

EXPERIMENTAL / LABORATORY STUDIES

Peripheral Facial Paralysis and Apoptosis Induced by Herpes Simplex Type I Virus: A Rat Study

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Abstract: Different results have been reported concerning the impact of agents used in the treatment of idiopathic facial nerve paralysis (FNP) generated by HSV type I (HSV-I) on the apoptotic process. We aimed at investigating the effects of different agents (steroids, acyclovir and interferon) used in the treatment of idiopathic FNP on the apoptotic process in animals in whom experimental FNP has been produced by HSV-I. After cleaning the auricles of 113 animals, linear injuries were produced, and then 25 micromole KOS HSV-I was inoculated with the solution containing 1.7×10^7 virus per milliliter. On the 6th day after the inoculation sixty animals (60/113, 53%) which developed facial paralysis were included in the study and were randomized into 4 groups consisted of 15 animals: steroid (SG), acyclovir (AG), interferon (IG) and control groups (CG). On the 21st day of the study (inoculation) and 15th day of the follow-up and treatment, the animals were decapitated. HSV-1 DNA was assayed with PCR technique on the ipsilateral brain stems and temporal cortexes obtained from the animals. The animals that were HSV-1 DNA positive were included in the study: CG group (5/15), SG (6/15), AG (4/15) and IG (4/15). To this end, flowcytometric analyses (apoptotic cell DNA index with propidium iodide stain and CD95 antibody stain) were evaluated in the ipsilateral brain stem and temporal cortex. The results demonstrated that the application of steroid to animals developing FNP resulted in significant suppression of CD95(+) CELLS ratios in the ipsilateral temporal cortex ($P < 0.05$), acyclovir or interferon application on the other hand did not create any significant change in the apoptotic process of either of the brain regions ($P > 0.05$). Although the apoptotic cell DNA index in the control group was found at a higher ratio than in treatment groups, the ratio was not significant ($P > 0.05$). This study demonstrates that; (i) in FNP generated by HSV-1, not only the facial nerve but also other brain regions including the brain stem and the temporal cortex might be affected, and (ii) steroids might have a limited effect on the total apoptotic process and therefore they may have neuroprotective effects.

Key Words: Facial nerve paralysis, HSV type I, apoptosis, steroids, acyclovir, and interferon

Introduction

Idiopathic facial nerve paralysis (FNP), namely, Bell's palsy or Antoni's palsy is a non-life threatening disorder resulting in significant functional, esthetic and psychosocial disturbances in the patient. Viral pathogenesis hypothesis is gaining acceptance in the current literature suggesting that herpes simplex virus might play a fundamental role in idiopathic FNP (1, 2). Sugita et al (3) were the first to succeed in producing acute and transient facial paralysis simulating Bell's palsy by inoculating herpes simplex virus into the auricles or

tongues of mice. In order to prove HSV infection related pathogenicity of the facial paralysis, animal experiments with inoculation of HSV into the facial nerve, tongue, nasal mucosa or auricle have been carried out (4-8). In FNP experimentally produced by HSV type I (HSV-I), different parts of the brain including the ipsilateral brain stem and temporal regions have been infected with HSV-I and even lesions have developed (9, 10, 11, 6). It is thought that in the neural lesions in which HSV-I localizes it results in wider involvements due to its neurotrophic features (9, 10). The apoptotic process has an important

contribution in injuries developing in neural tissue. CD95 is an important superficial marker in the triggering and progression of the apoptotic processes (12, 13).

Different results have been reported concerning the impact of the agents used in the treatment of idiopathic FNP on the apoptotic process (14-19). To this end, we aimed at investigating the effects of different agents (steroids, acyclovir and interferon) used in the treatment of idiopathic FNP on the apoptotic process that might develop in two different regions of the brain (ipsilateral brain stem and temporal cortex) which have anatomical and functional similarities with the facial nerve in animals in whom experimental FNP has been produced by HSV-I.

Material and Methods

Animals and viral inoculation

The study was conducted on 113 female rats each weighing 190-210 gr after obtaining the approval of hospital ethics committee. The facial functions of all the rats were evaluated and the ones with normal facial functions were recruited in the study. The criteria for normal facial nerve function were: symmetrical movements of whiskers during mastication and presence of eye blinking reflex after blowing pressurized air. Throughout the study we complied with the principles of experimental and surgical techniques in rats.

The HSV type-I (KOS strain) was used in our study. Solutions containing the virus were prepared by having 1.7×10^7 virus per milliliter of the solution (22). Before the inoculation, all the animals were injected with 87mg/kg ketamin hydrochloride (Ketalar, Eczacıbaşı, Turkey) and 13mg/kg xylazine hydrochloride (Rompun, Bayer, Turkey) intraperitoneally for anesthesia. After cleaning their auricles with 70% ethyl alcohol and drying, linear injuries were produced with a sterile technique using a 27 gauge needle. Then, 25 micromole KOS HSV Type I was inoculated into the injured site with the solution containing 1.7×10^7 virus per milliliter. After the inoculation, facial functions of the animals were evaluated three times a day. On the 6th day after the inoculation 60 animals (60/113, 53%) had unilateral loss of the whisker movements on the right, they were not able to close their eyes on the same side and the presence of these findings resulted in the diagnosis of peripheral facial paralysis. Clinically generated FNP was also evaluated electrophysiologically. For this purpose, one animal from

the treatment and one from the control group were given general anesthesia. Afterwards, the facial nerve was stimulated both on the affected right side and the intact left side at the point where it left the stylomastoid foramen. Muscle amplitudes that were generated and the response rates to the stimuli verified the diagnosis of FNP.

The animals that developed facial paralysis were included in the study and were randomized into groups. Each group consisted of 15 animals. These were steroid, acyclovir, interferon and control groups: Group 1- Control Group (CG): The animals in this group received 1 ml/kg intramuscular equal volume of physiologic saline. Group 2- Steroid Group (SG): the animals in this group received 1mg/kg intramuscular methyl prednisolone succinate once a day. Group 3- Acyclovir Group (AG): the animals in this group were weighed every day and total acyclovir dose of 5mg/kg was given intraperitoneally in 5 equal portions at equal time intervals. Group 4- Interferon group (IG): the animals in this group received 100.000 unit/kg interferon γ -2b intramuscularly every other day.

Perfusion and tissue preparations

On the 21st day of the study (inoculation) and 15th day of the follow-up and treatment, following the administration of high dose anesthetic substances, after the achievement of loss of consciousness, the animals were decapitated with a guillotine system providing decapitation with a single movement. HSV-1 DNA was assayed with the PCR technique on the ipsilateral brain stems and temporal cortexes obtained from the animals. The animals that were HSV-1 DNA positive were included in the study. There were five such animals in CG (5/15), six in SG (6/15), four in AG (4/15) and 4 in IG (4/15).

Afterwards, the apoptosis study was initiated. The cell suspension was prepared as follows. Tissue samples were transferred into Petri dishes and pellets were formed. Pellets were then transferred into test tubes containing EDTA and 1.8 ml of trypsin was added. The prepared material was kept in a 37 °C water bath for 7 minutes. The supernatant above the pellet was taken with a Pasteur pipette kept adjacent to the surface of the pellet. It was then centrifuged at 1250 rpm for 5 minutes. The supernatant was resuspended with 2 ml PBS and centrifuged twice. The supernatant was discarded with a Pasteur pipette. 0.5 ml of PBS was added and it was

filtered through a 40 micron filter. 100 microliter of an aliquot was taken from the prepared sample and mixed with 20 microliter of CD95 antibody stain (CD95, Beckman Coulter CD95 monoclonal antibody kit) in a test tube and kept at room temperature for 20 minutes in a dark environment. It was then assayed with flow cytometry with a relevant program.

Propidium iodide stain and flowcytometric analysis

For the DNA study, the filtered sample was mixed with 1 ml of 0.5% formalin for fixation and was kept at room temperature for 10 minutes. 100 microliter of the prepared sample was mixed with 10 microliter of propidium iodide (PI) and kept at room temperature for 20 minutes in the dark. It was then assayed with flow cytometry with a relevant program. COULTER Epics XL-MCL device EXPO 32 program utilizes PI stain (FlowTACS Apoptosis Detection Kit: Catalog number TA5354, R&D Systems, USA) for DNA analysis that fluoresces at 617 nm, linear signals were thus counted with the FL3 detector. First, control group samples were passed through the device, apoptotic and singlet cells were taken into two separate gates, doublet cells were left out of the gates. Peaks pertaining to apoptotic DNA fragments appeared between 0-200, therefore voltage adjustment was performed to have the X mean value of the peak at 200 for FL3 histogram. Samples were counted by using the same voltage adjustments.

Statistical analyses were carried out with the SPSS computer program (SPSS Inc, USA). Mean and standard errors of all the data were calculated.

Results

Facial nerve paralysis and HSV-1 DNA positivity

The study was completed in 19 animals (CG: n=5, 33%; SG: n=6, 40%; AG: n=4, 27% and IG: n=4, 27%) which were found to have developed FNP together with HSV-1 DNA positivity of the brain stem and temporal cortex and had inflammatory lesions in the histopathological examination. The ratio of HSV-1 DNA positivity in the cerebral regions of the control and treatment groups was not of statistical significance ($P > 0.05$).

Facial nerve paralysis, CD95(+) cells and apoptotic DNA index

The results demonstrated that the application of steroid to animals developing FNP resulted in significant suppression of CD95 (+) cells ratios in the ipsilateral temporal cortex ($P < 0.05$) (Table 1), acyclovir or interferon application on the other hand did not create any statistically significant change in the apoptotic process of either of the brain regions ($P > 0.05$). Also, with steroid treatment CD95 (+) cells ratios in the brain stem were mildly suppressed (CG: 26.6 ± 3.1 vs SG: 20.0 ± 1.4 and AG: 22.1 ± 3.6).

The results of propidium iodide stain and flowcytometric analysis were summarized in Table 1. Although the apoptotic cell DNA index (apoptotic cell DNA / total cell DNA) in the control group was found at a higher ratio than in treatments groups, the ratio was not significant ($P > 0.05$).

Table 1. The extent of CD95 and apoptotic DNA index in the groups (mean \pm SD).

Neural cells / Groups	CD95 (%)		DNA index (%)	
	Ipsilateral brain stem	Ipsilateral temporal	Ipsilateral brain stem	Ipsilateral temporal
Control	26.6 \pm 3.1	30.6 \pm 2.9*	25.9 \pm 3.9	29.0 \pm 1.3
Steroid	20.0 \pm 1.4	17.5 \pm 0.4*	23.4 \pm 3.8	25.5 \pm 1.9
Acyclovir	22.1 \pm 3.6	33.2 \pm 1.5	21.5 \pm 3.2	25.0 \pm 1.6
Interferon	26.6 \pm 3.5	25.2 \pm 4.1	23.7 \pm 5.9	24.8 \pm 3.7

* $P < 0.05$

Discussion

In different neural damages produced by HSV, not only the specific tissue but also adjacent and even non-adjacent cerebral tissues might be damaged. Similarly, our study revealed that in FNP generated by HSV-1, not only the facial nerve but also other brain regions including the brain stem and the temporal cortex were affected (9,10,11). In a study conducted by Ishii et al (6) nasal mucosa, tongue, oral muscles and auricular canal of the animals were infected with HSV-1; despite the fact that FNP did not develop, HSV related antigens have been isolated from facial nerve, trigeminal ganglia and pons; furthermore, these regions have demonstrated inflammatory cellular response, hemorrhage/degeneration and necrosis. This invasion of HSV-1 to more distant regions than the locally inoculated regions and its potential to create neural damage in those regions is mostly due to the neurotropic features of the virus (20).

One of the pathological processes initiated by HSV-1 in the neural tissue is the apoptotic damage (11, 17, 21). If the neuronal apoptotic process induced by HSV-1 can be suppressed by certain medications, this damage can be prevented to a certain extent.

Treatment in FNP should be based on the underlying pathophysiology. Studies on T and B lymphocytes (22, 23), immune complexes (24, 25), cerebrospinal fluid (4, 26, 27) and serum interferon levels (28) in patients with Bell's palsy suggest that the disease process might be a cellular immune response to a viral inflammatory process. If so, steroid treatment would be appropriate. Such treatment has already proved effective (29, 30, 31).

CD95 has been shown to mediate receptor dependent programmed cell death and to be expressed in the nervous system (12). It is now being recognized that CD95 signaling by immune cells mediates effects other than apoptosis (11, 12). In our study, amongst the medications shown to have positive effects on FNP treatment, only steroids were shown to suppress CD95 (+) cells ratios in the ipsilateral temporal cortex. In the studies that were carried out, in addition to the healing process of FNP (13), steroids were shown to have a

neuronal anti-apoptotic effect (14-16, 32). Furthermore, with steroid treatment CD95 (+) cells ratios in the brain stem were mildly suppressed, the situation being reflected in the apoptosis rates of both regions. This condition demonstrates that, in neural damage generated by HSV-1, steroids might have a limited effect in the total apoptotic process. The steroids were shown to exert their effects by increasing the expression of calbindin-D (CALB) which has neuro-protective effects (16) and by inhibiting the influx of calcium into the cell (32).

The results of the studies on the effects of acyclovir and interferon on FNP are controversial (17,18,19,21). Acyclovir is reported to have no effect on the apoptotic process on HSV-1 induced neuronal damage (17, 21), Interferon on the other hand might influence this process depending on the subtypes (18, 19). In our study, these two agents were not shown to have any effect on the apoptotic process in the neural damage accompanying FNP.

HSV-1 is known to be related to several neuropathological conditions (hemorrhagic encephalitis, FNP, chronic encephalitis, epileptogenic neural transformation) (33). Wu et al (34) reported that inoculation of HSV-1 to corneas of mice induced acute spontaneous behavioral and electrophysiological seizures and chronically increased hippocampal excitability together with susceptibility to seizures. HSV-1 induced FNP can also result in similar neuropathological conditions. Because of such possibilities, all HSV-1 induced neural lesions should preferably be treated and their long term follow-up would be crucial for the general health of the patient.

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