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Original Article

Subtypes and contribution of immunocytes in respiratory system of fatal hand, foot and mouth disease

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Abstract: Background and objective: Hand, foot, and mouth disease (HFMD) is a widespread disease which is common in childhood, especially in China. Even most of the cases are mild and self-limited, a very small number of HFMD children still suffer death. Immune reaction has been discovered to play a critical role in the regulation of the progression of HFMD. Our previous postmortem study also revealed that evident inflammation response was observed in the respiratory system of fatal HFMD cases. However, no detail of the subtypes and distribution of distinct immunocytes in the respiratory system of fatal HFMD have been unveiled. In the current study, the classification, quantity and location of immunocytes were determined in fatal HFMD children. Materials and methods: The formalin-fixed paraffin-embedded (FFPE) tissues of respiratory system were gathered from 15 autopsies of fatal HFMD children, as well as three children and eight adults of accidental suffocation. The hematoxylin-eosin (HE) staining was performed to observe the pathological morphology in all samples. Immunohistochemistry (IHC) was conducted to detect the expression of CD3, CD4, CD8, CD20, CD79a, CD57, CD68 and CD163 in all groups, which were specific biomarkers for different subtypes of immunocytes. Results: Inflammatory change was pronounced in the lungs of HFMD cases, including inflammatory cells infiltration, capillary congestion and tissue edema. The immunocytes in the lungs of HFMD were comprised of T and B lymphocytes, NK cells and macrophages, which displayed different distribution patterns. The numbers of $CD4^+$ T cells, $CD79a^+$ B cells, $CD57^+$ NK cells, $CD68^+$ and $CD163^+$ macrophages were significantly higher in HFMD group than those in the children control group ($P<0.05$). Conclusions: These observations provide evidence that main pulmonary pathological change can be characterized as virus pneumonia; and immunological cells are considered to be potentially crucial in the local immune response in lung tissues of fatal HFMD patients. Further study is required to explore the detailed and accurate relationship between local cellular immunity and the outcome of HFMD.

Keywords: Hand, foot and mouth disease (HFMD), EV71, immunocytes, T lymphocyte, B lymphocyte, naturel killing (NK) cell, macrophage

Introduction

Hand-foot and mouth disease (HFMD) is an acute infectious disease which is caused by the enteroviruses infection. Preschool children, especially those under 3 years old, are more prone to HFMD [1-4]. There are more than 20 subtypes of enteroviruses contributing to HFMD, among which, enterovirus 71 (EV 71) and Coxsackievirus A16 (Cox A16) are the most prevalent caus ative agents [5-9]. Our previous postmortem study revealed that the main symptoms of HFMD include rash on hands, feet and mouth. However, in a few cases, HFMD patients suffer meningitis, encephalitis, encephalomyelitis, pulmonary edema, circulatory disturbance, etc. And the death is mainly caused by brain stem encephalitis and neurogenic pulmonary edema [10]. The pathogenesis of HFMD hitherto has not been clarified, and it is hypothesized that the occurrence of HFMD may be associated with viruses directly infringing

tissue cells (e.g. neural tissue) or with sepsis causing abnormal immunity response. Researches of China and other countries have shown that HFMD-related viruses could lead to dysfunction of the immune system [11-14], but the role of immunity responsein the pathogenesis of severe HFMD has thus far remained unknown. Previous reports on the HFMD-caused immune system alteration focused on peripheral blood and cerebrospinal fluid [12, 14], whereas researches on focal immunity response in tissue were scarce. In this research, we detected the expression of CD3, CD4, CD8, CD20, CD79a, CD57, CD68 and CD163, in order to observe the distribution, quantity and significance of different types of immune cells in the tissues of respiratory system in autopsy cases of children who died of fatal HFMD by using immunohistochemistry (IHC). Furthermore, this study discussed the relationship between immune condition and the progression and prognosis of fatal HFMD, thus could present a theoretical base for treatment of HFMD by immune regulation.

Materials and methods

Clinical data

Fifteen autopsies of HFMD children from January, 2008 to August 2012 were collated in the study, age ranging from eight months to 3.6 years old (mean 1.72 years old). There were 12 boys and 3 girls in the group of HFMD. All the samples were pathologically confirmed as severe HFMD in accordance with the diagnostic criteria implemented by Ministry of Health (http://www.nhfpc.gov.cn/yzygj/s3593g/201306/6d935c0f43cd4a1fb46f8f71acf8e245.shtml) [5]. Three autopsy cases (two boys and one girl) of accidental suffocation in 2012 were selected as control group for children, age ranging from one month to five months (mean 0.3 years old). Another eight samples of normal lung tissues from adults in 2014 were also obtained, including seven males and one female with the age ranging from 47 to 74 years old (mean 55 years old). The formalin-fixed paraffin-embedded (FFPE) tissues from respiratory system were gathered from the Pathology Department of the First Affiliated Hospital of Guangxi Medical University. The Ethical Committee of the First Affiliated Hospital of Guangxi Medical University approved the current study and written informed consents were obtained by the families and clinicians to permit the usage of the samples for research.

Morphology and immunohistochemistry

FFPE sections on polylysine-coated slides were used for the morphology and IHC evaluation. The hematoxylin-eosin (HE) staining was performed to observe the pathological morphology in all samples. Immunohistochemical staining was implemented with EnVision™ detection system (Long Island, Shanghai, China) as previously reported [15]. The following monoclonal antibodies were incubated overnight in a humidity chamber at 4°C, including CD3, CD4, CD8, CD20, CD79a, CD57, CD68 and CD163 (Zhongshan Jinqiao Corp, Beijing, China). Blank control sections were incubated with only phosphate buffer solution (PBS) rather than primary antibody. Tissues of acute tonsillitis were used for the positive controls for CD3, CD4, CD8, CD20, CD79a and CD57, while normal hepatic tissues were taken as positive controls for CD68 and CD163.

The IHC positivity was assessed as specified based on the immunodetection of stain intensity. The staining pattern was yellow or brown color in the cytomembrane for CD3, CD4, CD8, CD20, CD79a and CD57, and in the cytoplasm for CD68 and CD163. The positive signaling could be focal, fine granular, linear or diffuse. The positive cells were identified and counted in 10 highmagnification (HPF 400×) randomly selected under a light optic microscope. The mean number of 10 visual fields was presentative for the result of a subtype of immunocytes, i.e., CD3, CD4 and CD8 for T lymphocytes; CD20 and CD79a for B lymphocytes, CD57 for natural killing (NK) cells, and CD68 and CD163 for the macrophages. All IHC staining wasexamined independently by three pathologists (HBP, SY and GC).

Statistical analyses

SPSS 20.0 for Windows was utilized for the statistical analysis. The amount of the positive cells was present as Mean ± Standard deviation (Mean ± SD) if the variable conformed to normal distribution, otherwise the data was expressed as Median (Inter-Quartile Range (IQR)) [M(*Q*)]. Student *t* test was applied to compare the difference of immunocytes among various groups if the data accorded with nor-

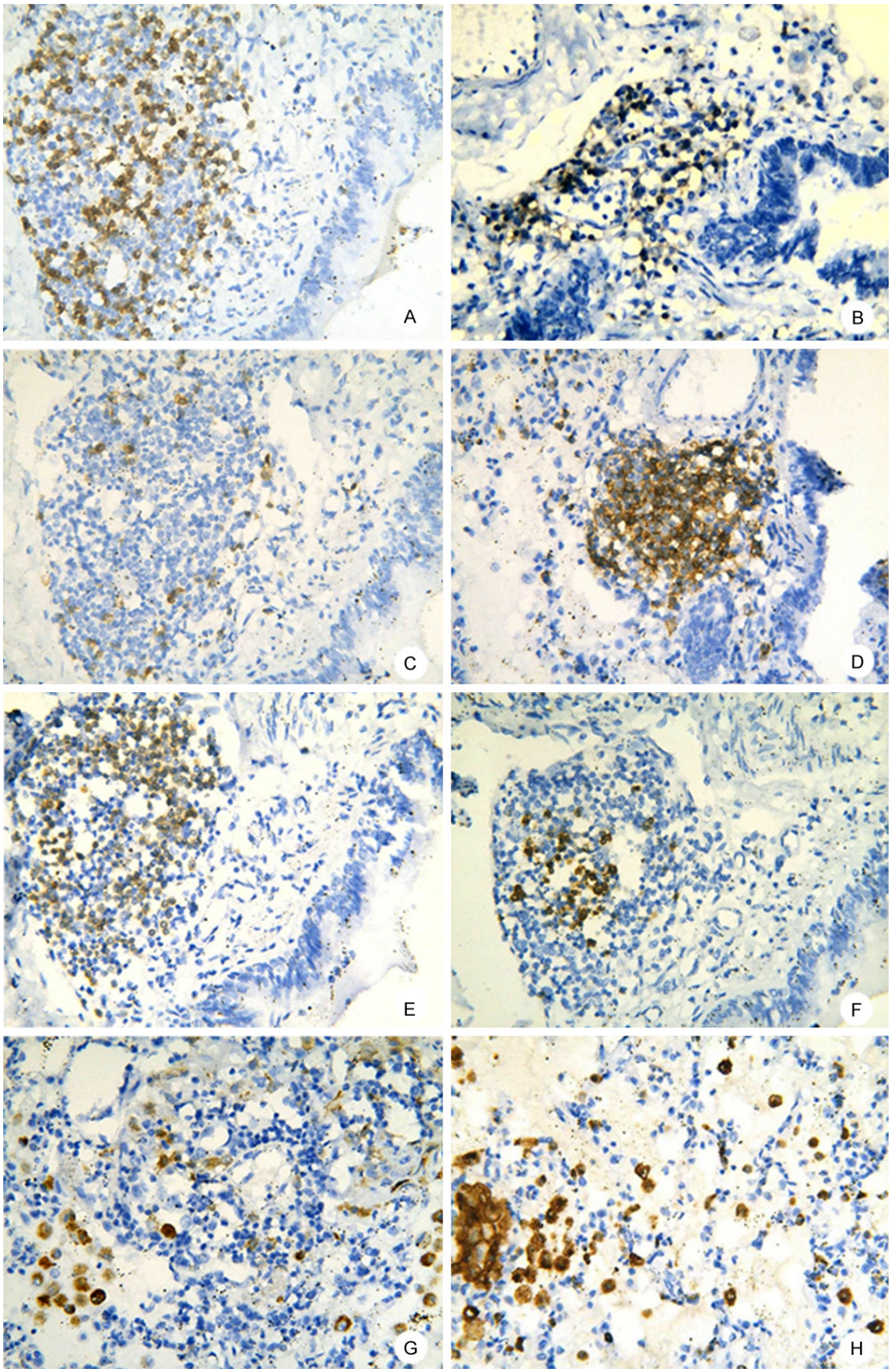
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Figure 1. Number and distribution of various immunocytes in lung tissues of HFMD. T lymphocytes: CD3 positive (A), CD4 positive (B), CD8 positive (C); B lymphocytes: CD20 positive (D), CD79a positive (E); Natural killing (NK) cells: CD57 positive (F); Macrophages: CD68 positive (G), CD163 positive (H). Immunohistochemistry, Supervision™, 400×.

Table 1. Comparison of different immunocytes between HFMD and control group [$\bar{X}$±S/M(Q)]

	HFMD (n=15)	Control (n=3)	t/Z value	*P* value
CD3	53.7±24.5	39.9±19.9	0.899	0.39
CD4	38.3±15.4	14.6±2.9	-2.165	0.03
CD8	28.1±15.2	23.7±6.4	0.482	0.64
CD20	62.9±35.6	25.1±4.1	-1.443	0.18
CD79a	59.4±29.0	19.9±8.8	2.270	0.04
CD57	14.4 (13.6)	9.2±1.7	2.020	0.04
CD68	16.9±3.3	11.0±2.9	2.798	0.02
CD163	19.0±2.2	10.6±1.5	6.110	0.00

mal distribution and homogeneity of variances, or else, Mann-Whitney U test would be employed. A value of $P<0.05$ was regarded as statistically significant.

Results

Pathological morphology of respiratory system in fatal HFMD

It was discovered by HE stain that inflammatory cells, mainly lymphocytes and mononuclear phagocytes, formed clusters around bronchi and bronchiole. Alveolar septum widened, where dilatation and congestion of the capillaries and venues were noted, and infiltration of lymphocytes and mononuclear phagocytes was also observed. In pulmonary alveoli, large amount of protein-rich edematous fluid, as well as lymphocytes and mononuclear phagocytes were noticed. A small number of neutrophils infiltrated in some cases. Notwithstanding the evidence, in a few cases, only pulmonary emphysema was observed since alveolar epithelium ruptured and alveoli merged.

The number and distribution of immunocytes in lung tissues of fatal HFMD

The immunocytes in the lungs of HFMD were comprised of T and B lymphocytes, NK cells and macrophages, which exhibited diverse distribution patterns. In HFMD group, CD3 positive ($CD3^+$), $CD4^+$, $CD8^+$ T lymphocytes and $CD20^+$, $CD79a^+$ B lymphocytes congregated around bronchi, bronchiole and thickened parts of alveolus walls. Most of $CD57^+$ NK cells largely scattered in the condensed alveolus walls. Only a few $CD57^+$ NK cells formed in clusters. $CD68^+$ and $CD163^+$ macrophages were mainly distributed in alveolar space. Compared with the children control group, the number of $CD4^+$ T cells, $CD79a^+$ B cells, $CD57^+$ NK cells, $CD68^+$ and $CD163^+$ macrophages was notablyhigher in HFMD group ($P<0.05$). However, there were no significant differences in the number of $CD3^+$, $CD8^+$ T cells and $CD20^+$ cells in both groups ($P>0.05$, **Figure 1**, **Table 1**).

Discussion

The current study discovered that the pathological morphology of lung tissue of lethal HFMD patients was in accordance with virus pneumonia accompanied with severe lung edema. Under microscope, typical inflammatory change could be noted, such as lymphocytes, mononuclear macrophages. Additionally, even neutrophils scattered; congestion of capillaries and venulesoccurred; and proteinic edemas fluid appeared. It was suggested that this type of pulmonary edema was neurogenic, which was caused by craniocerebral impairment or CNS diseasewithout cardiac and pulmonary disease [16]. Nevertheless, particular studies indicated that pathogenesis of pulmonary edema of severe HFMD patients still remained unknown, which might be associated with high pulmonary vascular permeability that was the result of systemic inflammation triggered by brain stem encephalitis and cytokine release [17, 18]. Ooiet al also confirmed that victims of fatal HFMD resulting from EV71 infection deceased from brain stem encephalitis and pulmonary edema [19, 20]. Therefore, it is essential to ascertain the pathological morphology of lung tissue of lethal HFMD patients, which will provide clinical evidence for early diagnosis of lethal HFMD and will bring advantage to early intervention, and better prediction of the progression and prognosis of HFMD.

Wang et al [21] and Chang et al [22] both observed the proinflammatory cytokines and immunological cells in sera and cerebrospinal

fluid of the EV71-infected patients, confirming that host immune response played a crucial part in the occurrence and development of HFMD. Our previous studies also proved that T cells, B cells and macrophages/microglia were all involved in the local immune response in the central nervous system (CNS) of severe HFMD patients (data on file). This study further explored the local immune status in lung tissues of HFMD patients by detecting the number and location of T cells, B cells, NK cells and macrophages in lung tissues of HFMD patients complicating virus pneumonia and pulmonary edema.

T lymphocytes are believed to mediate adaptive cellular immune response, also play an important assisting role in humoral immunoresponse. Mature T lymphocytes only expressed CD4 or CD8 molecules. $CD4^+$ help T cell can be further divided into three categories, Th1, Th2 and Th3. Our research results discovered that $CD3^+$, $CD4^+$ and $CD8^+$ T cells were distributed in clusters adjacent to bronchi and bronchiole, and the number of $CD4^+$ T cells was much greater than that in healthy controls. It was indicated that cellular immunity mediated by host T lymphocytes was connected with local immune response in lung tissues of HFMD patients. Th1 cells mediated cellular immunologic response by secreting interleukin-2 (IL-2), IL-γ and interferon-β (TNF-β); and Th2 cells assisted humoral immunoresponse by secreting IL-4, IL-5, IL-3, and IL-13. It was consistent with study of Yang et al [23] that the host T cells immunologic response played a crucial part in defending EV71 infection and inhibiting the development of the disease. There existed no significant difference regarding the counting of $CD3^+$ and $CD8^+$ T cells between HFMD group and control group, which contradicted the results in CNS conducted earlier by our research team (data not shown). We reconsidered the contradictory results and put forward one possible explanation that cellular immunity mediated by T cells might mostly act as an assisting role, rather than a major role, in lung tissues of fatal HFMD patients. Besides, it was observed that a considerable number of $CD3^+$, $CD4^+$, and $CD8^+$ T cells exuded from blood vessels, which implied that it might result in the decrease of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the peripheral blood samples of HFMD patients.

B lymphocytes function in the humoral immunity component of the adaptive immune system by secreting antibodies. B cell receptors (BCRs) were expressed on the cytomembrane of B lymphocytes, which consists of cell membrane immunoglobulin and Ig α(CD79a)/Ig β(CD79b) heterodimer. And BCRs permit the B lymphocytes to bind a specific antigen, against which it will initiate an antibody response. This research proved that $CD20^+$, $CD79a^+$ B cells scattered as T cells did. Compared with healthy control groups, the counting of $CD79a^+$ Bcells was noticeably higher in HFMD group. It was suggested that B cells-mediated cellular immunity played a part in the local immune response of lung tissue of the HFMD patient. After antigens combined with BCRs, signals were provided to be transmitted into the cells by CD79a/CD79b. And the signals cooperated with costimulatory signals generated by Th cells to activate B cells and thus play a vital role in the immune response. The current results contradicted our previous studies in CNS that the counting of $CD20^+$ cells was the same both in the lungs of HFMD and control groups. CD20 was the major surface marker for B cells. We attributed the differences to a small number of samples as control group. In next phase, more samples will be expected to confirm the conclusion.

Both NK cells and macrophages are classified as non-specific immune cells, which can destroy infectious target cells without the presensitization of antigens, and they are considered to be important in the immune system. This study showed that $CD57^+$ NK cells were disseminated in lung tissue of HFMD patients, despite some forming clusters; $CD68^+$ and $CD163^+$ macrophages were largely distributed in alveolar space, and the counting of all these cells was remarkably greater compared with healthy control groups. It was indicated that inherent immunity mediated by NK cells and macrophages was involved in the local immune response of lung tissue of HFMD sufferers. The result was inconsistent with previous conclusion that NK cells did not engage in the innate immune response in CNS (data not shown). It was inferred that EV71 infected lung tissues by way of respiratory passages and lung tissue was the infected organ in early stage. Innate immune response mediated by

NK cells and macrophages played a crucial role in the early phase of infection. In contrast, CNS was not the organ that was infected by EV71 in the early stage. Therefore, NK cells did not participate in the innate immune response in CNS of lethal HFMD cases.

In summary, the research detected the expression status of T cells, B cells, NK cells and macrophages in lung tissues of fatal HFMD cases, leading to conclusions that the main pathological change in lung tissues can be characterized as virus pneumonia; and that immunological cells are considered to be crucial in the local immune response in lung tissues. Further study remains required as to explore the detailed and accurate relationship between local cellular immunity and the outcome of HFMD.

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Disclosure of conflict of interest

None.

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