Role of Nitric Oxide in the Onset of Facial Nerve Palsy by HSV-1 Infection

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Bell palsy is an acute peripheral facial paralysis of unknown cause and usually involves only 1 side of the face. It is the most common cause of acute facial paralysis, with a reported annual incidence of 20 to 30 per 100,000 people in Japan. Although Bell palsy generally has a good prognosis, approximately 10% of patients do not respond well to treatment and experience profound denervation leading to severe sequelae such as synkinesis, contracture, and facial asymmetry. Recent molecular biological investigations have provided increasing evidence of an association with herpes simplex virus type 1 (HSV-1) reactivation. The presence of a latent HSV genome in the geniculate ganglion of the human facial nerve has been shown by polymerase chain reaction (PCR) at autopsy. In addition, active HSV sequences have been identified in the endoneurial fluid of the facial nerve during facial nerve decompression surgery in patients with Bell palsy. Although HSV-1 is a causative agent of facial paralysis, the precise mechanism of the paralysis remains unknown.

This study elucidated the role of nitric oxide (NO) in the incidence of facial nerve paralysis caused by HSV-1 in mice and to evaluate the possible role of edaravone, a free radical scavenger, in preventing the paralysis.

Levels of NO in the facial nerve were measured using high-performance liquid chromatography and absorption photometry. The incidence of facial palsy was assessed following administration of edaravone immediately after HSV-1 inoculation and daily for 11 days thereafter.

Before the onset of facial palsy, no substantial difference in the NO level was noted between the HSV-1–inoculated side and the control side. When facial palsy occurred, usually at 7 days after inoculation, the NO level was significantly higher on the inoculated side than on the control side. Following recovery from the palsy, the high NO level of the inoculated side decreased. No increase in the NO level was observed in animals without transient facial palsy. When edaravone was administered, the incidence of facial palsy decreased significantly.

These findings suggest that NO produced by inducible NO synthase in the facial nerve plays an important role in the onset of facial palsy caused by HSV-1 infection, which is considered a causative virus of Bell palsy. Hato and colleagues elucidate the role of nitric oxide in HSV-1–related facial nerve paralysis in mice and evaluate the role of edaravone, a free radical scavenger, in preventing the paralysis.
Nitric oxide (NO) plays important roles in regulating vasodilatation and protecting neuronal tissues. However, when a large amount of NO is produced by the enzymatic activity of inducible NO synthase (iNOS), which is expressed during infectious and inflammatory processes, cell membranes are destroyed by NO compounds such as nitrite (NO$_2^-$) and nitrate (NO$_3^-$), which form by reactions with superoxide species. Fujii et al. examined the involvement of NO in the pathology of viral infection using an encephalitis model developed by inoculating HSV-1 into the nasal cavity of rats. They concluded that HSV-1 inoculation led to the expression of iNOS in the inflammatory cells infiltrating the cerebellum. In addition, they showed that the administration of Nω-monomethyl-L-arginine, a NOS inhibitor, significantly improved the survival rate of the animals. Although NO is known to be related to the onset of viral neuropathy, the effects of NO on the onset of facial nerve paralysis due to HSV-1 infection remain unclear.

This study investigated (i) the NO level in the facial nerve and (2) the preventive effects of edaravone, a commercially available antioxidant for human use, on the onset of facial palsy.

Methods

Experimental Animals and Induction of Facial Paralysis by Inoculation With HSV-1

All experiments were conducted according to the Guidelines for Animal Experimentation at Ehime University School of Medicine. Four-week-old female Balb/c-Ajcl mice (Clea Japan), bred in a sterile environment and weighing 16 to 18 g, were used for the present study. They were bred in an animal center at the Ehime University School of Medicine. According to the method developed by Sugita et al., facial nerve paralysis was induced in mice under general anesthesia by scratching the back of the right auricle with a 27-gauge needle 20 times, followed by inoculation of the same site with 25 µL of HSV-1 (KOS strain, 1.4 × 10$^6$ plaque-forming units [PFU]/mL). The contralateral left auricle was inoculated with saline as the sham-treated (control) side. Facial nerve paralysis of the animals was assessed by daily observations of whisker movements and the blink reflex, according to Takahashi et al. Facial movements were classified into 5 grades, from normal (0/4) to complete paralysis (4/4). Facial nerve paralysis occurred only on the HSV-1-inoculated side, with no mouse showing facial palsy on the control side. Inoculation with HSV-1 and tissue collection were performed with the animals under deep anesthesia induced by intraperitoneal injection of pentobarbital (100 mg/kg).

Experiment 1: Measurement of NO

In a mouse model of facial nerve paralysis due to HSV-1 infection, paralysis occurred on days 6 to 9 after inoculation; most mice developed paralysis on day 7, and all mice showed recovery within 14 days after inoculation. In the present study, the day of HSV-1 inoculation was defined as day 0 for convenience. To examine the relationship between NO level and facial paralysis, NO was measured on day 4 (before paralysis), day 7 (at onset of paralysis), and day 14 (at recovery from paralysis). After inoculation of HSV-1, 32 mice were divided into 4 groups: group A, NO measured on day 4 (n = 8); group B, NO measured on day 7 without facial palsy (n = 8); group C, NO measured on day 7 with facial palsy (n = 8); and group D, NO measured on day 14 (n = 8). The ratio of the NO level on the HSV-1-inoculated side divided by the NO level on the control side was compared among the 4 groups.

The direct measurement of NO production is difficult in vivo because NO is unstable, has a short physiological half-life of 3 to 5 seconds, and is easily degraded into NO$_2^-$ and NO$_3^-$. An in vivo microdialysis and online high-performance liquid chromatography system was recently developed for the measurement of NOx (NO$_2^-$ and NO$_3^-$). Measurements of the NO level were performed using the following procedures. A mouse was anesthetized with a mixture of halothane and nitrous oxide, and the facial nerve was exposed at the foramen stylomastoideum via retroauricular skin incision. The epineurium was incised 1 mm in the direction of the nerve tract, and a microprobe (tip diameter, 220 µm; Eicom) for NO collection was inserted at this site. A dialysis membrane was attached to the tip of the microprobe, and endoneurial fluid was collected by perfusing lactated Ringer solution into the microprobe using a syringe pump. The specimens were automatically transferred through an automatic sample injector to a measuring device (ENO-20 NOx analyzer; Eicom). The NO was converted, through various NO compounds, into aqueous NO$_2^-$ and NO$_3^-$. The analyzer separated NO$_2^-$ and NO$_3^-$, and NO$_2^-$ was then reduced to NO$_3^-$, which reacts with naphthylethenediamine in the acidic solution contained in the device, producing azo compounds. The azo compounds are red, and the degree of color development was measured by absorption photometry (Figure). The absorbance of azo compounds is proportional to the NO level in the specimen. The measurement of NO levels requires use of this device for 10 minutes.
Nitric Oxide in HSV-1 Facial Nerve Palsy Onset

Experiment 1: Measurement of NO

The NO levels in the facial nerve were compared among the 4 groups using the NO ratio, which was calculated as the NO level of the inoculated side divided by that of the control side. The results are summarized in Table 1. The NO ratio measured on day 4, before the onset of paralysis (group A), was 0.991, and the NO ratio measured on day 7 in mice without facial palsy (group B) was 1.021. Thus, the NO level did not differ between the inoculated (right) and control (left) sides in these 2 groups. In contrast, the NO ratio measured on day 7 in mice that developed facial palsy (group C) was 1.681, showing a significant increase in the NO level on the HSV-1-inoculated side compared with the control side. The NO ratio measured after the mice had recovered from facial palsy (group D) was 0.973, indicating that there was no difference in the NO level between the right and left sides after recovery.

Experiment 2: Effects of Edaravone Administration

As a commercially available free radical scavenger for human use, edaravone (Radicut; Mitsubishi Pharma) detoxifies harmful free radicals, including NO. Clinically, it is widely used to treat acute ischemic stroke. Since edaravone protects against cell membrane damage caused by NO, the effects of edaravone when administered immediately after HSV-1 inoculation may provide indirect evidence for an association between NO and the onset of facial palsy due to viral infection.

Thirty mice inoculated with HSV-1 were divided into 3 groups: group a (edaravone, 1 mg/kg/d; n = 10), group b (edaravone, 10 mg/kg/d; n = 10), and group c (phosphate-buffered saline, 0.1 mL/d; n = 10). Immediately after the HSV-1 inoculation, 0.1 mL of edaravone or phosphate-buffered saline was administered into the peritoneal cavity, and the treatment was continued once a day for 11 days, from day 0 to day 10.

After the HSV-1 inoculation, facial muscle movements, including the blink reflex, were observed daily. A paralysis score of 2/4 or higher, determined according to Takahashi et al., was taken to indicate facial nerve paralysis. The incident rates of facial palsy were compared among the 3 groups.

Statistical Analysis

The t test was used for the statistical analysis of differences in experiment 1; the Fisher exact test was used for the statistical analysis of differences in experiment 2. P < .05 was deemed to indicate statistical significance.

Results

Table 1. Ratios of Nitric Oxide Levels, HSV-1–Inoculated Side to Control Side

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Ratio, Mean (SD), %</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (day 4 after inoculation, before paralysis onset) (n = 8)</td>
<td>0.991 (0.126)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Group B (day 7 after inoculation, without paralysis) (n = 8)</td>
<td>1.021 (0.090)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Group C (day 7 after inoculation, with paralysis) (n = 8)</td>
<td>1.681 (0.402)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Group D (day 14 after inoculation, recovered from paralysis) (n = 8)</td>
<td>0.973 (0.068)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Table 2. Effects of Edaravone and Placebo Administration

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Rate of Facial Nerve Paralysis, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group a (edaravone, 1 mg/kg) (n = 10)</td>
<td>5 of 10 (50)</td>
</tr>
<tr>
<td>Group b (edaravone, 10 mg/kg) (n = 10)</td>
<td>2 of 10 (20)</td>
</tr>
<tr>
<td>Group c (phosphate-buffered saline) (n = 10)</td>
<td>6 of 10 (60)</td>
</tr>
</tbody>
</table>

Discussion

Many studies have been conducted using the mouse model of facial nerve paralysis induced by HSV-1 infection as an animal model of Bell palsy. In this mouse model, facial nerve paralysis occurs at around 1 week after inoculation and resolves spontaneously within 2 weeks. Histological examinations of the facial nerve have revealed vacuolar formation and inflammatory cell infiltration in the facial nerve from the onset of facial palsy until 2 weeks after recovery from the palsy. This temporal dissociation between the paralysis and the histological findings indicates that factors other than the virus may be involved in the development of paralysis. Based on chronological observations of HSV-1–neutralizing antibody titers in the mouse model and the results of an immune transfer study using anti–HSV-1 and sensitized T cells, our group previously concluded that the neurological damage was not caused by an autoimmune mechanism. Honda et al. performed electrophysiological and histological studies of the facial nerves and suggested that facial nerve paralysis in this model may result from neuropraxia due to demyelination. Furthermore, Wakisaka et al. reported that the facial nerve underwent demyelination centrally from the geniculate ganglion and that the damage, as observed by transmission electron microscopy, appeared to be mild. Apparently, HSV-1 causes facial neuritis in this model; however, the detailed mechanism underlying the demyelination caused by HSV-1 remains unclear.

In the present study, the NO level was increased on the HSV-1-inoculated side only when facial palsy occurred. According to Honda et al., facial nerve demyelination could be observed in paralyzed mice immediately after the onset of paralysis and disappeared completely after resolution of the paralysis. As an increase in the NO level was evident in the facial nerves of mice experiencing palsy (group C), we considered the NO level to be a sensitive indicator of neuronal injury.
that the demyelination observed in this animal model could be due to Schwann cell damage by the toxic actions of NO, NO$_2^-$, and NO$_3^-$. The NO is produced by the enzymatic activity of 3 NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and iNOS. Both eNOS and nNOS are constitutively expressed, and NO produced by these enzymes exerts physiological actions on the circulatory and nervous systems. In contrast, iNOS expression is induced during infectious, inflammatory, and immunoreactive processes, and it produces a larger amount of NO. Macrophages, vascular endothelial cells, glial cells, and neurons express iNOS in response to inflammation. Although the precise mechanism underlying NO-induced neuronal damage is still unclear, the toxic effect of NO is known to be greatly enhanced by reaction with another toxic agent, especially superoxide, to form peroxynitrite. Profuse NO formed through iNOS was thought to play an injurious role to contribute to neuronal damage through the production of peroxynitrite, which could destroy Schwann cell membranes.

The administration of the free radical scavenger edaravone at 10 mg/kg reduced the incidence of facial palsy in the present study. The increased expression of iNOS associated with HSV-1 inoculation would produce an excessive amount of NO, which can react with superoxide to form the free radicals NO$_2^-$ and NO$_3^-$. These free radicals are potent initiators of cell membrane lipid peroxidation and result in Schwann cell damage. By inhibiting the peroxidation of membrane lipids, edaravone may effectively protect cells against cell membrane damage. The present findings suggest that the administration of edaravone prevented Schwann cell damage and the resultant nerve demyelination, probably by inhibiting the actions of NO$_2^-$ and NO$_3^-$. The present study also indicated that NO may not be an essential factor for the development of facial palsy: 1 mouse in group C did not exhibit an elevated NO level despite the onset of facial palsy. Furthermore, 3 of 10 mice with facial palsy did not express iNOS in the facial nerve, and 2 mice developed facial palsy even after the administration of edaravone (10 mg/kg). Higher doses of edaravone should be investigated in future studies because the effect of edaravone is known to be dose dependent. Thus, in addition to direct neurological damage caused by the virus and secondarily by NO, unknown factors may also be involved in the onset of facial palsy.

Based on the results of the experiments presented here, we propose that NO plays an important role in the incidence of facial palsy due to HSV-1 infection, although the details of the mechanism remain unclear.

In conclusion, this study revealed an increase in the NO level in the facial nerve when facial palsy occurred in response to HSV-1 inoculation in mice. The administration of edaravone effectively reduced the incidence of facial palsy in this mouse model. These findings suggest that NO is an important factor in the development of facial palsy.

**REFERENCES**