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.

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

Panos Pictures

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

Expert Opinion

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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

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 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 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 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 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

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 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 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 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 (88%) in the treatment group recovered completely, as compared with 38 patients (68%) in the control group ($p < 0.05$). 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 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 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 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), 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 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 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 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 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

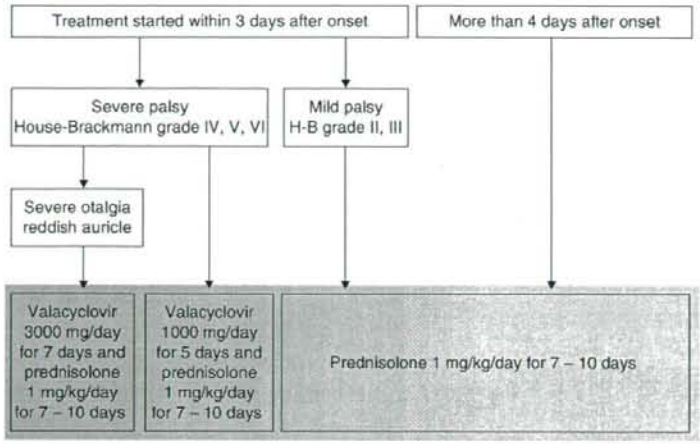

Figure 1. Summary of recommendation for initial treatment of Bell's palsy.

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.

5. Expert opinion

Fortunately, Bell's palsy is largely benign, especially when compared with Ramsay Hunt syndrome, and only 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 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 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, oral administration of prednisolone (1 mg/kg/day for 7 – 10 days) is sufficient because such cases typically show 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, 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 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.

Declaration of interest

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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

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-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.

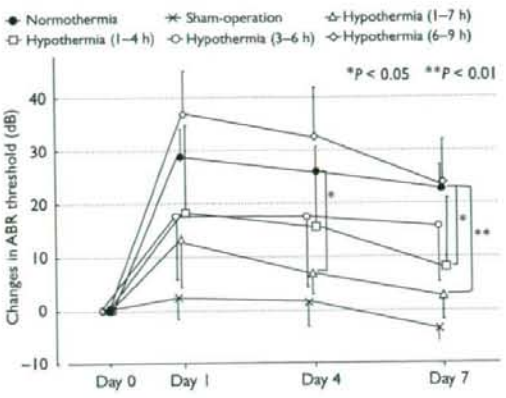

Fig. 1 Plots of brain stem response threshold over 7 days in the normothermic, sham-operated and hypothermic animals. Note that threshold elevations were greatest in the 6–9 h hypothermic animals, then normothermic animals, and lowest in the sham-operated controls. Intermediate thresholds were found 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.

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

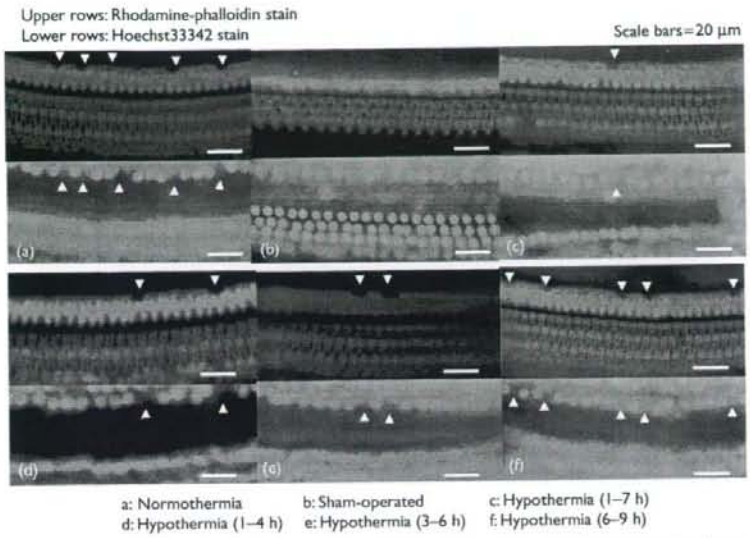

Fig. 2 Epifluorescence images of labeled organ of Corti from animals in each group 7 days after inducing ischemia or the sham operation. The specimen from the normothermia group shows that the stereocilia and nuclei of some inner hair cells (IHCs) had disappeared. In contrast, fewer dead cells were found 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.

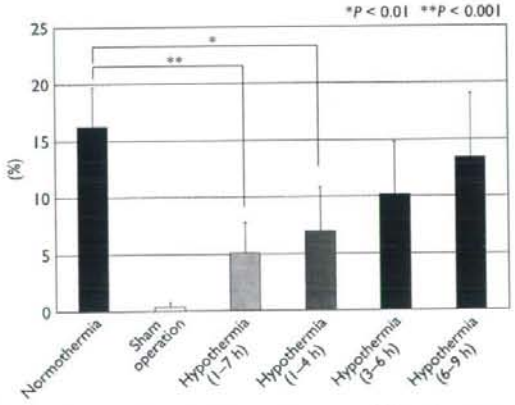

Fig. 3 The percentage of inner hair cells (IHCs) lost 7 days after surgery compared with the total number of IHCs at the basal turn in each group. These data indicated that the ratio of dead IHCs at the basal turn in hypothermic animals was fewer than that in normothermic animals. The protective effects 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^-),

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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ORIGINAL ARTICLE

Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs

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Abstract
Conclusion: Local application of hepatocyte growth factor using biodegradable gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Objectives:* To develop an inner ear drug delivery system using gelatin hydrogels that is capable of a sustained delivery of growth factors to the cochlea. We examined the efficacy of the local application of gelatin hydrogels containing hepatocyte growth factor (HGF) in protecting cochlear hair cells from noise-induced damage. *Materials and methods:* A piece of gelatin hydrogel previously immersed in either HGF or saline was placed on the round window membrane of a guinea pig 1 h after noise exposure (4 kHz octave band noise at 120 dB sound pressure level for 3 h). Auditory function was monitored using auditory brainstem responses (ABRs), and the loss of hair cells was evaluated quantitatively. *Results:* Local HGF treatment significantly reduced the noise exposure-caused ABR threshold shifts and the loss of outer hair cells in the basal portion of the cochleae.

Keywords: *Cochlea, drug delivery, growth factor, protection, hair cell*

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities. However, available therapeutic options are limited to hearing aids and cochlear implants. Therefore, many investigations have concentrated on finding novel therapeutic molecules that could possibly be used in the treatment of SNHL. These studies have discovered several agents that exhibit therapeutic activity against SNHL. Despite such basic research progress, the translation of these basic findings into useful therapeutic clinical agents has yet to be achieved. One considerable obstacle to the development of such clinical applications revolves around the current lack of a safe and effective method for drug delivery to the cochlea. As a way of resolving this, we have developed a new method for local inner ear treatment that uses gelatin hydrogel as the inner ear drug delivery system [1]. Biodegradable gelatin hydrogel has been used previously for the sustained release of proteins or peptides, including growth and trophic factors [2]. We have previously demonstrated the efficacy of gelatin hydrogels in the sustained delivery of brain-derived neurotrophic factor [3] and insulin-like growth factor 1 (IGF-1) [4,5] in animal experiments. In addition, we are currently performing a clinical trial designed to examine local IGF-1 therapy that uses gelatin hydrogels for treating acute SNHL (http://www.kuhp.kyoto-u.ac.jp/~ent/ClinicalTrial/Gel_Eng.html).

Hepatocyte growth factor (HGF) was originally identified as the protein that is responsible for stimulating hepatocyte proliferation [6]. It is present in various cells and is a paracrine cellular growth and morphogenetic factor [7,8]. Hearing impairment caused by aminoglycosides is ameliorated after the transfer of the HGF gene to the inner ear via an

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intrathecal injection of the viral vector [9]. The HGF gene transfer for the treatment of SNHL has been published and patented (US Patent 7390482). Thus, local, sustained application of rhHGF might be effective for the treatment of SNHL and could potentially be approved for clinical applications in the near future.

Previous reports have documented the potential use of gelatin hydrogel for a sustained release of HGF [2,10]. Therefore, based on the previous reported data, we designed the current study to examine the efficacy of using gelatin hydrogels for local rhHGF application to treat noise-induced hearing loss (NIHL) in guinea pigs.

Materials and methods

Experimental animals

A total of 18 male 4-week-old adult Hartley guinea pigs weighing 300–350 g (Japan SLC, Hamamatsu, Japan) served as the experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at the Kyoto University Graduate School of Medicine. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Biodegradable gelatin hydrogels

The biodegradable hydrogels were prepared as described previously [3–5]. Since other studies have analyzed the in vitro HGF release profiles from hydrogels and demonstrated that a hydrogel made with 10 mM glutaraldehyde allows for optimal HGF delivery [2,10], we designed the present study to use the same type of hydrogel.

Noise exposure and drug application

Baseline auditory brainstem response (ABR) thresholds were measured just before the noise exposure. Animals were then exposed to a 4 kHz octave band noise at 120 dB sound pressure level for 3 h in a ventilated sound exposure chamber. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity.

A 2 mm^3 piece of hydrogel was immersed in 20 µl physiological saline that contained either 1.0 µg/µl rhHGF or physiologic saline alone (control). Under general anesthesia using midazolam (2 mg/kg, intramuscular; Astellas, Tokyo, Japan) and xylazine (2 mg/kg, intramuscular; Bayer, Tokyo, Japan), the piece of hydrogel was then placed on the round window membrane in the left ear of the animals 1 h after the noise exposure ($n = 6$ for each group).

Functional analysis

ABRs were measured to assess the auditory function, with the ABR threshold measurements performed at the 4, 8, and 16 kHz frequencies. ABRs were obtained before and after exposure to the noise, and on days 3, 7, 14, and 21 after the drug application. Animals were anesthetized using midazolam and xylazine and kept warm using a heating pad. Generation of acoustic stimuli and the recordings of the evoked potentials were performed using a PowerLab/4sp (AD Instruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (0.1 ms cos^2 rise/fall with a 1 ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) that was connected to a funnel fitted to the external auditory meatus. To record bioelectrical potentials, subdermal stainless steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active) and contralateral to the measured ear (reference). Stimuli were calibrated against a ¼-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). Responses between the vertex and mastoid subcutaneous electrodes were amplified using a digital amplifier (MA2; Tucker-Davis Technologies, Alachua, FL, USA). Thresholds were determined from a set of responses at varying intensities with 5 dB SPL intervals. Electrical signals were averaged for 1024 repetitions. Thresholds at each frequency were verified at least twice.

Histological analysis

On day 21 after the drug application, animals were deeply anesthetized with midazolam and xylazine and the cochleae were exposed. After removal of otic vesicles, 4% paraformaldehyde in 0.01 mol/l phosphate-buffered saline (PBS) at pH 7.4 was gently introduced into the perilymphatic space of the cochleae. Temporal bones were then excised and immersed in the same fixative at 4°C for 4 h. After rinsing with PBS, cochleae were dissected from temporal bones and subjected to histological analysis in whole mounts. To quantitatively assess the hair cell loss, we examined three regions of the cochlear sensory epithelia that were at a distance of 40–60%, 60–80% or 80–100% from the apex.

Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were performed to label the surviving inner hair cells (IHCs) and outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience, Ramona, CA, USA) was used as the primary antibody, and Alexa-546-conjugated anti-rabbit goat IgG (1:500; Molecular Probe, Eugene, OR, USA) was used as the secondary antibody. Following immunostaining for myosin VIIa, specimens were then stained with FITC-conjugated phalloidin (1:300; Molecular Probe). Specimens were viewed under a confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany). To test the non-specific labeling, the primary antibody was omitted from the staining procedures. Three authors (T.I., T.N., and Y.S.K.) counted the numbers of IHCs and OHCs in 0.2 mm long regions of the apical, middle or basal portions of the cochleae. The average of the values was used as the data for each animal.

Statistical analysis

Overall effects of rhHGF application on ABR threshold shifts were examined using a two-way factorial analysis of variance. When interactions were significant, multiple comparisons with Fisher's protected least significant difference (PLSD) were used for pairwise comparisons. Differences in the IHC and OHC numbers for each region of the cochlea between the rhHGF- and saline-treated cochleae groups were examined using a Student's t test. Values of $p<0.05$ were considered statistically significant. Values are expressed as the mean $\pm$ the standard error.

Results

Auditory function

Time courses of the alterations in the ABR threshold shifts at 4, 8, and 16 kHz after the application of rhHGF or saline are shown in Figure 1. Local application of rhHGF showed a significant effect on the reduction of the ABR threshold shifts at the 16 kHz frequency ($p=0.030$). There was also a significant difference in threshold shifts on day 21 between the rhHGF- and saline-treated animals, as shown by the Fisher's PLSD test ($p=0.045$). No significant differences were found for the threshold shifts between the two groups at 4 or 8 kHz.

Histological protection

Immunostaining for myosin VIIa and phalloidin staining demonstrated partial degeneration of the OHCs in the 60–80% distance regions from the apex

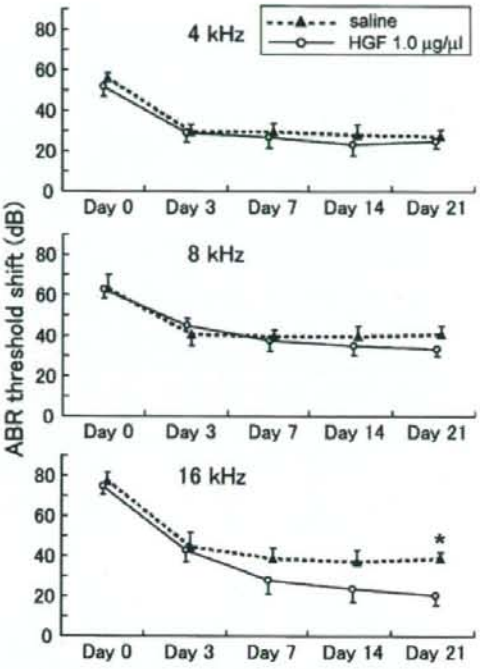

Figure 1. ABR threshold shifts after noise exposure in saline- and HGF-treated animals. An overall effect of HGF application is significant at 16 kHz (two factorial ANOVA, $p=0.030$), not at 4 or 8 kHz. The difference in threshold shifts between saline- and HGF-treated animals is significant on day 21 at 16 kHz. *$p=0.045$, Fisher's PLSD.

in the saline-treated cochleae (Figure 2A). The same region for the 1.0 μg/μl rhHGF-treated cochleae exhibited almost normal morphology (Figure 2B). In both experimental groups, OHC loss was not apparent in the 40–60% or 80–100% distance regions from the apex. IHCs were well maintained in every region of the cochleae in both groups. Quantitative assessments revealed a significant difference in OHC numbers in the 60–80% distance region from the apex between the saline- and rhHGF-treated cochleae (Figure 3, $p=0.003$). No significant differences in OHC numbers were observed in the 40–60% or 80–100% distance regions. There were also no significant differences in the IHC numbers noted in any of the cochleae regions between the two experimental groups.

Discussion

Our findings indicate that local application of rhHGF using biodegradable gelatin hydrogels is effective in the attenuation of OHC damage due to noise trauma, resulting in the reduction of ABR

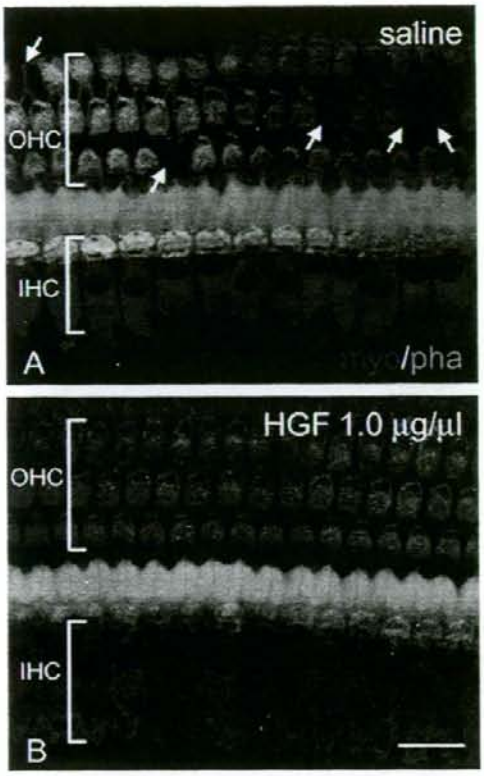

Figure 2. Immunostaining for myosin VIIa (myo) and phalloidin staining (pha) demonstrated loss of outer hair cells (OHC) in the upper basal portion of the saline-treated cochlea (A) and preservation of OHC in that of the HGF-treated cochlea (B). Arrows indicate loss of OHC. IHC, inner hair cells. Scale bar represents 20 μm.

thresholds. ABR measurements demonstrated that post-traumatic local application of rhHGF via gelatin hydrogels had a significant effect on the attenuation of threshold shifts at 16 kHz. Histological analyses demonstrated significant protection of the OHCs in the 60–80% distance from the apex, which is the region responsible for the 10–20 kHz hearing range [11].

Our previous study using IGF-1 indicated that there was a significant reduction of ABR threshold shifts at 4 or 8 kHz [9]. The present findings demonstrated that local HGF treatment caused significant effects at 16 kHz. The spread of the growth factors from the base to the apex of the cochlea occurred by diffusion. Thus, the molecular weights of growth factors could influence the distribution of these factors within the cochlea. The molecular weight of HGF is 69 kDa for the α-subunit and 34 kDa for the β-subunit, while that for

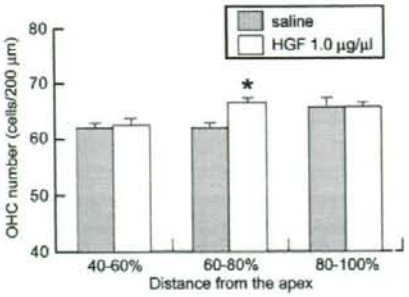

Figure 3. Means of numbers of surviving outer hair cells (OHCs) in saline- and HGF-treated cochleae. In the 60–80% distance region from the apex, the value of HGF-treated cochleae is significantly higher than that of saline-treated cochleae. $^{*}p = 0.003$, t test. Bars represent standard errors.

IGF-1 is 7.6 kDa. Therefore, HGF may be abundantly distributed in the more basal portions of the cochlea as compared with that seen for the IGF-1 distribution.

Previous studies have demonstrated that several agents ameliorate NIHL when they are applied before noise exposure; however, only limited agents including IGF-1 [5] show protective effects by post-exposure administration. Local application of D-Jun N-terminal kinase-1 (D-JNK-1) peptide, an inhibitor of c-Jun N-terminal kinase, 12 h after noise exposure attenuates NIHL [12]. The efficacy of D-JNK-1 peptide has been demonstrated by application via an osmotic mini-pump or a hyaluronic acid gel. In the current study, we used the gelatin hydrogel for sustained delivery of rhHGF into the cochlea. This system may also be utilized for local delivery of D-JNK-1 peptide, because the gelatin hydrogel is suitable for sustained delivery of peptides [1,2]. The efficacy of local D-JNK-1 peptide application via gelatin hydrogels will be evaluated in the near future. Post-exposure administration of edaravone, a free radical scavenger, also rescues cochleae from NIHL [13]. Locally applied edaravone via an osmotic mini-pump can rescue OHCs even when it is applied 21 h after noise exposure. Edaravone is clinically available; however, how to deliver edaravone into the cochlea continuously is an obstacle for clinical use. Gelatin hydrogels are not suitable for sustained delivery of edaravone, because edaravone is not soluble in water [1,2]. Therefore, drug delivery systems that fit for edaravone should be developed before clinical application of local edaravone treatment.

The mechanisms of cochlear hair cell protection by HGF are not well understood. The cochlear hair cells are degraded through the process of apoptosis after exposure to intense noise [14]. Exposure to intense sound causes production of hydroxyl radicals

in the cochlear hair cells [15], which leads to peroxidation of the mitochondrial membrane and the release of cytochrome *c* from the mitochondria to the cytosol. The Bcl-2 family proteins, Bcl-xL and Bak, are produced in the hair cells following noise exposure, and it is the balance of these two proteins that is responsible for the regulation of this process [16]. Predominance of Bcl-xL, which is an anti-apoptotic member of the Bcl-2 family, results in the suppression of the cytochrome *c* release, whereas a predominance of the pro-apoptotic member, Bak, leads to the promotion of the cytochrome *c* release. HGF is known to up-regulate Bcl-xL, which is mediated by the phosphorylation of STAT3 [17]. Therefore, OHCs might be protected against noise through the same pathway. HGF also has anti-oxidant activity [18], which contributes to the protection of cells from apoptosis. This mechanism could possibly involve the same mechanism of protection provided by HGF for the OHCs. In the mechanisms of NIHL, disruption of afferent dendrites attached to IHCs is also involved [19]. Therefore, a regrowth of the nerve fibers and a re-afferentiation of the IHC is important for recovery of hearing after noise trauma. After spinal cord injury, HGF promotes axonal regrowth resulting in functional recovery [18]. This mechanism could also be involved in the significant reduction of ABR threshold shifts observed in the present study. In order to be able to elucidate the HGF distinct mechanism for the protection of auditory systems, further investigations are required.

In conclusion, the present findings suggest that HGF potentially has a role as a protector of OHCs from noise trauma. We are currently in the process of developing a clinical treatment for SNHL that administers local IGF-1 via gelatin hydrogels. Present results strongly suggest that HGF is the next therapeutic candidate that can be used as a local treatment agent via gelatin hydrogels in SNHL clinical trials.

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A Mouse Model for Degeneration of the Spiral Ligament

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ABSTRACT

Previous studies have indicated the importance of the spiral ligament (SL) in the pathogenesis of sensorineural hearing loss. The aim of this study was to establish a mouse model for SL degeneration as the basis for the development of new strategies for SL regeneration. We injected 3-nitropropionic acid (3-NP), an inhibitor of succinate dehydrogenase, at various concentrations into the posterior semicircular canal of adult C57BL/6 mice. Saline-injected animals were used as controls. Auditory function was monitored by measurements of auditory brain stem responses (ABRs). On postoperative day 14, cochlear specimens were obtained after the measurement of the endocochlear potential (EP). Animals that were injected with 5 or 10 mM 3-NP showed a massive elevation of ABR thresholds along with extensive degeneration of the cochleae. Cochleae injected with 1 mM 3-NP exhibited selective degeneration of the SL fibrocytes but alterations in EP levels and ABR thresholds were not of sufficient magnitude to allow for testing functional recovery after therapeutic interventions. Animals injected with 3 mM 3-NP showed a reduction of around 50% in the EP along with a significant loss of SL fibrocytes, although degeneration of spiral ganglion neurons and hair cells was still present in certain regions. These findings indicate that cochleae injected with 3 mM 3-NP may be useful in investigations designed to test the feasibility of new therapeutic manipulations for functional SL regeneration.

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INTRODUCTION

Sensorineural hearing loss (SNHL) is a common disability in industrialized countries. Recent studies using human temporal bones and animal models have highlighted the importance of the spiral ligament (SL) in the pathogenesis of SNHL. Degenerative changes of the SL have been observed in human temporal bones with acoustic neuroma (Mahmud et al. 2003), endolymphatic hydrops (Vasama and Linthicum 1999), or presbycusis (Kusunoki et al. 2004; Ohlemiller 2004). Several animal models have also indicated the involvement of SL degeneration in the mechanisms for SNHL that are related to aging (Hequembourg and Liberman 2001; Spicer and Schulte 2002) or excessive noise (Hirose and Liberman 2003). The SL is located in the cochlear lateral wall and is composed of fibrocytes. The fibrocytes in the SL form a gap junction network, which is closely related to the maintenance of the endocochlear potential (EP; Xia et al. 1999). In addition, a recent study has indicated that the cochlear gap junctions also play a role in the transport of energy and nutrient supplies (Zhao 2005). A mouse model for DFN3, an X-chromosome-linked non-syndromic mixed deafness, exhibits a significant reduction in the immunoreactivity for the gap junction protein, connexin 26 (Cx26), in the SL fibrocytes, which results in a profound hearing loss (Minowa et al. 1999; Xia et al. 2002). Therefore, during SNHL treatment, the SL fibrocytes should be viewed as therapeutic targets.

Treatment options for SNHL are currently limited to cochlear implants and hearing aids. Thus, there is a need for the development of alternative means of biological therapy, such as cell and/or gene therapy. In fact, recent studies have demonstrated the potential of cell and/or gene therapy for the treatment of SNHL (Izumikawa et al. 2005; Okano et al. 2005, 2006). Such therapeutic strategies may be applicable for the regeneration of SL fibrocytes. However, in order to examine the efficacy of such novel therapeutic strategies for the regeneration of SL fibrocytes, an appropriate model for SL degeneration is required. Recently, a rat model for the selective loss of SL fibrocytes has been reported (Hoya et al. 2004; Okamoto et al. 2005). In this model, a direct application of 3-nitropropionic acid (3-NP) onto the round window membrane causes damage to the SL fibrocytes. 3-NP is an inhibitor of succinate dehydrogenase, an enzyme of the citric acid cycle on the mitochondria. However, in a series of rat experiments, EPs have not been evaluated, although the measurement of the EP is crucial for the evaluation of SL function. To this end, the aim of the present study was to establish a mouse model for selective loss of SL fibrocytes that also exhibited a significant reduction of the EP, which would make it possible to use this model as the basis for the development of therapeutic strategies for SL regeneration. We injected 3-NP into the posterior semicircular canal (PSCC) of C57BL/6 mice and monitored the cochlear function using measurements of auditory brain responses (ABRs) and EPs, followed by subsequent histological analyses of the cochleae.

METHODS

Animals

Female C57BL/6 mice (Japan SLC Inc., Hamamatsu, Japan) aged 6–10 weeks (n=59) were used as experimental animals. All of the animals were maintained at the Institute of Laboratory Animals, Kyoto University Graduate School of Medicine, Japan. The experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine and were conducted in accordance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental groups

3-NP (Sigma, St. Louis, MO, USA) was dissolved into physiological saline at a concentration of 1, 3, 5, or 10 mM and adjusted to pH 7.4 with NaOH. We divided the experimental animals into four groups according to the applied concentration of 3-NP (1 mM, n=13; 3 mM, n=14; 5 mM, n=12; 10 mM, n=12). Animals administered physiological saline were used as controls (n=12).

3-NP administration

We used an injection of 3-NP into the semicircular canal as a method for drug application into the mouse cochlea, similar to previously reported studies (Nakagawa et al. 2003; Lee et al. 2003; Iguchi et al. 2004; Okano et al. 2006). This method makes it possible to administer drugs or place cell transplants into the cochlear fluid spaces without functional and/or histological damage to the cochlea. Under general anesthesia with midazolam (10 mg/kg), medetomidine (37.5 μg/kg) and butorphanol tartrate (0.5 mg/kg), a retroauricular incision was made in the left ear, and the PSCC was exposed. A small hole was then made in the bony wall of the PSCC. Using microscopy, a fused silica glass needle (Eicom, Kyoto, Japan) was then inserted into the perilymphatic space of the PSCC, and substrates were injected at a rate of 0.5 μl/min for 3 min using a microsyringe pump (Eicom). Thereafter, the hole was plugged with a fat graft and covered with fibrin glue. The animals that showed ABR threshold shifts less than 30 dB at the frequency of 40 kHz 1 day after 3-NP application were eliminated from experiments. One animal was eliminated from the 1 or 3 mM 3-NP group, and two animals were eliminated from the 5 mM 3-NPgroup.

ABR recording

An ABR recording was used to monitor the auditory function of the experimental animals. The right cochleae were mechanically destroyed in order to avoid cross hearing. Under general anesthesia, ABR measurements were performed as has been previously described (Shiga et al. 2005). The generation of acoustic stimuli and the subsequent recording of the evoked potentials were performed using a PowerLab/4sp (AD Instruments, Castle Hill, Australia). The acoustic stimuli, consisting of tone burst stimuli (0.1-ms cos2 rise/fall and 1-ms plateau) were delivered monaurally through a speaker (ES1spc, Bioresearch Center, Nagoya, Japan), which was connected to a funnel that was fitted into the external auditory meatus. In order to record the bioelectrical potentials, subdermal stainless steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active) and contra-lateral to the measured ear (reference). The stimuli were calibrated against a 0.25-in. free-field mike (ACO-7016, ACO Pacific Inc., Belmont, CA, USA) that was connected to an oscilloscope (DS-8812 DS-538, Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111, Ono

Sokki, Yokohama, Japan). The responses between the vertex and the mastoid subcutaneous electrodes were amplified with a digital amplifier (MA2, Tucker-Davis Technologies, Alachua, FL, USA). The ABRs were recorded before drug application and on postoperative days (PODs) 1, 7, and 14. Thresholds were determined for the frequencies of 10, 20, and 40 kHz from a set of responses at varying intensities with 5-dB SPL intervals. When no response was present at the highest sound level available, the threshold was designated as being 5 dB greater than that level so that statistical tests could be done.

Measurement of endocochlear potentials

EP recording was performed under general anesthesia on POD 14. A silver–silver chloride reference electrode was placed under the skin of the dorsum. An incision was then made in the inferior portion of the left postauricular sulcus, with the bulla perforated in order to allow for exposure of the stapedial artery and the basal turn of the cochlea. Thinning of the bone over the SL followed by the creation of a small opening with a pick made it possible to access to the scala media of the basal turn. A micropipette electrode (10–40 MO) filled with 150 mM KCl was advanced through the bony aperture into the SL. The entry of the electrode tip into the endolymph was characterized by fast changes of the recorded potentials. The electrode was advanced until a stable potential was observed. At the point of the stable potential, there were no alterations that were dependent upon the electrode depth. The signal was amplified through an amplifier (Duo 773, World Precision Instruments, Sarasota, FL, USA). As the electrode was advanced, the DC potentials were recorded via an A-D converter (PowerLab 4sp, AD Instruments) coupled to a desktop computer.

Tissue preparation

On POD 14, after measurements of EPs, the animals were deeply anesthetized with a lethal dose of anesthetic drugs followed by intracardial perfusion with physiological saline. Subsequently, 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4 was infused. To decalcify the excised temporal bones, they were immersed in the same fixative at 4°C for 4 h and then placed into 0.1 M ethylenediaminetetraacetic acid in PBS. The number of sample subsets used for the evaluation of the hair cell damage in the whole mounts included control, n=4; 1 mM, n=4; 3 mM, n=5; 5 mM, n=4; and 10 mM, n=4. Remaining samples were utilized for morphometric analysis of frozen sections (n=8; 1, 3, 10 mM, n=6; 5 mM). Samples were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C. For histological analysis, 10-μm-thick mid-modiolar sections of the cochleae were obtained and then prepared.

Immunohistochemistry

Immunohistochemistry for Na,K-ATPase α and Cx26 were employed in order to determine the sites that were affected by the local 3-NP application in the cochlear lateral wall. The primary antibodies were mouse anti-Cx26 antibody (1:500; Zymed Laboratories, South San Francisco, CA, USA) and rabbit monoclonal anti-Na, K-ATPase α antibody (1:500; Epitomics, Inc., Burlingame, CA, USA). Alexa-488 conjugated anti-rabbit goat IgG (1:500; Molecular Probes, Eugene, OR, USA) and Alexa-568 conjugated anti-mouse goat IgG (1:500; Molecular Probes) were used as the secondary antibodies.

Immunohistochemistry for βIII-tubulin (TuJ1) was performed to identify the spiral ganglion neurons (SGNs) in the Rosenthal's canal. The primary antibody was rabbit anti-βIII-tubulin antibody (1:250; Covance Research Products, Berkeley, CA, USA), and Alexa-568 conjugated anti-rabbit goat IgG (1:500; Molecular Probes) was used as the secondary antibody followed by nuclear staining with 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes).

Immunohistochemistry for myosin VIIa and F-actin staining by phalloidin were performed to identify cochlear hair cells or the locations in which hair cells were present in whole mounts. The primary antibody was rabbit anti-myosin VIIa antibody (1:500; Proteus Bioscience, Ramona, CA, USA), with Alexa-568 conjugated anti-rabbit goat IgG (1:500; Molecular Probes) used as the secondary antibody. Samples were then stained using fluorescein-isothiocyanate-conjugated phalloidin (3 μg/ml; Sigma-Aldrich, Inc.).

Quantitative assessments for histological damage

Morphometric assessments of the SL, the stria vascularis (SV) and the SGNs were performed for each cochlear turn as has been previously described (Suzuki et al. 2006). The sections stained with hematoxylin–eosin (HE) were used for the SL and the SV, and those stained by immunohistochemistry for βIII tubulin were used for the SGN. The cochlear specimens were observed using a light microscope (Olympus BX50, Tokyo, Japan). The images were acquired with a CCD camera connected to a personal computer. The areas of the SL, the SV, Rosenthal's canal, and the cochlear turn were quantified by measuring their cut surfaces using ImageJ software (http://www.nist.gov/lispix/imlab/prelim/dnld.

html). The total numbers of nuclei in the SL were counted for each cochlear turn. For the SGN, βIII tubulin- and DAPI-positive cells were counted. The cell density of the SL (SL density) and the SGN (SG density) and the ratio of the SV area (SV ratio) were used to reduce the variance caused by differences in the cutting directions among the cochlear specimens. The SL density was determined as the number of cell nuclei per 1 μm^2 of the SL area, and the SG density was determined as the number of cell nuclei per 1 μm^2 of Rosenthal's canal. The SV ratio was determined by dividing the SV area by that of the cochlear turn. We calculated the SL density, the SG density, and the SV ratio in two mid-modiolar sections separated by 40–50 μm from each cochlea, with the average defined as the data for the animal.

To reveal details of SL degeneration, a loss of five types of SL fibrocytes was examined, respectively. The types of SL fibrocytes were classified into type I–V fibrocytes according to their location in the SL (Spicer and Schulte 1991; Schulte and Steel 1994; Xia et al. 1999; Hirose and Liberman 2003). The density for each type was determined as the number of cell nuclei per square micrometer of the SL area.

Quantitative analyses for the numbers of remaining inner hair cells (IHCs) and outer hair cells (OHCs) were performed on whole mounts. The 20–40% distance portions from the apex were defined as being apical, the 40–60% distance portions were defined as upper basal, and the 60–80% distance portions were defined as basal. The numbers of IHCs and OHCs were counted in a 0.2-mm-long region of the apical, the upper basal, and the basal portion of the cochleae.

Statistical analyses

The overall effect on ABR threshold shifts of the local 3-NP applications was examined by a two-way factorial analysis of variance (ANOVA). When the interaction was significant, multiple comparisons with the Tukey–Kramer test were performed for pairwise analyses. Differences between the recorded time points for the EP, the SL or SG density, or the SV ratio among the experimental groups and in ABR thresholds in the control animals were examined by a single factorial ANOVA with the Scheffe's test. A p value less than 0.05 was considered statistically significant. All data are represented as the mean±standard error.

RESULTS

ABR threshold shift

In the control animals that were treated with saline, no significant elevations of the ABR thresholds were observed for any of the frequencies. In the experimental animals, local 3-NP applications of each of the concentrations used in this study caused significant ABR threshold shifts in a dose-dependent manner (Fig. 1A–C). The animals injected with 5 or 10 mM 3-NP exhibited approximately 80- to 90-dB threshold shifts on POD 1, with no recovery of the ABR thresholds noted during the observation period. The animals injected with 3 mM 3-NP showed a remarkable threshold shift on POD 1 that was similar to the 5 or 10 mM model, although there was a trend for recovery of the ABR threshold over time. The differences in the ABR threshold shifts between POD 1 and POD 7 or 14 were significant at 10 and 20 kHz. On POD 14, the 3 mM model exhibited significant threshold shifts for each of frequencies tested (50.4± 3.0 dB at 10 kHz, 56.2±2.9 dB at 20 kHz, 61.9±2.9 dB at 40 kHz). The animals injected with 1 mM 3-NP showed significant threshold shifts on POD 1, while on POD 14, the ABR thresholds at 10 and 20 kHz had returned to levels that were seen for the control animals (Fig. 1A,B). A significant ABR threshold shift (22.9±5.2 dB) was only found at 40 kHz on POD 14 in the animals injected with 1 mM 3-NP (Fig. 1C).

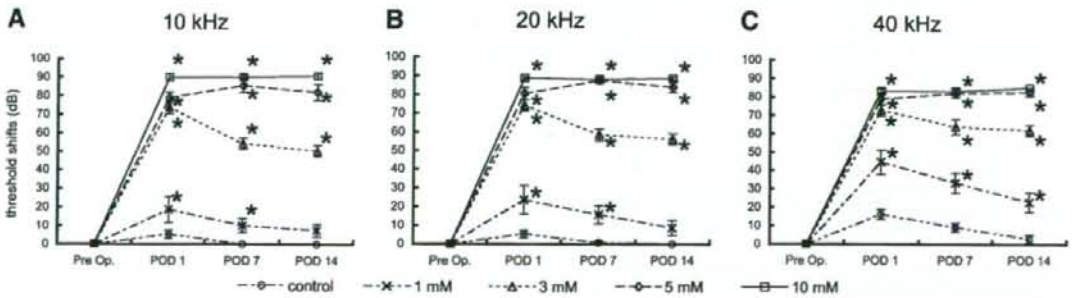

FIG. 1. Means of ABR threshold shifts at 10 (**A**), 20 (**B**), and 40 kHz (**C**) following local saline or 3-NP application on postoperative days (*POD*) 1, 7, and 14. Local applications of 3-NP have significant interactions with threshold shifts at all the frequencies ($p<0.0001$, two-way factorial ANOVA). *Asterisks* indicate significant differences to the control group in threshold shifts by multiple comparisons with the Tukey–Kramer test. *Bars* represent standard errors.