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자연살해세포의 인터페론 감마 분비 활성도와 정신적 스트레스가 대상포진 발병에 미치는 영향

Influence of interferon gamma secretory activity of natural killer cells and psychological stress on herpes zoster

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의학과 미생물학전공

김 춘 관

Abstract

Influence of interferon gamma secretory activity of natural killer cells and psychological stress on herpes zoster

Choon Kwan Kim

Microbiology Major

Graduate school of Medicine

Seoul National University

Background: Herpes zoster (HZ) results from reactivation of varicella-zoster virus (VZV) which is latent in dorsal root ganglia. Several risk factors of VZV reactivation has been identified such as older age, female gender, psychological stress and decreased cellular immunity. The pathogenesis of HZ is closely linked to reduced varicella-zoster virus-specific cell-mediated immunity. However, little is known about the role of natural killer (NK) cells in the pathogenesis of HZ. Psychological stress is known to be associated with reduced cell mediated immunity and might impair the function of NK cells in HZ. This study aimed to investigate possible associations among NK cells, T cells and psychological stress in HZ.

Materials and methods: Case-control study was done in order to investigate the association between psychological stress and the function of NK cells in HZ. Forty-four case subjects with recent history of HZ were matched to 44 control subjects without history of HZ at the ratio of 1:1 for age and gender. The function of NK cells was measured by the levels of interferon-gamma secretion from NK cells in 1ml of whole blood with a stimulant. Interferon-gamma secretion from NK

cells, psychological stress events, stress cognition scale scores and cytomegalovirus-specific cell-mediated immunity were compared.

Results: A significantly lower median level of interferon-gamma secreted by NK cells was observed in patients with a recent diagnosis of HZ than in control subjects (582.7 pg/ml vs. 1783 pg/ml; P=0.004), whereas cytomegalovirus-specific cell-mediated immunity was not associated with HZ. Psychological stress events and high stress cognition scale scores were significantly associated in patients with HZ (P<0.001 and P=0.037, respectively). However, reduced interferon-gamma secretion from NK cell and psychological stress were not associated.

Conclusion: Patients with a recent diagnosis of HZ display reduced interferon-gamma secretion from NK cells and frequent previous psychological stress events compared with controls. However, reduced NK cell activity is not an immunological mediator between psychological stress and HZ.

Keyword: NK cell, interferon-gamma, psychological stress, herpes zoster, cytomegalovirus, varicella-zoster virus

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Chapter 1. Introduction

1.1. Study Background

1.1.1. Epidemiology of herpes zoster

Varicella-zoster virus (VZV) is a medically important human-specific virus which belongs to the subfamily *Alphaherpesviridae*. This subgroup, which also includes herpes simplex virus 1 and 2, has a group-defining characteristic to persist in sensory neurons [1]. When VZV primarily infects younger individual, it causes chicken pox. After a primary VZV infection, herpes zoster (HZ) results from reactivation of VZV latent in dorsal root ganglia. Over 95% of adults, more than 50 years old, are seropositive for VZV, therefore, at risk of developing HZ. The estimated average overall incidence of HZ is about 3.4 - 4.82 per 1000 person-years which increases to more than 11 per 1000 person-years in those aged at least 80 years according to the epidemiologic data reported from several countries [2]. In Korea, the overall incidence of HZ was 10.4 per 1000 person-years according to the data of National Health Insurance Service (NHIS). Female gender also influenced on HZ incidence in Korean data; 12.6 cases per 1000 person-years in women and 8.3 cases per 1000 person-years in men [3].

1.1.2. Risk factors of herpes zoster

To date several risk factors for HZ have been identified, such as older age, depressed cell-mediated immunity (CMI), diabetes, genetic susceptibility, trauma, recent psychological stress, female gender and European ethnicity [4]. Among above-mentioned risk factors, its associations were not clearly elucidated. Even the factor of older age may not be a primary risk factor considering HZ also occurs in young adult, especially in stressful circumstances such as military camp [5].

Despite many studies reported female preponderance of HZ [6], it is also uncertain because several reports did not show statistically significant gender difference in the incidence of HZ [7]. In the light of pathogenesis of HZ, women were more vulnerable to psychological stress and show resistance to glucocorticoids feedback explaining the gender difference in HZ incidence [8].

1.1.3. Immuno-pathogenesis of herpes zoster

While the role of adaptive immunity has been well investigated, the role of innate immunity has not been elucidated yet in pathogenesis of HZ. Generally, B cell immunity is important for viral neutralization of the cell-free virus. Despite of the formation of VZV-specific antibodies after varicella infection, this is not necessary for recovery from varicella. Because VZV is a cell-associated virus, virus-specific CMI is needed to eliminate intracellular pathogens. Previous studies also reported that reduced VZV-specific CMI is the most important risk factor for HZ [9-11]. VZV-specific CMI is also essential for the maintenance of latency in dorsal root ganglia. The incidence of HZ has been reported high in cancer patients, bone marrow transplantation and it did not correlate with the levels of VZV-specific antibodies [12]. The variation of VZV-specific CMI was also investigated during occurrence of HZ showing VZV-specific effector T cell activity peaking at 1-3 weeks after the onset of HZ and decreasing rapidly thereafter [13].

1.1.4. Role of NK cells in herpes zoster

It is also well known that innate immune responses such as macrophage is primarily activated before virus-specific T cell immune response occurs under circumstances of primary viral infection. As natural killer (NK) cells also play important roles in the early stage of viral infection [14], NK cell activity might affect the pathogenesis of HZ. The NK cell activity is important for the initial

control of VZV and to trigger the induction of VZV-specific immunity. Activated NK cells are major source of interferon-γ to recruit virus specific T cells and can mediate to amplify initial T cell proliferation. Although NK cell activity varies with age and gender among healthy individuals, only small variations are observed over a long period of time in each of individual, and individuals have been subdivided into consistently high and low groups [15]. In other aspects, significant functional impairments of NK cells have been reported in both direct cytotoxicity and the secretion of cytokines with age [16]. In elderly subjects, the impairment of NK cell secretory activity might be associated with an increased incidence of HZ.

1.1.5. Role of psychological stress in herpes zoster

There has been a common belief that HZ more frequently occurs in stressful individuals. Although one study reported that psychological stress event is common in the patient with HZ [17], the association between psychological stress and HZ has not been clearly proven because the surrogative markers for measuring psychological stress has not standardized scientifically and the cognitive aspect of psychological stress has an individual variation even on the same stressful circumstances. Many previous studies reported that psychological stress increases neuroendocrine hormones, particularly glucocorticoids and catecholamines and eventually reduces overall immune function [18]. The mechanisms of reduced immune functions by psychological stress also suggested that the action of stress hormones has detrimental effects on immune function, including reduced NK cell activity, lymphocyte proliferation, antibody production and reactivation of latent viral infections [19, 20]. Recently, the serotonergic systems and neuromodulators such as cytokines have been raised as the other possible mediators between psychological stress and depressed immunity [21, 22]. However, the interplay

among psychological stress, NK cell activity and HZ has not been clearly elucidated yet, while NK cell activity is reduced in response to or during psychological stress [23, 24].

1.1.6. Cytomegalovirus-specific CMI in HZ

Psychological stress can induce reactivation and shedding of cytomegalovirus (CMV) [25], and CMV infection might be a trigger for VZV reactivation in young adults [26]. It is well known that VZV-specific antibody has no role in preventing HZ and VZV-specific cell mediated immunity is important. However, VZV-specific cell mediated immunity is not easily measurable *in vivo* and major epitope for the prevention of VZV reactivation was not identified. Taken these findings, CMV-specific immunity may be an important variable in HZ pathogenesis, and a surrogate marker of overall T cell immunity in Korea where CMV sero-positivity is almost 100% in adults [27].

1.1.7. The measurement of NK cell activity

Although measurements of NK cell cytolytic activity such as the ^{51}Cr-release assay is considered the standard measuring method of NK cell activity, it is not useful in common clinical settings due to its complicated protocol and hazardous radioactive material. Several flow cytometric methods for NK cell cytotoxicity have been developed using various fluorescent dye, which still need cancer cell lines such as K562 cells [28, 29]. In addition to cytolytic activity assays, IFN-γ secretory activity can be used to evaluate NK cell function. Recently, a commercially available kit for IFN-γ secretory activity of NK cell was developed that requires only 1 ml of whole blood and measure the level of IFN-γ release from NK cells [30, 31]

1.1.8. The hypothesis of this study

Based on above-mentioned findings, the hypothesis of this study that NK cells might serve as an immunological mediator between psychological stress and HZ pathogenesis is established. Specifically, the first hypothesis of this study is that NK cell function, measured by IFN-γ secretory activity, is reduced in HZ patients compared with control subjects. The second hypothesis is that reduced NK cell function is an immunologic mediator between the psychological stress and HZ occurrence.

1.2. Study purpose

First, the possible role of NK cells in the pathogenesis of HZ was investigated by comparing NK cell activity between patients with a recent HZ diagnosis and control subjects without a history of HZ. In order to elucidate the exact influence of NK cell function on HZ occurrence, large observational cohort in which NK cell functions of each subjects had been measured before HZ occurrence is needed. In this case-control study, however, NK cell activity was measured after HZ occurrence. Therefore, the aim of this study would be limited to investigate the association of HZ occurrence and NK cell activity, especially IFN-γ secretory activity without the causal relationship. Because NK cell functions might be decreased with age and different by sex, the control subjects were matched to age and gender. Also, because VZV-specific cell mediated T cell immunity is decreased with age and its difficulty of measurement, CMV-specific cell mediated immunity was investigated by measuring the levels of IFN-γ secretion in order to adjust or compare between HZ and control subjects.

Second, the relationship between psychological stress and NK cell activity was investigated in HZ. The psychological stress was evaluated by investigating whether psychological stress event had occurred in recent time and how psychological stress was recognized in individual subjects by measuring psychological stress cognition scales quantitatively.

Chapter 2. Materials and Methods

2.1. Study Subjects

Study participants were prospectively enrolled at the Veterans Health Service (VHS) Medical Center in Seoul, Korea from March 2016 to September 2016. This study was approved by the Institutional Review Board of the Veterans Health Service Medical Center [file number BOHUN 2015-12-002-001]. Written informed consent was obtained from the enrolled patients with HZ and control subjects. The inclusion criteria were the following: Adults >18 years of age who were clinically diagnosed with HZ by dermatologists or infectious diseases specialists within 6 months of HZ onset. Subjects were excluded if they had one or more of the following conditions: Fever ≥38.3°C, suspected or confirmed infection with diseases other than HZ, a VZV IgG seronegative status, current malignancy, human immunodeficiency virus infection, pregnancy or recent use of chemotherapeutic agents or immunosuppressive drugs within the past 6 months. Control subjects without a history of HZ were individually matched with the subjects with HZ at the ratio of 1:1 for age and gender. Control subjects included patients who visited the outpatient clinic in the VHS medical center and agreed to enroll in this study. The HZ histories of control subjects were evaluated according to patient memory and medical records greater than 10 years in length (the VHS adopted electronic medical records in 2006). HZ stage was simply classified as the eruptive stage, in which the skin displayed a vesicular rash, and the healing stage, in which the skin displayed a crust or healed scar. The initial clinical manifestations were determined based on interviews of subjects with HZ or were obtained from the electronic medical records. The size of skin lesions was classified as <20 cm or ≥20 cm.

Numeric pain rating scale scores (1-10) were reported by patients with HZ and classified as 1-5 or 6-10. All enrolled patients with HZ and control subjects were questioned about HZ vaccination, previous surgeries, chronic illnesses, a previous history of malignancy and body mass index. Blood tests were performed to measure leukocyte counts, the levels of C-reactive protein (CRP), blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and serum immunoglobulin (Ig), and the CMV and VZV serologic statuses at the time of enrollment. Interferon-gamma release assays (IGRAs) for NK cells and CMV-specific T cells were performed simultaneously.

2.2. NK cell IGRA

One milliliter of whole blood was collected, immediately transferred to a NK-Vue™ test tube (ATgen™, Seongnam, Korea) and incubated at 37°C for 17 h. The NK-Vue™ test tube was coated with Promocar™ which was a mixture of cytokines including IL-2, IL-12, IL-15, IL-18 with stabilizing peptides to stimulate the NK cells. Due to the small background activities compared with the test tubes, the negative control tubes were not used, as recommended in the manufacturer's instructions. Supernatants from the incubated tubes were stored at -70°C. An IFN-γ ELISA was performed on the thawed supernatants according to the manufacturer's instructions (ATgen™, Seongnam, Korea). The thawed supernatants were centrifuged at 11,500g for 1 min at room temperature immediately before loading them into microplate wells. Each vial of lyophilized IFN-γ standard, high positive control and low positive control was reconstituted with 500 μL of deionized or distilled water, respectively. Final IFN-γ concentrations were 4000 pg/ml for the

IFN-γ standard, 1000 pg/ml for high positive control and 165 pg/ml for low positive control. After aliquoting of 50 μL of diluent into each well, 50 IFN-γ of each dilution of IFN-γ standard curve, 50 μL of each low and high positive controls, and 50 μL of each sample were added to wells containing diluents. After incubation for 1 hr at room temperature, each well of microplate was washed with 300 μL of wash buffer four times. The detection solution was prepared by dilution of biotin conjugate (biotin-conjugated murine monoclonal antibody against human IFN-γ) and streptavidine HRP 1:99 into conjugate diluent. After adding 100 μL of detection solution to each well, the microplate was incubated for 1 hour 30 min at room temperature and washed four times. After adding 100 μL of TMB (tetramethyl benzidine) solution, the microplate was incubated for 30 min at room temperature in the dark. After adding stop solution to each well, the absorbance of each well was measured at 450 nm. All data processing and calculations were carried out using software packages with microwell plate readers. The lower limit of detection for the NK cell IGRA was 40 pg/ml, and the upper limit was set to 2000 pg/ml for convenience.

2.3. CMV-specific T cell IGRA

Three milliliters of whole blood were collected and immediately transferred to each of the following 3 CMV-Quantiferon™ tubes (Qiagen, Hilden, Germany): a negative control tube (nil), a positive control tube (mitogen) and a CMV antigen (Ag) tube. The antigen tube contained a variety of CMV peptides, including pp65, IE-1, IE-2, pp50 and gB. After 24 h of incubation at 37°C, the supernatants were collected and stored at -70°C. An IFN-γ ELISA was performed on the thawed

supernatants according to the manufacturer's instructions. All plasma samples and reagent, except for conjugate 100x concentrates (murine anti-human IFN-γ HRP), were brought to room temperature before use. The plasma samples harvested from blood collection tubes and subsequently frozen or stored for more than 24 h prior to assay were thoroughly mixed before addition to the ELISA well. 50 μL of each freshly prepared working strength conjugate and plasma samples, and standards were added to ELISA wells. After the conjugates and plasma sample were mixed using microplate shaker for 1 min, the microplate was incubated at room temperature for 2 h. Each of wells was washed with 400 μL of working strength wash buffer for at least 6 cycles using automated plate washer. After 100 μL of enzyme substrate solution was added to each well, the microplate was incubated at room temperature for 30 min. Following incubation 50 μL of enzyme stop solution was added. The optical density (OD) of each well was measured within 5 minutes of stopping the reaction using microplate reader fitted with 450 nm filter and with a 650 nm reference filter. The background IGRA value from the nil tube was subtracted from the value of CMV Ag tube, and the resulting value was used in the subsequent analysis. The upper limit for the CMV-specific T cell IGRA was set to10 IU/ml for convenience.

2.4. Stress events and stress cognition scales

Psychological stress events were classified into more than 14 life categories[1], such as health, financial, residential, familial, occupational, educational, sexual, religious, marital, social, abuse, separation, recent loss, self-esteem and

[1] Korean version in addendum 1

expression problems. Subjects were interviewed to determine whether they had experienced the abovementioned stress events within the previous 6 months. The stress cognition scale was measured using a questionnaire[2] comprising 21 questions or statements, such as 'I feel like crying', 'I have become more suspicious', 'I feel like breaking something', 'I can't do anything', 'My life is meaningless', ' I should be perfect at everything', ' I can't pull myself out of one particular thought', I don't feel like talking', and 'I feel like hitting someone' etc. This questionnaire was validated by correlating the stress cognition scale score with three pre-existing measures related to stress, such as the Korean version of Symptom Checklist-90-Revised (SCL-90-R), the Korean version of the global assessment of recent stress (GARS) scale, and the perceived stress questionnaire (PSQ) [32]. Each question was weighted with scores of 0-4 points according to a Likert-style answer: 'not at all' (0), 'somewhat' (1), 'moderately' (2), 'very much' (3), and 'absolutely' (4). The sum of each score was used as the stress cognition scale score, with a possible range of 0 to 84 points.

2.5. Statistical analysis

Means of continuous variables for the subjects' characteristics and laboratory parameters were compared among groups using two-sided Student's t-tests for normally distributed variables, and medians were compared using the Mann-Whitney U test for non-normally distributed variables, such as age, stress cognition scale scores, and CMV-specific T cell and NK cell IFN-γ secretion. Median values for NK cell IFN-γ secretion among the 3 groups were compared

[2] Korean version in addendum 2.

using the Kruskal-Wallis test and adjusted using Dunn's multiple comparison test. The distribution of categorical variables between groups was compared using Fisher’s exact test. A logistic regression analysis of individual risk factors for herpes zoster was performed. GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA), SPSS version 24 (IBM, Armonk, NY, USA) and the R program (version 3.4.3) were used for statistical analyses.

Chapter 3. Results

3.1. Study population

Forty-four subjects with HZ and 44 control subjects were enrolled in the present study. The demographics of the study population are shown in Table 1. The median ages of the subjects with HZ and control subjects were 71 and 71 among the HZ and control subjects, respectively. The male-to-female ratio was high (37:7) because the study subjects were enrolled at a hospital for veterans. Patients with HZ and control subjects were not matched according to chronic illnesses or histories of malignancy. Although patients with current cancer were excluded from the present study, the frequency of patients with a history of malignancy was greater in the HZ group than in the control subjects (P=0.039). Based on the assumption that HZ vaccination does not affect NK cell activity, the history of HZ vaccination was not adjusted between the patients with HZ and control subjects. Therefore, fewer patients with HZ had received the HZ vaccination (N=6) than the control subjects (N=16). The median time intervals from vaccination to study enrollment were 22 months (range: 10-58 months) and 12 months (range: 2-24 months) in patients with HZ and control subjects, respectively. The subjects with HZ displayed more frequent psychological stress events (P<0.001) and higher stress cognition scale scores (P=0.037) than the control subjects. Histories of smoking and alcohol consumption; previous surgeries; the mean body mass index; and chronic illnesses, such as hypertension, diabetes, ischemic heart disease and chronic renal failure, were not different between patients with HZ and control subjects.

Table 1. Characteristics of Patients with HZ and Control Subjects.

Characteristics	Number of patients		*P* values
	HZ (N=44)	Controls (N=44)	
Age	71.0 [67.0; 75.5]	71.0 [68.0; 75.5]	0.927
Men/women	37/7 (84.1%)	37/7 (84.1%)	1.0
Chronic illness	31 (70.5%)	29 (65.9%)	0.819
History of malignancy	11 (25.0%)	3 (6.8%)	0.039
History of HZ vaccination	6 (13.6%)	16 (36.4%)	0.025
Smoking	5 (11.4%)	2 (4.5%)	0.434
Alcohol consumption	15 (34.1%)	17 (38.6%)	1.0
History of surgery	6 (13.6%)	2 (4.5%)	0.266
Psychological stress events	27 (61.4%)	5 (11.4%)	<0.001
Stress cognition scale scores	14.0 [7.5; 18.0]	9.0 [5.0; 14.0]	0.037
Body mass index (kg/m^2)	24.5±2.8	23.9±3.0	0.2616

P values for continuous variables were calculated using a two-sided Student's t-test or the Mann-Whitney U test. Descriptive data are presented as the means±standard deviations (SD) for normally distributed variables and medians [interquartile ranges (IQR)] for non-normally distributed variables. Fisher's exact test was used for categorical variables.

3.2. Laboratory parameters

The white blood cell counts, lymphocyte counts, and the AST, ALT, CRP, and serum IgG, IgA and IgM levels were not different between the patients with HZ and control subjects (Table 2). All enrolled patients with HZ and control subjects were seropositive for CMV IgG and VZV IgG. All study subjects were CMV IgM-seronegative, and 4 of the 44 (9.1%) subjects with HZ were VZV IgM-seropositive. A higher median VZV IgG level was observed in subjects with HZ than in the control subjects (4000 mIU vs. 1883 mIU/ml, $P<0.001$), whereas the CMV IgG level was not different between the patients with HZ and control subjects.

Table 2. Comparison of Laboratory Parameters Between Patients with HZ and Control Subjects.

Parameters	Number of patients		*P* values
	HZ (N=44)	Control (N=44)	
WBC (cells/μl)	5800 [5115; 7365]	5695 [5015; 6815]	0.904
Lymphocytes (cells/μl)	1760 [1430; 2110]	1905 [1450; 2315]	0.374
hs CRP (mg/l)	0.90 [0.39; 3.20]	0.75 [0.40; 1.45]	0.332
AST (U/l)	25.0 [22.0; 28.5]	25.0 [21.0; 30.5]	0.799
ALT (U/l)	23.0 [17.0; 32.5]	21.0 [17.0; 26.5]	0.579
Creatinine (mg/dl)	0.95 [0.81; 1.16]	1.03 [0.82; 1.15]	0.567
IgG (g/l)	12.5 ± 2.8	12.4 ± 2.2	0.903
IgA (g/l)	2.42 [1.73; 2.96]	2.34 [1.99;2.98]	0.686
IgM (g/l)	0.76 [0.62; 1.07]	0.80 [0.58; 1.07]	0.851
VZV IgG levels (mIU/l)	4000 [2334.5; 4000.0]	1883 [1263.5; 2499.0]	<0.001

P values for continuous variables were calculated using a two-sided Student's t-test or the Mann-Whitney-U test. Descriptive data are presented as the means±SD for normally distributed variables and medians (IQRs) for non-normally distributed variables. Abbreviations and normal ranges: WBC, white blood cell (4-10.0 $\times 10^3$ cells/μl); lymphocytes (1.5-4 $\times 10^3$ cells/μl); hs CRP, high-sensitivity C-reactive protein (<3.0 mg/l); AST, aspartate aminotransferase (13-33 U/l); ALT, alanine aminotransferase (8-42 U/l); creatinine (0.7-1.2 mg/dl); IgG (7-16 g/l); IgA (0.7-4.0 g/l); IgM (0.4-2.3 g/l); VZV, varicella-zoster virus.

3.3. NK cell and CMV-specific T cell IGRAs

First, the levels of IFN-γ secreted from NK cells between the patients (N=44) with HZ and the control subjects (N=44) were compared to investigate the association between NK cell activity and HZ (Fig 1). A significantly lower median level of IFN-γ was secreted from NK cell in subjects with HZ than from those of the control subjects (582.7 pg/ml vs. 1783 pg/ml, P=0.004), whereas the median level of IFN-γ secreted from CMV-specific T cells was not significantly different between the patients with HZ and control subjects (0.955 IU vs. 4.645 IU, P=0.3310). This result showed there was no significant T cell dysfunctions both HZ and control subjects, despite of slight tendency to low IFN-γ secretion from CMV-specific T cells of HZ subjects. Considering negative findings of CMV IgM, there were no recent CMV reactivations in all of study participants. Therefore, IFN -γ secretions from CMV-specific T cells could reflect the overall T cell immunity.

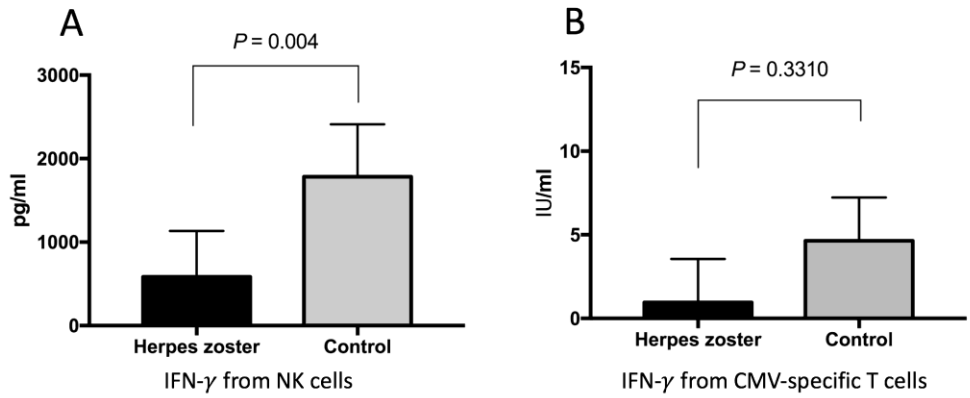

Fig 1. Comparison of IFN-γ Levels Secreted from NK cells (A) and CMV-specific T cells (B) from Patients with HZ and Control Subjects. Median values are depicted with a 95% confidence interval bar. The *P* value was calculated using the Mann-Whitney U test.

The levels of IFN-γ secreted from NK cells according to the HZ stage were analyzed in a subgroup analysis (Fig 2). Forty-four patients with HZ were classified into the eruptive stage group (N=18) and the healing stage group (N=26). The median level of IFN-γ secreted from NK cells was statistically significantly different among three groups, including the control group (P=0.0079). Although the median level of IFN-γ secreted from NK cells was not different between the eruptive stage group and the healing stage group (762.5 pg/ml vs. 496 pg/ml, P>0.999), the levels were statistically significantly different between the healing stage group and the control group (496 pg/ml vs. 1783 pg/ml, P=0.0107).

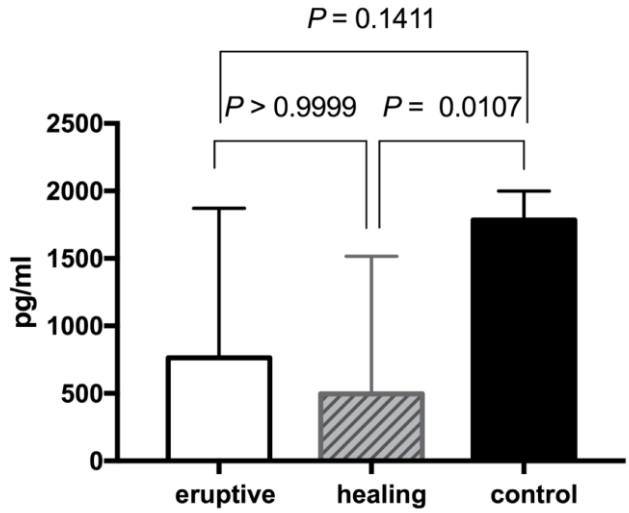

Fig 2. NK cell IFN-γ Secretion according to HZ stage.

The difference in levels of IFN-γ secreted from NK cells from subjects in the eruptive stage (N=18), healing stage (N=26) and control groups (N=44) are shown. Median values are depicted with a 95% CI bar and were compared among three groups using the Kruskal-Wallis test (*P*=0.0079). Median values were compared between two groups, and *P* values were adjusted using the Dunn's multiple comparison test.

3.4. NK cell IFN-γ secretion and psychological stress

The association between NK cell IFN-γ secretion and psychological stress was analyzed in 88 enrolled subjects. The group (N=32) that had previously experienced a psychological stress event had higher psychological stress cognition scale scores than the subjects (N=56) who had not experienced a stress event (median 14.5 vs. 9.5, *P*=0.0250, Fig 3A). The levels of IFN-γ secretion were compared according to psychological stress event (Fig 3B) and psychological stress scale scores (Fig 3C) which was categorized by <14 (N=49) and ≥14 (N=39). Neither the psychological stress event (median 1288 pg/ml vs. 965.6 pg/ml, *P*=0.3994, Fig 3B) nor the psychological stress cognition scale score (median 1515 pg/ml vs. 959 pg/ml, *P*=0.0982, Fig 3C) was associated with NK cell IFN-γ secretion. However, there was a trend toward reduced NK cell IFN-γ secretion in subjects with high psychological stress cognition scale score which could reflect current psychological stress status.

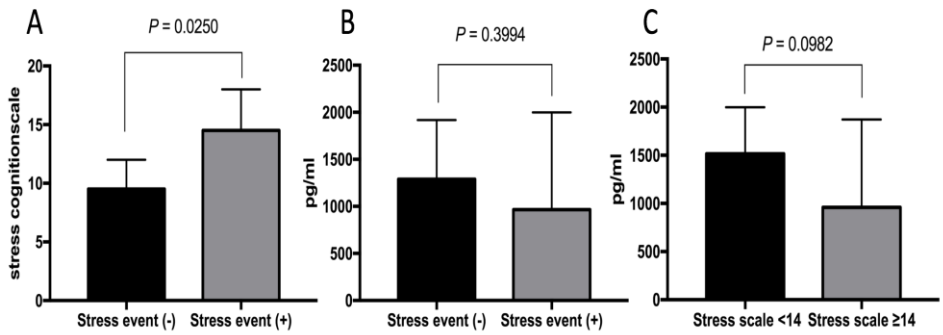

Fig 3. Association Between Psychological Stress and Levels of IFN-γ Secretion from NK Cells.

The stress cognition scale scores were compared (Fig. 3A) between the subjects with psychological stress event (N=32) and the subjects without psychological event (N=56). The levels of IFN-γ secretion from NK cells were compared between the subjects with psychological stress event and the subjects without psychological stress event (Fig. 3B). The levels of IFN-γ secretion from NK cells were compared between the subjects (N=49) with psychological stress cognition scale scores <14 and the subjects (N=39) with psychological stress cognition scale scores ≥14 (Fig. 3C). The median values are depicted with 95% CIs. *P* values were calculated using the Mann-Whitney U test.

3.5. Risk factors for HZ

Multivariate logistic regression analyses were performed to identify risk factors associated with HZ compared with the control subjects. Odds ratios (ORs) and 95% CIs were estimated using logistic regression models. All variables that were identified as significant in the univariate analysis were included in the logistic regression model (Table 3, model 1). The VZV IgG levels were not included as a variable because they were tested after the occurrence of HZ. Stress cognition scale scores and NK cell IFN-γ secretion were categorized by cut-off values of 14 and 600 pg/ml, respectively, considering the small number of subjects and their distributions. The effects of the independent variables associated with herpes zoster by estimating the ORs using a stepwise logistic regression model (Table 3, model 2) were identified. The following independent risk factors for HZ were identified in model 2: a history of malignancy (OR 5.403), psychological stress events (OR 20.356) and NK cell IFN-γ levels less than 600 pg/ml (OR 6.775). The variable of psychological stress cognition scale scores was dependent on the variable of psychological stress events.

Table 3. Logistic Regression Analysis of Factors Associated with HZ.

Risk factors	Model 1 (All variables that were significant in the univariate analysis)			Model 2 (Backward stepwise variable selection from model 1)		
	OR	95% CI	*P* values	OR	95% CI	*P* values
History of HZ vaccination	0.320	0.070-1.471	0.143	0.305	0.067-1.386	0.124
History of malignancy	5.63	1.103-28.701	0.038	5.403	1.111-26.266	0.037
Psychological stress events	17.87	4.431-72.099	<0.001	20.356	5.164-80.238	<0.001
Stress cognition scale score<14	0.49	0.147-1.655	0.252			
NK cell IFN-γ secretion<600 pg/ml	6.4	1.72-23.804	0.006	6.775	1.876-24.465	0.004

P values, OR and CI were calculated using the R program.

3.6. Factors affecting NK cell IFN-γ secretion

The levels of IFN-γ secretion from NK cells was compared among 44 subjects with HZ stratified according to the following factors: age, gender, chronic illness, history of malignancy, HZ vaccination, alcohol consumption, psychological stress event, stress cognition scale scores, size of the skin lesion and initial pain scale scores (Table 4). There was no statistically significant difference in NK cell IFN-γ secretion according to age and gender. However, the trend toward age-associated declining of NK cell IFN-γ secretion was shown. Patients with a skin lesion of more than 20 cm had lower levels of NK cell IFN-γ secretion than patients with a skin lesion less than 20 cm (P=0.032). The other factors, including psychological stress events and stress cognition scale scores, were not related to NK cell IFN-γ secretion. Among a total of 88 study subjects, the NK cell IFN-γ levels were also compared (Table 5). Subjects who had received HZ vaccination had significantly higher NK cell IFN-γ secretion levels than did unvaccinated subjects (P = 0.005). However, its clinical significance was undetermined because HZ vaccination was more frequently done in 44 control subjects than in HZ subjects (6/44 vs 16/44).

Table 4. Factors Affecting IFN-γ Levels in NK Cells Among 44 HZ Patients.

Factors	No. of patients	Median levels of IFN-γ secretion from NK cells (pg/ml)	*P* values
Age (years)			0.169
<70	19	958.5	
≥70	25	391.9	
Gender			0.759
Male	37	599.7	
Female	7	543.2	
Chronic illness			0.414
Yes	31	599.7	
No	13	404.8	
History of malignancy			0.692
Yes	11	599.7	
No	33	565.7	
HZ vaccination			0.352
Yes	6	1148.70	
No	38	554.45	
Alcohol consumption			0.431
Yes	16	471.05	
No	28	1016.80	
Psychological stress events			0.325
Yes	27	958.5	
No	17	391.9	
Stress cognition scale scores			0.348
<14	18	1085.45	
≥14	26	540.25	
Size of skin lesions			0.032
<20 cm	26	1020.25	
≥20 cm	18	271.05	
Initial pain scale scores (NRS[a])			
1-5	22	582.7	0.887
6-10	22	685.6	

[a]NRS: numeric rating scale. *P* values were calculated using the Mann-Whitney U test.

Table 5. The factors affecting NK cell IFN-γ levels among total of 88 patients

Factors	No. of patients	Median NK cell IFN-γ secretion (pg/ml)	*P* values
Age (years olds)			0.628
<70	38	1142	
≥70	50	1050	
Gender			0.434
Men	74	1161	
Women	14	889.8	
Chronic illness			0.545
Yes	60	1034	
No	28	1428	
History of malignancy			0.953
Yes	14	1241	
No	74	1058	
HZ vaccination			0.005
Yes	22	2000	
No	66	984.5	
Alcohol intake			0.695
Yes	33	1026	
No	55	1189	
Psychological stress events			0.399
Yes	32	965.6	
No	56	1288	
Stress cognition scores			0.098
<14	49	1515	
≥14	39	958.5	

P values were calculated using the Mann-Whitney U test.

3.7. Receiver operating characteristic (ROC) curve analysis of NK cell IGRA data

A ROC curve analysis was used to determine the utility of NK cell and CMV-specific T cell IGRAs for the diagnosis of recent HZ (Fig 4). At a cut-off value of 600 pg/ml, the sensitivity and specificity for HZ were 50% and 86.4%, respectively, and at a cut-off value of 1000 pg/ml, the sensitivity and specificity for HZ were 59.1% and 78.2%, respectively. Therefore, due to its low sensitivity and high specificity for HZ, the NK cell IGRA might be useful for differentiating between patients with recent HZ and patients without a history of HZ (area under curve: 0.685, 95% CI: 0.573-0.798, *P*=0.003), whereas the CMV-specific T cell IGRA was not useful (area under the curve: 0.557, 95% CI: 0.436-0.679, *P*=0.356).

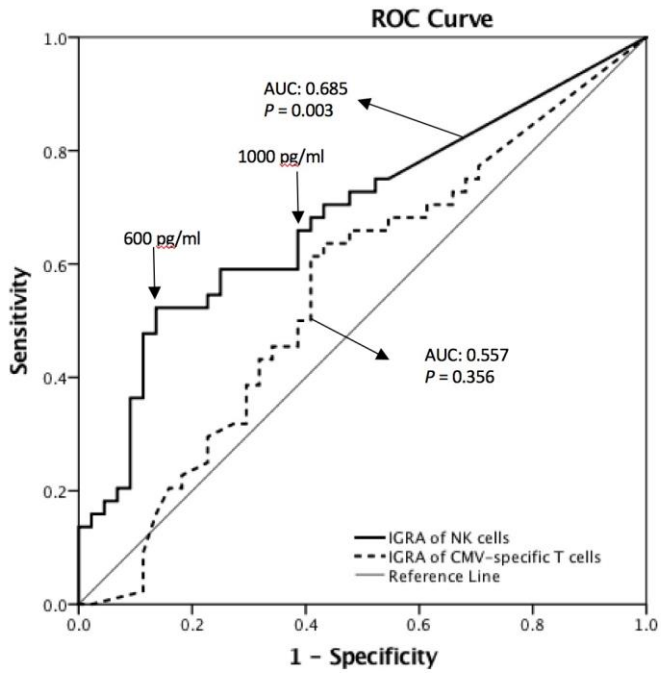

Fig 4. ROC Curves for IGRAs Using NK (continuous line) and CMV-specific T (dotted line) Cells from Subjects with HZ.

The points corresponding to 600 pg/ml (the most distant point from the diagonal line) and 1000 pg/ml (the smallest square of the distance between the left upper corner and the curve) for the NK cell IGRA are indicated on the ROC curve. The area under the curve (AUC) and asymptotic *P* value were calculated for each test based on the assumption of nonparametric data and the null hypothesis (true area=0.5) using SPSS software. IGRA: IFN-γ-release assay, AUC: area under the curve

Chapter 4. Discussion

4.1. Immunologic mechanism of VZV reactivation

To date the pathogenesis of HZ, the process of latent VZV reactivation, has not been clearly elucidated. With recent advance of VZV viral genome studies, it is known that the ORF61 gene product is related with the conversion between the latency state and lytic state; 6 genes are regularly expressed in latent stage and 71 genes are expressed in lytic stage [33, 34]. Current understanding of pathogenesis of HZ runs into limitations when the following question is raised: What is the immunological triggering factor of latent VZV reactivation? While B cell immunity plays some role in protection after varicella vaccination, T cell immunity is an essential component in HZ vaccination. In many clinical reports, e.g. in patients with HIV infection or immunosuppressive therapy but levels of antibody to VZV are relatively saved, the incidence of HZ was markedly increased [35, 36]. Based on these studies, it has been hypothesized that the reactivation of VZV is associated with a decline below some unknown host-specific threshold level of VZV-specific CMI. This hypothesis has also limitations when a following question is confronting: Why do some of young individuals, immunologically competent, come down with HZ?

In the initial stage of viral infection or reactivation, innate immunity such as macrophages and NK cells might play roles in inhibiting viral replication and recruitment of virus-specific adaptive immune cells. With the paucity of post-mortem ganglion samples with active stage of HZ, the precise roles of NK cells during initial reactivation has been difficult to investigate. In several histologic studies from post-mortem dorsal root ganglia of patient with zoster, VZV viral

particles, and infiltration of T cells and NK cells were observed and the roles of $CD4^+$, cytolytic $CD8^+$ T cell were suggested in HZ and post-herpetic neuralgia [37, 38]. Because VZV is a human-specific virus, there has been no reliable animal model for VZV infection. Recently, a xenograft model in mice with severe combined immunodeficiency enable the initial VZV infection to skin, T cells, dorsal root ganglia [39]. However, xenograft model also has a limitation to investigate the roles of NK cells and T cells in initial stage of HZ. Therefore, the measurement of NK cell cytotoxic activity from peripheral blood might be useful for the study of the role of NK cells in HZ.

4.2. Significance of reduced NK cell activity in HZ

The difficulty of measurement of NK cell activity contributed to the paucity of studies about the role of NK cell function in HZ. Although the measurement of NK cell activity used in this study was robust, it was recently developed; it reflects *in vivo* NK cell secretory function by assessing whole blood supernatant and short term *in vitro*-stimulated (17 h) NK cells instead of PBMCs stimulated for 72 h with IL-2. However, the levels of IFN-γ secretion from peripheral blood measured in this study could not exactly reflect the activity of NK cells of ganglion. Despite of the pitfall of the measurement of NK cell activity, several studies examining the associations between the activity of NK cells and tumorous diseases were recently performed using this method [40, 41].

In contrast to general concepts of NK cell activation during viral infection, low levels of IFN-γ secretion from NK cell of patients with HZ compared with controls without a history of HZ were observed. These findings are supported by

the results of two previous studies of NK cell activity in patients with HZ: NK cell cytotoxicity was reduced during HZ infection in immunocompromised hosts [42], and IFN-γ production by peripheral blood mononuclear cells (PBMCs) was decreased during the early stages of HZ infection [43]. Based on these findings, it is suggested VZV activates the immune system and simultaneously uses several immune evasion mechanisms to limit NK cell activity. Following elucidation of the complex mechanism of NK cell activation in the past few decades [44], VZV infection was recently shown to modulate the expression of a ligand for the natural killer group 2D (NKG2D), a potent NK cell-activating receptor, and therefore to limit NK cell activity [45, 46]. After VZV is reactivated in the dorsal ganglion and skin, the overall activity of NK cells of peripheral blood also might be reduced through the modulation of NKG2D expression by unknown mediators from the reactivated VZV of neuronal or skin tissues. Another possible explanation for reduced IFN-γ secretions of NK cell from patients with recent HZ could be that HZ patients already had the reduced NK cell activity and kept its low activity before and after HZ occurrence. However, we are unable to determine whether low levels of IFN-γ secretion from NK cell was a predisposing factor for HZ or secondary consequence of the immunomodulatory effects of VZV in the present study. Multiple sequential tests over a long period are required in the same patients with HZ to clarify the change in NK cell IFN-γ secretion after VZV reactivation.

4.3. Interplay among psychological stress, NK cell activity and HZ

Moreover, psychological stress events were more common and stress cognition scale scores were higher in patients with HZ than in the controls. Although there is a common belief that psychological stress provokes the occurrence of HZ, this link is difficult to prove scientifically because HZ itself causes psychological stress [47]. This study investigated both psychological stress events before HZ and the stress cognition scale scores after HZ to overcome self-reporting bias and clarify the unclear temporal relationship between psychological stress and HZ. The present study determined that a psychological stress event provokes HZ and influences the psychological stress cognition scale score after HZ. In addition, NK cell IFN-γ secretion was not reduced in the HZ patients with stress events compared with the HZ patients without stress events. Although this finding contradicts findings from previous studies showing that psychological stress suppresses NK cell cytotoxicity and IFN-γ production from PBMCs [48], it at least suggests that reduced NK cell activity does not mediate the association of HZ and psychological stress. According to several recent studies, psychological stress simultaneously reduces perforin expression on NK cells and raises blood cortisol levels [49], and glucocorticoids suppress NK cell cytolytic activity, constitutive production of cytokine and perforin, and stimulated production of IL-6, TNF-α, IFN-γ [50]. It is unclear why psychological stress was not related with NK cell IFN-γ secretion in the present study. Although the cortisol levels between patients with HZ and control subjects was not compared in the present study due to the diurnal variations in these levels, the association between cortisol levels and NK cell IFN-γ secretion in patients with HZ should be investigated in a future study.

4.4. Association between HZ symptoms and NK cell activity

Disseminated HZ involving more than 3 dermatomes can occur in immunocompromised hosts, and thus, the severity of HZ symptoms is associated with the robustness of host immune function. In fact, severe forms of varicella infection have been reported in patients with NK cell deficiency [51]. As expected, a correlation between reduced NK cell IFN-γ secretion and the size of the skin lesion was identified. Thus, NK cell immunity plays a role in the clinical manifestations of HZ. The interplay between NK cell activity and VZV-specific T cell immunity should be investigated, to determine the clinical significance of reduced levels of IFN-γ secretion from NK cell.

4.5. No relationship between CMV specific immunity and HZ

In the present study, IFN-γ levels in CMV-specific T cells did not differ significantly between patients with HZ and control subjects, suggesting that patients with HZ and control subjects are equally affected by immune senescence because CMV-specific CMI decreases prominently with age [52]. Although CMV-specific CMI might represent overall T cell immunity in CMV-seropositive patients, the results from the present study suggests that it does not directly reflect VZV-specific CMI in patients with HZ. Otherwise, the number of subjects in this study might be insufficient to show a statistically significant difference in the levels of IFN-γ secretion from CMV-specific T cells between patients with HZ and control subjects. Given previous studies about the declined VZV-specific CMI in HZ, this small difference in CMV-specific CMI subjects might be exaggerated in VZV-specific CMI between patients with HZ and control.

4.6. Factors affecting NK cell IFN-γ secretion

The present study did not find any affecting factors on NK cell IFN-γ secretory activity including age and gender. One old study revealed that NK cells from male blood donors showed more robust cytotoxic activity than those from female blood donors using ^{51}Cr release assay [15]. This gender difference was not robust between postmenopausal aged woman and similar aged men, suggesting estrogen plays inhibitory roles in NK cell activity. However, a recent study showed a contradictory result that elderly women have more robust NK cell activity than do elderly men by measuring cytotoxic granule exocytosis, chemokine synthesis [53]. Therefore, further study about the associations among estrogen, gender and NK cell activity is needed by a standardized measurement method for NK cell activity.

4.7. The limitations of this study

This study had three major limitations. First, it could not evaluate the interplay among NK cell activity, VZV-specific CMI and HZ because precise VZV-specific CMI measurements were difficult to perform. Although the ELISPOT (enzyme-linked immunospot) assay has been used as a standard method for measuring VZV-specific CMI measurement [54], there is no known VZV antigen or epitope available for predicting protective VZV-specific CMI. Second, subjects with HZ were clinically diagnosed based on limited serological evidence and not viral isolation or PCR, and the control subjects who participated in the present study may represent a source of bias due to incomplete memories regarding their HZ histories. Third, because the tests for NK cell activity were not performed

before HZ occurred, the precise role of NK cells in HZ pathogenesis was not elucidated.

According to recent studies, NK cells play roles in modulation of T cell immunity as well as antimicrobial, antitumoral defenses [55]. Because this complex immune interaction between adaptive and innate immune cells in addition to hormones, cytokines and neurotransmitters, there might as well be no straightforward relationship among psychological stress, NK cell activity and VZV-specific CMI. Further study might only differentiate the strong interaction from the weak interaction among the immune interaction complex which comprised a lot of immune mediators.

Chapter 5. Conclusion

Based on the result of this study, the following conclusions can be drawn. First, psychological stress is associated with HZ and previous psychological event is a risk factor of HZ occurrence. Second, NK cell IFN-γ secretion activity is reduced in HZ patients. However, it is not certain that this change occurs after or before HZ contraction. Third, the immunologic link between psychological stress and herpes zoster is not NK cells but might be VZV-specific T cells through neuroendocrine pathways or neuromodulators such as stress hormones and cytokines. In conclusion, patients with a recent HZ diagnosis display reduced NK cell IFN-γ secretion activity and more frequent previous psychological stress events compared with controls. However, the reduced NK cell activity is not an immunological mediator between psychological stress and HZ pathogenesis.

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Addendum

1. Questionnaire for recent psychological stress event in Korean

최근 6 개월 이내에 다음과 같은 스트레스 유발 요인이 있었습니까?

사건	예	시기
경제적, 금전적 문제		
건강상의 문제(대상포진 제외)		
주거환경 문제		
부모 혹은 자녀와의 갈등		
학업 문제		
직장 문제		
배우자와의 갈등		
사회 생활중 갈등		
성적(性的) 문제		
종교 문제		
자존감 상실의 문제		
자신의 생각 표현 어려움		
최근의 상실 사건(배우자, 자녀, 부모 사별)		
학대 사건(신체적, 성적, 정신적)		
기타		

2. Questionnaire for psychological stress cognition scales in Korean

인지적 스트레스 반응 척도

다음 문항들은 여러분이 일상생활에서 스트레스를 받았을 때 일어날 수 있는 생각들 입니다. 각 문항을 주의깊게 읽으면서 오늘을 포함하여 지난 일주일(7일) 동안에 어느 정도로 경험했는지를 해당되는 빈칸에 ○표를 하십시오. 문항을 하나도 빠뜨리지 말고 반드시 한 곳에만 표시하십시오.

문 항	전혀 그렇지 않다	약간 그렇다	웬만큼 그렇다	상당히 그렇다	아주 그렇다
01. 울고 싶다					
02. 의심이 많아졌다					
03. 무엇인가 부수고 싶다					
04. 나는 무능한 사람이다					
05. 삶의 의미를 잃어 버렸다					
06. 어떤 일을 하던지 간에 완벽해야 한다					
07. 한 가지 생각에서 헤어나지 못한다					
08. 말하기 싫다					

09. 누군가를 때리고 싶다					
10. 나는 아무 쓸모가 없는 사람이다					
11. 자신감을 잃었다					
12. 누구에게도 욕을 먹어서는 안된다					
13. 죽고 싶다					
14. 사람들이 나를 싫어한다					
15. 잘 하는 게 하나도 없다					
16. 내가 하는 일에 전망이 없다					
17. 일하기 싫다					
18. 아무런 생각을 하고 싶지 않다					
19. 누군가를 죽이고 싶다					
20. 나는 인생의 낙오자 (또는 실패자)다					
21. 나는 내자신을 싫어한다					

Abstract in Korean (한글 초록)

자연살해세포의 인터페론 감마 분비 활성도와 정신적 스트레스가 대상포진 발병에 미치는 영향

서론: 대상포진의 면역학적 병인으로서 수두대상포진 바이러스 특이적 T 세포면역의 저하는 잘 연구되어 있다. 그러나, 대상포진과 선천면역과의 관련성, 특히 자연살해 세포 활성도와의 관련성에 대한 연구는 매우 적다. 또한, 정신적 스트레스에 노출되면, 적응 혹은 선천면역의 저하가 발생할 수 있고, 결과적으로 바이러스 감염에 쉽게 이환될 수 있음은 잘 알려져 있다. 또한, 정신적 스트레스에 노출되면 자연살해세포의 활성도가 감소한다는 몇몇 연구가 있었으나, 정신적 스트레스가 대상포진 발병에 어떤 면역학적 기전으로 영향을 미치는지는 연구된 적이 없다. 이 연구는 대상포진 발병에서 정신적 스트레스의 역할과 정신적 스트레스와 자연살해 세포 활성도의 관련성 분석을 통해, 자연살해세포의 활성도가 정신적 스트레스와 대상포진 발병의 면역학적 매개 역할을 하는지 알아보고자 하였다.

방법: 최근에 대상포진을 앓은 44 명의 환자와 대상포진을 앓은 적이 없는 44 명의 건강 대조군을 성별과 나이를 매칭하여 연구 대상으로 등록한 후, 자연살해세포의 인터페론 감마 분비능, 최근의 정신적 스트레스 사건여부, 현재의 정신적 스트레스 인지점수, 거대세포바이러스 특이적 세포의 인터페론 감마 분비능을 비교하였다.

결과: 자연살해세포의 인터페론 감마 분비능은 건강 대조군보다 대상포진 환자군에서 의미있게 낮았다(582.7 pg/ml vs. 1783 pg/ml; P=0.004). 이에 반해 거대세포바이러스 특이적 세포의 인터페론 감마 분비능은 두군에서 차이가 없었다. 정신적 스트레스 사건은 대상포진 군에서 빈번하였고(P<0.001), 정신적 스트레스 인지점수도 대상포진 군에서 통계적으로 의미있게 더 높았다(P=0.037). 그러나, 감소된

자연살해세포의 인터페론 감마 분비능은 정신적 스트레스와 관련성이 없었다.

결론: 최근에 대상포진을 앓은 환자군은 자연살해세포의 인터페론 감마 분비능은 감소되고 빈번한 정신적 스트레스 사건을 가지고 있다. 그러나, 자연살해 세포의 감소된 인터페론 감마 분비능은 정신적 스트레스와 대상포진 발병과의 면역학적 매개체로 간주할 수 없다.

주요어 : 자연살해 세포, 인터페론 감마, 정신적 스트레스, 대상포진, 수두 대상포진 바이러스, 거대세포 바이러스

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