpes simplex virus and varicella zoster virus, and inhibit viral replication. The doses differ between viruses—ie, 1000 mg valaciclovir or 1000 mg aciclovir per day for 5 days for herpes simplex virus and 3000 mg valaciclovir or 4000 mg aciclovir per day for 7 days for varicella zoster virus. The timing also differs between antiviral drugs and steroids. Because antivirals cannot destroy viruses, but rather only prevent further viral replication, such drugs should be administered within 3 days of the onset of facial palsy.[10] By contrast, steroids are effective over a longer period and work when neural oedema peaks 7–10 days after onset.[11]

The apparently conflicting findings between our study[6] and the Scottish study[7] might have been caused by several factors. Because 1000 mg valaciclovir is insufficient to treat varicella zoster virus, we used serological and PCR studies to exclude patients infected with varicella zoster virus. By contrast, Sullivan and colleagues[7] did not assess virological status, and so patients infected with varicella zoster virus might have been included in their study, because 2000 mg acyclovir is also insufficient to treat this virus. Gilden and Tyler[12] noted that the severity of facial palsy affects the prognosis of the paralysis. The patients in Sullivan's study seemed to have milder facial palsy than our patients, because the mean House-Brackmann score for their patients was 3·6 of 6 (the most severe palsy scores as 6), whereas our patients had a mean score of 4·3 of 6. This difference in the scores is consistent with the different populations: our study was in tertiary medical centres whereas Sullivan recruited patients in primary care. Additionally, the methods used to assess paralysis differed between the two studies. We assessed facial paralysis by recording voluntary facial movements, whereas Sullivan used four photographs for assessment, which might have masked small functional defects. Furthermore, we used valaciclovir, a prodrug of aciclovir with higher antiviral activity than aciclovir. These factors might have led to the different conclusions about efficacy.

The treatment strategy for Bell's palsy is to accelerate recovery to prevent further worsening of facial palsy in patients who are only mildly affected, and to avoid sequelae, such as synkinesis, contracture, and facial asymmetry, in more severe cases. The most important issue in the treatment of acute facial palsy is the existence of infection with varicella zoster virus in Bell's palsy. Additionally, Ramsay-Hunt syndrome can be misdiagnosed as Bell's palsy when a patient does not show zoster or vestibulocochlear dysfunction. In our study[13] zoster oticus appeared well after the onset of paralysis in 34% of patients with Ramsay-Hunt syndrome. Because varicella zoster virus is more virulent and causes more severe facial paralysis than herpes simplex virus, differential diagnosis is important to select the appropriate treatment: varicella zoster virus needs higher doses of antiviral than herpes simplex virus. Increasing the dose of antiviral for all patients seems unwise, because higher doses can increase the risk of side-effects, such as acute renal failure and neurological disorders. Higher doses also increase the cost of drug treatment. Various attempts have been made for the prompt detection of DNA fragments of varicella zoster virus by real-time PCR, for example, from the auricular scratch exudates. However, the sensitivity of this test is not high enough at present.

There are no established guidelines for the treatment of Bell's palsy. On the basis of the current published work, we believe that Bell's palsy should be treated by the following protocol. In moderate paralysis, oral administration of prednisolone is sufficient, because such patients usually show excellent prognosis. In severe paralysis, treatment should differ by the timing of initial medication. When treatment starts within 3 days of the onset of paralysis, we prescribe valaciclovir in addition to prednisolone, because of the high incidence of viral infection. In most cases, 1000 mg per day of valaciclovir is given for 5 days, whereas a higher dose of valaciclovir (ie, 3000 mg per day for 7 days) is needed in patients who have the preceding symptoms of reactivation of varicella zoster virus, such as severe auricular pain, stiff neck, and reddish auricle. When treatment starts more than 4 days after the onset of paralysis, we prescribe prednisolone, but antiviral drugs are not used. We believe that the key point in improving the prognosis of Bell's palsy is to diagnose infection with herpes

simplex or varicella zoster viruses earlier and to treat the infection promptly with an adequate dose of an antiviral in combination with steroids.

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Progress or backsliding on HIV and illicit drugs in 2008?

2008 could be a landmark year for progress on a vexing global public-health problem, the use of illicit drugs. Beginning in March, member states of the UN have been asked to engage in a year of reflection to gauge progress in the decade since the 1998 UN General Assembly Special Session on the problem of drugs worldwide.[1]

The 1998 session was convened under the catch-phrase "A drug-free world—we can do it". The declaration from the meeting emphasised the goal of complete eradication of opium poppy, coca, and cannabis production and illicit drug use, with a focus on policing and criminal law.[2] However, the declaration was largely silent on measures to address HIV infection and other health problems for people who are unable or unwilling to stop using drugs.

The printed journal includes an image merely for illustration

Panos Pictures

Drug user at Pakistan Society for Injecting Drug Users, a drop-in centre in Karachi

In 2008, the UN must do better. Epidemics of HIV and hepatitis C driven by injected-drug use should weigh heavily in the considerations of member states on whether they really can—or should try to—achieve a drug-free world.

Despite the political appeal of zero-tolerance policies, UN member states have recognised that measures other than prohibition are required to address massive mortality and morbidity among drug users. For example, the unanimous declaration from the first HIV/AIDS Special Session of the General Assembly in 2001 included a pledge to ensure access to sterile injection equipment for people who use drugs,[3] which was reiterated in 2006. UN agencies have also developed guidance on HIV prevention measures, including sterile-syringe programmes, and WHO added methadone and buprenorphine to its list of essential medicines in 2005.[4]

But some UN-supported drug-control efforts undermine recognition of the importance of HIV prevention measures for people who use drugs. The International Narcotics Control Board (INCB), a

Postischemic mild hypothermia alleviates hearing loss because of transient ischemia

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The effect of postischemic mild hypothermia on the inner ear has not been clarified. In this study, we investigated whether hypothermia after transient ischemia could prevent cochlear damage and its therapeutic time window. Mongolian gerbils were divided into six groups: a sham-operation group, a normothermia group, and four hypothermia groups in which hypothermia was induced 1–7, 1–4, 3–6, and 6–9 h after reperfusion. Animals subjected to postischemic mild hypothermia within 3 h after reperfusion had attenuated hearing loss and inner hair cell loss. The protective effect was greater when hypothermia was induced earlier and had a longer duration. This implies that mild hypothermia after ischemia could have therapeutic effects for inner ear ischemic damage. *NeuroReport* 19:1325–1328 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: cochlear ischemia, hair cell loss, hearing loss, mild hypothermia

Introduction

Hypothermia is thought to be an efficient procedure to alleviate ischemic damage of the brain. Deep hypothermia, however, causes complications such as lowering of blood flow, increased incidence of bacterial infection, and decreased cellular immunity [1–3]. In an experimental study with rats, Busto *et al.* (1989) demonstrated that ischemia-induced damages of the brain were prevented by the application of mild hypothermia [4]. Since then, many investigators studied the protective effects of mild hypothermia on the brain and reported similar results [5,6]. According to a recent clinical study, mild hypothermia increased the chance of recovery in comatose survivors of cardiac arrest [7]. These findings suggested that mild lowering of the body temperature might be sufficient to attenuate ischemic damage of neuronal tissue.

Over the last 10 years, we have studied the inner ear damage because of transient cochlear ischemia in gerbils. In our earlier study, we demonstrated that pre-ischemic mild hypothermia (32°C) was effective in preventing cochlear damage because of ischemia [8]. The effects of mild hypothermia applied after ischemia have, however, not been investigated. The purpose of this study was to determine if postischemic hypothermia could prevent cochlear damage, and if so, to reveal further the effective timing and duration of this procedure.

Materials and methods

The following experiments were conducted in accordance with the Guidelines for Animal Experimentation at Ehime University School of Medicine. Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60–80 g were used. Anesthesia was induced with a mixture of 3% halothane and nitrous oxide/oxygen (7:3) gas and maintained with a mixture of 1% halothane gas. The animals were artificially ventilated using a small tube inserted through the mouth. The tidal volume was set to 1 ml and the rate to 70 times per minute. The Mongolian gerbil congenitally lacks the posterior cerebral communicating arteries and the cochlea receives their blood supply from the bilateral vertebral arteries by way of the basilar artery and the labyrinthine artery. Therefore, simultaneous occlusion of the bilateral vertebral arteries causes cochlear ischemia on both sides. The vertebral arteries were exposed bilaterally and dissected free from the surrounding connective tissues through a ventral midline incision of the neck [9]. Then, 4-0 silk ligatures were loosely looped around each artery. Cochlear ischemia was induced by pulling the ligatures with 5 g weights. After 15 min of ischemia, the sutures were removed to allow recirculation, which was confirmed by microscopic observation.

Hypothermia was introduced by placing the animal in a supine position on an ice bag. The temperature of the animal was monitored with a thermocouple probe (PTI-200, Unique Medical Corporation, Tokyo, Japan) inserted in the rectum. It was controlled by a thermo controller (TACT-2DF, Physitemp Corporation, New Jersey, USA) using a heating plate (HP-1M, Physitemp Corporation) and an ice bag. The rectal temperature was kept at 37 ± 1°C in normothermia, whereas it was kept at 32 ± 1°C in mild hypothermia. The

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animals were divided into four groups on the basis of the timing of the start and end of hypothermia after reperfusion, 1–7 (n=6), 1–4 (n=14), 3–6 (n=8), and 6–9 h (n=8). As controls, some animals were subjected to sham operation (n=4) or normothermia after ischemia (n=16).

The hearing of animals was assessed before and 1, 4, and 7 days after ischemia. Under halothane anesthesia, auditory brain stem responses (ABRs) were recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). The stimulus was introduced into the ear canal via a tiny polypropylene tube; thus each ear was stimulated separately. Recording needle electrodes were placed at the vertex and the retroauricle. In this study, we measured ABRs to 8000 Hz tone burst (0.5 ms rise/fall time and 10 ms duration), as hearing acuity at higher tones is more vulnerable to ischemic insult [10]. Responses to 300 consecutive stimuli were averaged, and the threshold of ABR was determined by measuring responses in 5 dB steps.

For histological study, animals were decapitated under deep anesthesia 7 days after ischemia. After the removal of the otic bullae, the cochleae were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 into the scalae and postfixed for 2 h with the same fixative at 4°C. The specimens were immersed in phosphate-buffered saline (PBS) and the organ of Corti was dissected out by means of surface preparation technique under an operative microscope. The entire lateral and medial wall of the cochlear capsule was opened without disrupting the organ of Corti. Then, the basal turn of the organ of Corti was isolated and photographed with a camera (PM10SP, Olympus, Tokyo, Japan) through a microscope (BX60, Olympus). The specimen was then stained with rhodamine-phalloidin (Molecular Probes, Eugene, Oregon, USA) diluted 250 times in PBS containing 0.25% Triton X-100 and 1% bovine serum albumin for 30 min at room temperature. After rinsing in PBS, it was further stained with Hoechst 33342 (Calbiochem-Navabiochem Corporation, La Jolla, California, USA) dissolved in PBS, in a dark room for 1 h. It was again rinsed in PBS and mounted in carbonate-buffered glycerol (one part 0.5 M carbonate buffer at pH 9.5 to nine parts glycerol) containing 2.5% 1,4-diazabicyclo[2,2,2]octane to retard bleaching of the fluorescent signal. Fluorescence was detected using an Olympus BX60 microscope equipped with a green (BP 546, FT 580, LP 590 nm) and UV (BP 365, FT 395, LP 397 nm) filter. The number of intact and dead hair cells in the basal turn of the cochlea was quantified and the percentage of dead hair cells to whole hair cells was expressed. Earlier, we investigated the number of hair cells at each turn and the average number of inner hair cells (IHCs) at basal turn was about 300 in the gerbils [11]. In this study, we counted only the specimens that had at least 200 IHCs at the basal turn. We evaluated at least 200 of the IHCs at the basal turn.

Statistical differences of the data between each group were evaluated with Kruskal–Wallis test followed by Dunn's multiple comparison test. All values were represented as mean±SD. A P value of less than 0.05 was considered statistically significant.

Results

Sequential changes in ABR thresholds in the six groups are summarized in Fig. 1. In this figure, ABR threshold before the treatment was defined as 0 dB, and the subsequent increase of threshold is shown on the ordinate. In sham-

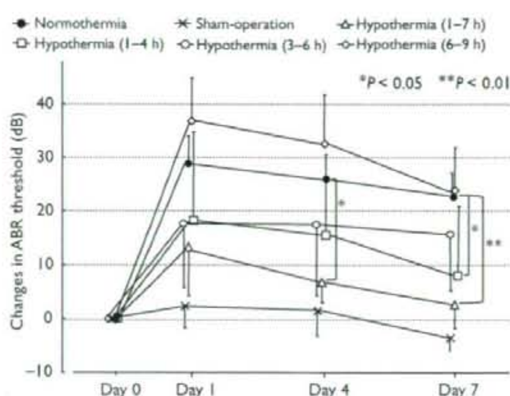

Fig. 1 Plots of brain stem response threshold over 7 days in the normothermic, sham-operated and hypothermic animals. Note that threshold elevations were greatest in the 6–9 h hypothermic animals, then normothermic animals, and lowest in the sham-operated controls. Intermediate thresholds were found in the animals exposed to hypothermia earlier in the procedure. The latter showed greater recovery than either normothermic or 6–9 h hypothermic animals. The data were represented by mean ± 1 SD.

operated controls, no substantial change in ABR was noted after the operation. In the normothermia group, the increase in the ABR threshold was 29.7±9.0 dB (mean±1 SD) on day 1 with a slight recovery on day 4, and recovered to 23.4±8.9 dB on day 7. In the hypothermia groups, the results depended on the timing and duration of hypothermia. When hypothermia was applied within 3 h after reperfusion, the increases in the ABR threshold were attenuated. The alleviating effects were more prominent when the postischemic cooling was initiated promptly and/or applied longer. The average increases in ABR threshold on day 7 in 1–7 h group, 1–4 h group, and 3–6 h group were 2.5±4.2, 7.9±13.4, and 15.6±10.2 dB, respectively. In contrast, a protective effect was not found in 6–9 h group; the average increase on day 7 was 23.8±4.4 dB, which was almost the same as in the normothermia group.

Representative areas of the organ of Corti in each group on day 7 are shown in Fig. 2. Rhodamine-phalloidin staining permits the observation of the hair cell stereocilia, whereas Hoechst 33342 reveals the nuclei. In normothermia group, stereocilia and nuclei of the IHCs disappeared sporadically, as indicated by arrowheads. In contrast, such death of the IHCs was less in hypothermia groups. Sham-operated animals did not show substantial hair cell damage.

Ratios of dead IHCs on day 7 were compared among six groups. As shown in Fig. 3, the mean percentage of IHC loss was 16.3±3.5% in the normothermia group, whereas it was 0.3±0.4% in the sham-operated group. In hypothermia groups, damage of the hair cell was less than in the normothermia group, including the 6–9 h group. The percentages of dead IHCs in the 1–7 h group, 1–4 h group, 3–6 h group, and 6–9 h group were 5.1±2.7, 7.0±3.8, 10.1±4.7, and 13.4±5.6%, respectively. Statistically significant differences were noted between the 1–7 h group and the normothermia group (P<0.001), and between the 1–4 h group and the normothermia group (P<0.01). It should be noted that even 6–9 h of hypothermia was effective in reducing the number of dead IHCs. In all animals, loss of

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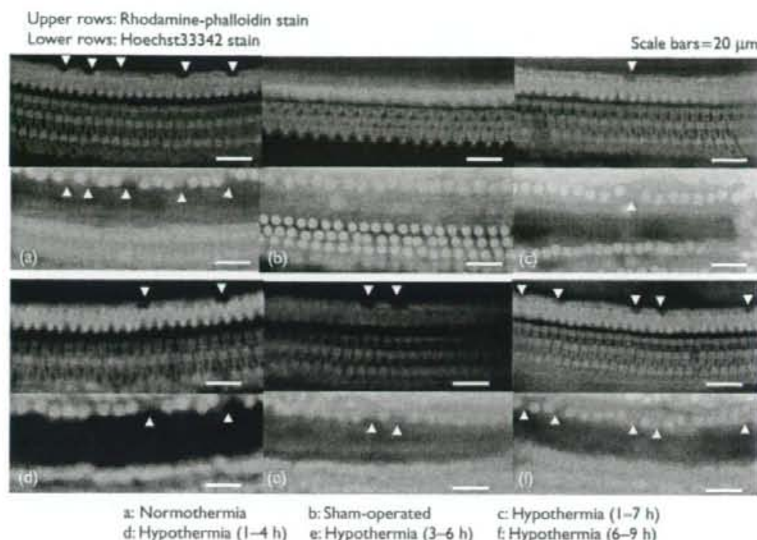

Fig. 2 Epifluorescence images of labeled organ of Corti from animals in each group 7 days after inducing ischemia or the sham operation. The specimen from the normothermia group shows that the stereocilia and nuclei of some inner hair cells (IHCs) had disappeared. In contrast, fewer dead cells were found in the groups subjected to hypothermia from 1 to 7, 1 to 4, and 3 to 6 h. However, the damage in the group that started hypothermia 6 h after reperfusion (i.e. 6–9 h) was similar to that in the normothermia group. Note the outer hair cells were remained almost intact. Scale bar=20 μm. Arrowheads indicate damaged IHCs.

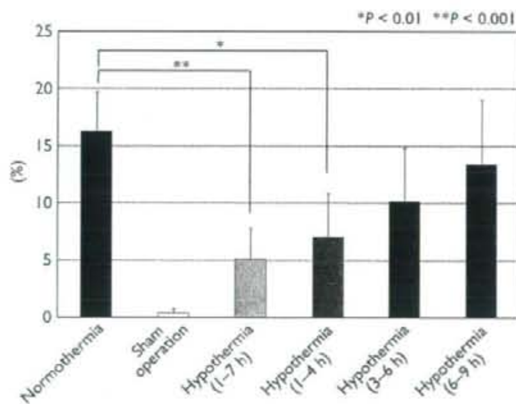

Fig. 3 The percentage of inner hair cells (IHCs) lost 7 days after surgery compared with the total number of IHCs at the basal turn in each group. These data indicated that the ratio of dead IHCs at the basal turn in hypothermic animals was fewer than that in normothermic animals. The protective effects were more prominent after earlier and/or longer application of hypothermia. It should be noted that even 6–9 h of hypothermia was effective in reducing the number of dead IHCs. The data were represented by mean ± 1 SD.

the outer hair cells was fewer than 3% on day 7 (data not shown). No statistical differences in the ratio of outer hair cell loss among the six groups were observed.

Discussion

In this study, postischemic mild hypothermia was effective in alleviating ischemic damage of the cochlea. At present, the protective mechanisms of hypothermia on inner ear tissue remain unclear.

Glutamate, an excitatory neurotransmitter in the cochlea, is thought to play an important role in the pathogenesis of ischemia-induced cochlear damage. In our studies of transient cochlear ischemia in the gerbil [12], we sequentially measured glutamate concentration in the perilymph after loading of ischemia and showed that glutamate levels abruptly increased immediately after ischemia but decreased rapidly thereafter. Watanabe *et al.* [8] demonstrated that hearing loss and the inner ear damage were completely prevented by pre-ischemic mild hypothermia. Hyodo *et al.* [13] considered that such protective effects were primarily through reduction of glutamate efflux, as the increase of glutamate concentration was completely prevented by pre-ischemic hypothermia. In this study, the postischemic hypothermia was started more than 1 h after ischemia when glutamate concentration was supposedly no longer high. This indicates that some mechanisms other than glutamate efflux may be involved in the protective effects of postischemic hypothermia. It may be that the protective mechanism of postischemic hypothermia is different from that of pre-ischemic hypothermia.

Inducible nitric oxide synthase (iNOS), which is generated by ischemic insult and produces excessive amounts of nitric oxide (NO), is considered responsible for delayed neuronal injury [14]. In the brain, the maximal enzymatic activity of iNOS has been noted to occur 1 day after ischemia [15]. Morizane *et al.* [16] showed that immunostaining for iNOS was positive in the organ of Corti and the spiral ganglion 1–4 days after transient ischemia. They further determined that NO concentration in the scala tympani was high on day 1, and returned to pre-ischemic level on day 7. Excessively produced NO leads to the formation of superoxide (O_2^-),

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peroxynitrite ($ONOO^-$), and hydroxyl (OH^-) radicals [17], which are toxic especially to neuronal cells by working as oxidative stresses. According to recent in-vivo studies, hypothermia has been shown to inhibit all of these processes in the brain [18–21]. Thus, the cochlea might be protected by postischemic mild hypothermia through the attenuation of oxidative stress as in the brain.

As revealed in this study, postischemic mild hypothermia attenuated hearing impairment and hair cell loss when the body was cooled within 3 h after reperfusion. The protective effects were more prominent after earlier and/or longer application of hypothermia. Mild hypothermia of 6–9 h attenuated loss of IHCs, although it failed to prevent substantial increase in ABR threshold. The discrepancy between the ABR threshold and hair cell loss is probably because of the fact that the animals could not tolerate long-term experiments and produced weak ABR responses. The effects of postischemic mild hypothermia on the brain have also been studied in experimental cerebral infarction in rats. Ohta *et al.* reported that significant protective effects were observed when hypothermia was started within 4 h after reperfusion; they believe that treatment within 4 h is the therapeutic time window of postischemic mild hypothermia [22]. On the basis of these findings, we believe that mild hypothermia should be applied within 3–4 h after ischemic insult; later application of the procedure would not work well.

From a clinical point of view, mild hypothermia might be a promising option in the treatment of idiopathic sudden sensorineural hearing loss, as cochlear ischemia is considered one of the most possible causes of this disease [23–25]. Furthermore, hypothermia might also be applied in the treatment of other sensorineural hearing losses such as aminoglycoside ototoxicity, traumatic inner ear damage, noise-induced hearing loss, etc. This is because hypothermia has been shown to alter a variety of mechanisms, including metabolic enzymatic activity, inflammatory process, production of reactive oxygen species, and the expression/down-regulation of various genes [18]. Further study will be necessary to elucidate the effects of mild hypothermia on various types of inner ear damage because of causes other than ischemic insult.

Conclusion

Postischemic mild hypothermia is effective in attenuating hearing impairment and hair cell loss when the body was cooled within 3 h after reperfusion. The protective effects are more prominent by earlier and/or longer application of hypothermia. The present results suggested that mild hypothermia might be a promising procedure to attenuate inner ear damage because of idiopathic sudden sensorineural hearing loss that is supposed to be caused by cochlear ischemia.

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Bone Marrow-Derived Cells Expressing Iba1 Are Constitutively Present as Resident Tissue Macrophages in the Mouse Cochlea

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Immune-mediated inner ear disorder has been well established as a clinical entity; however, the innate immune system of the inner ear is a poorly understood area of research with high clinical and immunological importance. Although the presence of resident tissue macrophages in the inner ear has been suggested, there has been some controversy. In this study, we analyzed the origin of cochlear resident macrophages and the contribution of hematopoietic bone marrow (BM) to the recruitment of macrophages in the cochlea. To visualize the localization of BM-derived cells, BM chimeric mice were made by transplantation of hematopoietic stem cells, which were genetically labeled with enhanced green fluorescent protein, into lethally irradiated C57BL/6 mice. The distribution and characteristics of BM-derived cells in the mouse cochlea were studied immunohistochemically. We successfully identified the constitutive presence of tissue resident macrophages in the spiral ligament and spiral ganglion that are derived from BM in larger numbers than previously reported. Moreover, cochlear resident macrophages gradually turn over for several months during steady-state replacement by BM-derived cells, and the number of cochlear macrophages immediately increased in response to local surgical stress. The present findings demonstrate the hematopoietic origin of cochlear resident and infiltrating macrophages. Our study provides a novel anatomical and immunological basis for the inner ear and indicates that the cochlear resident macrophages would be a therapeutic target in inner ear disorders. © 2008 Wiley-Liss, Inc.

Key words: hematopoietic stem cell; microglia; innate immunity; inner ear

Macrophages are generally considered to be derived from circulating monocytes and roughly classified into two categories; 1) infiltrating macrophages, which migrate from the circulation into tissues in response to inflammatory signals, and 2) resident tissue macrophages, which are present in tissues during steady-state conditions. Resident tissue macrophages take up residence in virtually every tissue of the body and have a broad role in the innate immune system. Recent studies have demonstrated multiple key functions of resident tissue macrophages not only in phagocytosis of foreign bodies or senescent cells but also in the production and secretion of cytokines and the regulation of specific immune responses (Gordon and Taylor, 2005).

The inner ear was once believed to be an immunoprivileged organ isolated by the blood–labyrinthine barrier similar to the central nervous system (CNS) and the cornea and retina of the eye. Although immune-mediated inner ear disorders have been well established as a clinical entity with progressive and fluctuating bilateral sensorineural hearing loss (SNHL), the innate immune system of the inner ear is a poorly understood area of research with high clinical and immunological importance. Recently, Hirose et al. (2005) reported the existence of mononuclear phagocytes in the spiral ligament of nonnoise-exposed $CX3CR1^{GFP/GFP}$ transgenic mice, and the density of CD45-positive cells in the cochlea was quite different between wild-type and transgenic mice used in the study. Lang et al. (2006) also reported that bone marrow (BM)-derived cells are present in the noninjured inner ear of BM chimeric mice and that 5% of BM-derived cells are macrophages dual-labeled with CD45R and/or F4/80. However, the phenotypes shown by BM-derived cells in the inner ear are as yet only partially understood. Whereas previous studies have reported macrophages infiltrating into the coch-

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lear fluid space in various experimental models, or suggested the presence of resident tissue macrophages in the inner ear, there has been some controversy on the distribution and phenotype of resident tissue macrophages in the inner ear.

A good understanding of the origins and distribution of the resident tissue macrophages in the inner ear, as well as the timing and context of their recruitment, will be essential to understanding the pathogenensis of inner ear immune disorders in which a loss of tissue homeostasis might result from dysfunction of resident tissue macrophages. The aims of the present study were to establish the distribution and phenotype of resident macrophages in the cochlea and to test the contribution of hematopoietic BM to the recruitment of cochlear macrophages.

BM chimeric mice were made by transplantation of hematopoietic stem cells (HSCs) from enhanced green fluorescent protein (EGFP)-transgenic mice into irradiated adult wild-type mice, and the distribution of BM-derived cells in the cochlea was traced. Immunohistochemistry was employed to determine the phenotype of BM-derived cells. The mobilization of cochlear macrophages was tested by using systemic application of macrophage colony-stimulating factor (M-CSF), the primary regulator of the activation of mononuclear phagocytes. We also examined the response of cochlear macrophages to local surgical invasion, used as a model for an acute, local, exogenous stress on the inner ear.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc (Shizuoka, Japan). EGFP transgenic mice [B6;C3-Tg(ACtb-EGFP)CX-FM139Osb] were used as a source of HSCs (Okabe et al., 1997). The animals were maintained in a specific pathogen-free microisolator environment in the Institute of Laboratory Animals, Kyoto University Graduate School of Medicine. All experimental protocols were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine, and conducted in accordance with the NIH *Guide for the care and use of laboratory animals*.

BM Chimeric Mice

HSCs were collected as lineage marker (Lin)-negative, c-kit (CD117)-positive, stem cell antigen 1 (Sca1)-positive cells from the BM of EGFP transgenic mice via cell sorting on a FACS Vantage (Becton-Dickinson, San Jose, CA) as described previously (Yoshimoto et al., 2003). Briefly, whole BM cells were isolated from the femurs and tibiae of EGFP transgenic mice (8–12 weeks of age). BM mononuclear cells were labeled with a primary antibody cocktail (BD Pharmingen, San Diego, CA) for CD3 (145-2C11), B220/CD45R (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), and TER119 (TR119). Lineage-depleted cells (Lin^- cells) were obtained by using auto-MACS (Militenyi Biotec, Bergish Gladbach, Germany). Lin^-/c-kit^+/$Sca1^+$ cells were collected by cell sorting on a FACS Vantage as HSCs using R-PE-conjugated anti-mouse Ly-6A/E (Sca-1; clone: E13-161.7; BD Pharmingen) and APC-conjugated anti-mouse CD117 (c-Kit; clone: 2B8; BD Pharmingen). C57BL/6 mice (n = 6, 10–12 weeks of age) were irradiated with 9.5 Gy gamma rays (Gamma Cell 40 Exactor; MDS Nordion Inc., Ottawa, Ontario, Canada) and each received 5×10^3 HSCs through the tail vein. At 1 (four ears from two animals), 2 (four ears from two animals), or 4 weeks (four ears from two animals) and 3 (6 ears from 6 animals), or 6 months (six ears from six animals) after transplantation, the temporal bones were dissected out under overdose anesthesia. Peripheral blood samples were also collected from the animals 3 months after transplantation to determine the chimeric ratio of blood cells.

Systemic Application of M-CSF

C57BL/6 (10 weeks of age, n = 5) mice received an intraperitoneal injection of M-CSF (Kyowa Hakko Kogyo, Tokyo, Japan) dissolved in physiological saline once per day for 7 consecutive days (total dose 7×10^5 units). The dose of M-CSF was equivalent to that used in a clinical setting, relative to body weight. On the day after M-CSF application, the temporal bones were collected and prepared for cryostat sections. Animals (n = 5) receiving physiological saline instead of M-CSF were used as controls.

Local Surgical Treatment

C57BL/6 mice (10 weeks of age) were injected with 3 μl physiological saline into the posterior semicircular canal (PSCC). This minimally invasive treatment was a modified protocol used in previous studies when administrating drugs (Lee et al., 2003; Nakagawa et al., 2003; Kim et al., 2005) or cells (Iguchi et al., 2003; Okano et al., 2006) into the inner ear of mice. With animals under general anesthesia with ketamine (75 mg/kg) and xylazine (9 mg/kg), a retroauricular incision was made in the left ear, and the PSCC was exposed. A small hole (approximately 180 μm in diameter) was made in the bony wall of the PSCC with a 26-G needle. A fused silica glass needle (170 μm outer diameter; EiCOM, Kyoto, Japan) was then inserted into the perilymphatic space of the PSCC, and the solution was injected at a rate of 1 μl/min for 3 min using a Micro Syringe Pump (EiCOM). The hole in the PSCC was plugged with connective tissue and covered with fibrin glue. On days 1 (n = 4), 7 (n = 5), and 28 (n = 5), the left temporal bones were collected and prepared as cryostat sections to study the density of Iba1-positive cells in the cochlea. Temporal bones collected from age-matched normal animals were used as preoperative controls (n = 5).

The auditory function of experimental animals was monitored by auditory brainstem response (ABR) recording as described previously (Shiga et al., 2005). ABRs were recorded before treatment and on days 1, 7, and 28 after surgery. Thresholds were determined for frequencies of 10, 20, and 40 kHz.

The mobilization of Iba1-positive cells from BM to the cochlea were also examined in BM chimeric mice (n = 8) that had received EGFP-labeled HSCs 3 months before. Four animals received a saline injection into the PSCC as described

above, and the cochleae were collected 7 days after surgery. The remaining four animals were preserved as controls, receiving no surgical treatment.

Immunohistochemistry

Under general anesthesia with ketamine and xylazine, animals were perfused intracardially with ice-cooled phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer. The temporal bones were collected and immersed in the same fixative for 4 hr at 4°C. The samples were decalcified with 10% EDTA in PBS and cryoprotected with 30% sucrose. Specimens were prepared as cryostat sections (10 μm in thickness). Midmodiolar sections were provided for histological analyses.

Cryostat sections were immersed in blocking solution containing 10% goat serum for 30 min and incubated with a primary antibody at 4°C overnight. Characteristics of BM-derived cells were examined by immunostaining for leukocyte common antigen CD45; ionized calcium binding adapter molecule 1 (Iba1), which is specific for microglia/macrophages (Imai et al., 1996); microglia/macrophage-specific glycoprotein F4/80; and macrosialin CD68, which is specifically expressed by tissue macrophages. Immunohistochemistry for Ki67, a nuclear protein expressed in proliferating cells, was performed on the BM chimeric mice to determine the proliferation of BM-derived cells in situ. The primary antibodies used in this study were rat anti-mouse CD45 (1:50; 30-F11; BD Pharmingen), rabbit anti-Iba1 (1:1,000; Wako Pure Chemicals, Osaka, Japan), rat anti-mouse F4/80 (1:10; Cl:A3-1; Serotec, Oxford, United Kingdom), rat anti-mouse CD68 (1:1,000; FA-11; Serotec), and rabbit anti-Ki67 (1:200; SP6; Lab Vision, Fremont, CA). The localization of primary antibodies was visualized using secondary antibodies conjugated with Alexa Fluor 488, 555, or 633 (1:500; Molecular Probes, Eugene, OR). Nuclei were counterstained by 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Negative controls lacked primary antibody labeling. Specimens were viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) or a Leica TCS-SP2 confocal laser scanning microscope (Leica Microsystems, Tokyo, Japan) with a digital image-capture system.

Quantification

To determine the chimeric ratio in peripheral blood, smears of blood samples were made on slides. Total cells with nuclei were based on nuclear counts with DAPI staining observed with a fluorescence microscope. The ratio of EGFP-positive cells to the total number of cells was calculated. At least 200 nuclei were counted in each sample.

For the quantitatification of BM-derived cells or Iba1-positive cells, four sections were selected randomly from the 12 most midmodiolar sections for each experimental or control animal. To assess the distribution of BM-derived cells in the cochlea, the number of cells derived from engrafted HSCs in one midmodiolar section from base to apex was counted in six animals at 6 months after HSC transplantation. All BM-derived cells defined by coexpression of EGFP and DAPI within the cochlea were counted by two double-blinded examiners. The number of HSC-derived cells coexpressing Iba1, F4/80, CD45, or CD68 was also counted for examination of the phenotype of BM-derived cells. The ratio of EGFP-positive cells labeled with Iba1, F4/80, CD45, or CD68 to the total number of EGFP-positive cells was calculated. To study the replacement of Iba1-positive cells in the cochlea by engrafted EGFP-positive cells, the number of cells dual-labeled with EGFP and Iba1 in one midmodiolar section was counted. The ratio of the expression of EGFP to the total number of Iba1-positive cells was calculated in the cochleae harvested at 1, 2, or 4 weeks and 3, or 6 months after HSC transplantation. To investigate the dynamics of EGFP- or Iba1-positive cells in the cochlea, the density of EGFP- or Iba1-positive cells in SG was calculated by a modified method as described previously for evaluating the density of SG neurons (Shinohara et al., 2002). All EGFP- or Iba1-positive cells with nuclei stained for DAPI within each profile of Rosenthal's canal from the midbasal portion of the cochlea were counted. The outline of Rosenthal's canal profile was then traced under a brightfield image to generate the area of SG in Image J software (http://www.nist.gov/lispix/imlab/prelim/dnld.html). The density of EGFP- or Iba1-positive cells in SG was expressed as the cell number for an area of 10,000 μm^2. The density of EGFP- or Iba1-positive cells in SL of midbasal portion of the cochlea was also calculated by tracing the outline of the SL region occupied by type I-V fibrocytes and expressed as the cell number for an area of 10,000 μm^2. The number of total cells in the area of interest was determined by counting nuclei on DAPI-stained sections in Image J software. The number of proliferating macrophages in the cochlea was determined by counting the colocalization of Ki67 and DAPI. The ratio of EGFP-positive cells labeled with Ki67 to the total number of CD68-positive cells per section was also calculated.

Statistcal Analysis

Statistical analyses were performed by using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer's test, for the analysis of ABR thresholds, alteration of Iba1-positive cells by EGFP-positive cells, and effect of local surgical stress. An unpaired *t*-test was used in other statistical analyses. $P < 0.05$ was considered statistically significant. All data are presented as the mean ± SE.

RESULTS

BM-Derived Cells Are Widely Distributed in the Spiral Ganglion and the Spiral Ligament

Six months after BM transplantation, numerous EGFP-positive cells were found widely within the cochlea of transplanted mice (Fig. 1a). A large population of EGFP-positive cells is located in the connective tissue of SL and auditory nervous system, including SG and acoustic nerve (AN; Fig. 1b–d). In SL, EGFP-positive cells were observed predominantly in its lower portion, corresponding to the type II and IV fibrocyte regions (Fig. 1b). In the auditory nervous system, EGFP-positive cells were observed along nerve fibers both in SG and

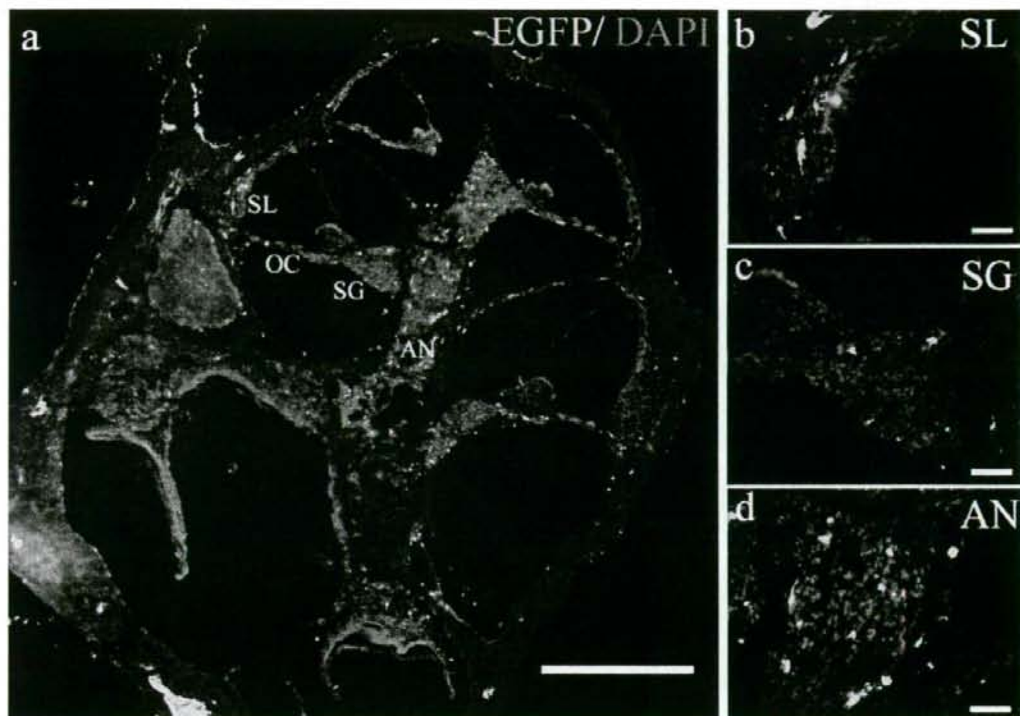

Fig. 1. Distribution of hematopoietic cell-derived cells in the cochlea. **a:** Distribution of hematopoietic cell-derived cells is shown in a midmodiolar section obtained from bone marrow (BM) chimeric mice 6 months after transplantation. BM-derived cells expressing EGFP were distributed from the base to the apex of the cochlea. Blue fluorescence shows nuclear staining with DAPI. **b–d:** In SL, EGFP-positive cells were observed predominantly in the lower portion of SL occupied by type II and IV fibrocytes (b). In the auditory nervous system, EGFP-positive cells were observed along nerve fibers in both SG (c) and cochlear modiolus (d). AN, auditory nerve; OC, organ of Corti; SG, spiral ganglion; SL, spiral ligament. Scale bars = 500 μm in a; 50 μm in b–d.

AN (Fig. 1c,d). In one midmodiolar section obtained at 6 months after transplantation, 90.3 ± 6.9 cells expressing EGFP were observed, 35.6% ± 3.1% of which were in the cochlear connective tissues of SL and the spiral limbus and 62.2% ± 3.0% in the cochlear nervous system, including SG and AN. The proportion of BM-derived cells to the total cells was 7.7% ± 0.9% in SL and 5.5% ± 0.9% in SG, which was compatible with the study by Lang et al. (2006). EGFP-positive cells were occasionally identified on the undersurface of the basilar membrane in the scala tympani and in the stria vascularis (data not shown). No EGFP-positive cells were observed within the cochlear sensory epithelium.

More Than 80% of BM-Derived Cells in the Cochlea Demonstrated the Phenotype of Macrophages

The following analyses of immunohistochemistry were carried out to characterize hematopoietic BM-derived cells in the adult mouse cochlea. Most EGFP-positive cells expressed F4/80 (Fig. 2a), Iba1 (Fig. 2b), or CD68 (Fig. 2c), indicating that cochlear HSC-derived cells have differentiated into the macrophage lineage. Cells dually labeled with EGFP and Iba1, EGFP, or F4/80 or with EGFP and CD68 were localized both in the cochlear connective tissue and the cochlear nervous system. The expression of Iba1 was found in 92.5% ± 3.1% of EGFP-positive cells, and F4/80 expression was observed in 87.3% ± 4.2%. Although more than 80% of BM-derived cells in the cochlea demonstrated the phenotype of macrophage, immunoreactivity for CD45, a common leukocyte antigen, was identified in only 7.4% ± 0.5% of EGFP-positive cells in the cochlea of BM chimeric mice. The number of cells doubly stained with Iba1 and CD45 was limited to one or two in one section (0.9% ± 0.3% of the total number of Iba1-positive BM-derived cells) except for cells in BM of the temporal bone. In contrast, CD68 expression was found in 81.7%

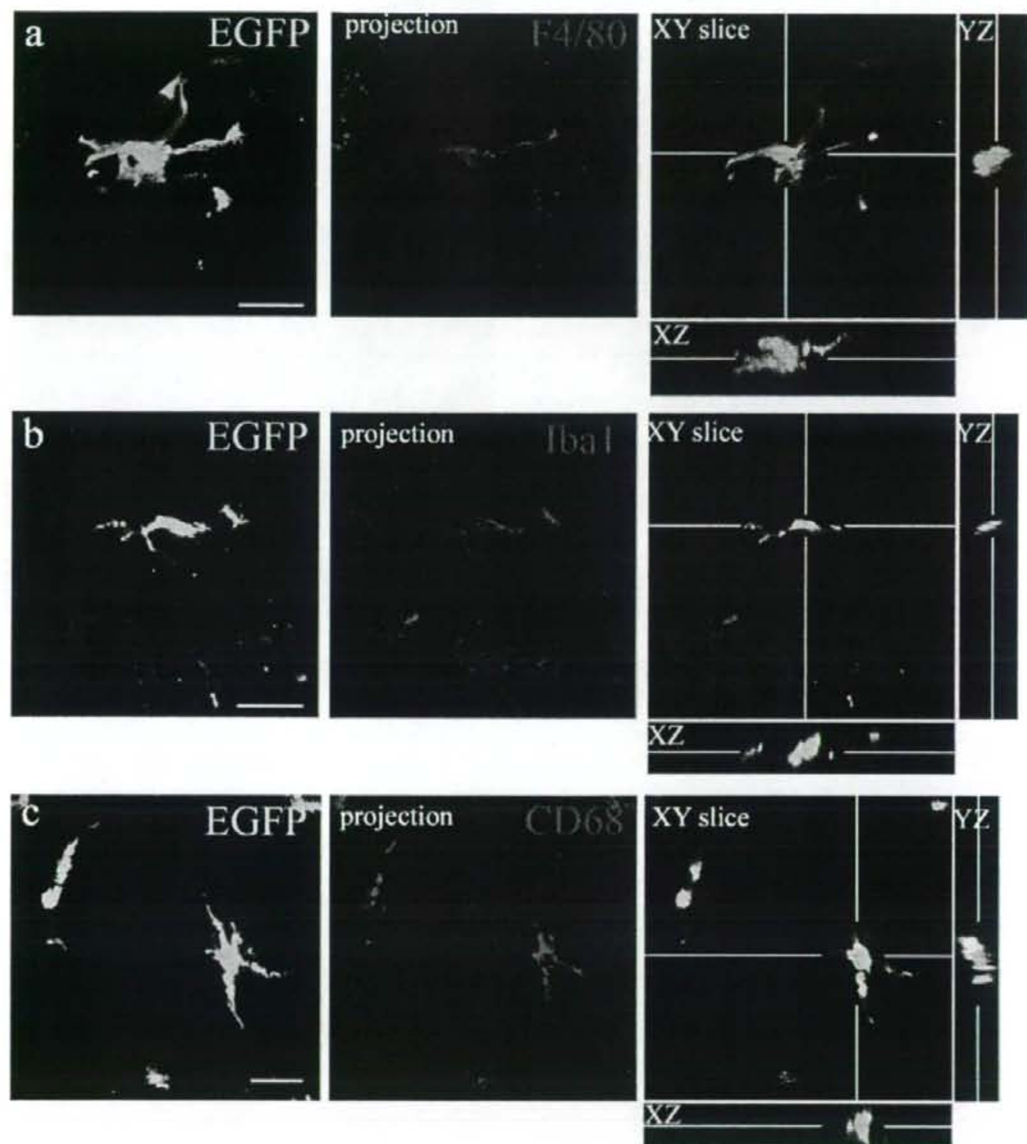

Fig. 2. Immunohistochemistry for F4/80, Iba1, and CD68 in the cochleae of bone marrow chimeric mice 6 months after transplantation. **a:** Photomicrographs obtained by confocal microscopy demonstrate colocalization of EGFP and F4/80 in the cells derived from transplanted HSCs within the spiral ganglion (SG). Immunoreactivity for F4/80 was frequently observed in HSC-derived cells in the lower part of spiral ligament (SL) and the SG. **b:** Iba1 expression was also found in HSC-derived cells in the SL and SG. With confocal microscopy, HSC-derived cells in SL are found to be dually labeled with EGFP and Iba1. **c:** CD68 was also colocalized in BM-derived cells expressing Iba1 in SL. Scale bars = 20 μm.