Death Mechanisms of Pulmonary Alveolocytes in Mice Infected with Influenza Viruses A/H1N1/California/04/2009 and A/H5N1/Goose/Krasnoozerskoye/627/05

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In CBA mice infected with influenza viruses A/H1N1/California/04/2009 and A/H5N1/Goose/Krasnoozerskoye/627/05 in a dose of 10 MLD₅₀, the mechanisms of death of pulmonary alveolocytes over 10 postinfection days were studied by light microscopy, immunohistochemistry, and morphometry. In mice infected with A/H1N1, alveolocytes died predominantly via necrosis, while apoptosis mostly employed the mitochondrial pathway. In mice infected with A/H5N1, apoptosis was the dominant mechanism of alveolocyte death proceeded via membrane receptor signaling followed by switching to FAS-mediated pathway via activation of FADD, the apoptotic signal transduction protein.

Key Words: *alveolocytes; influenza A viruses; apoptosis; necrosis; caspases 9, 3*

All influenza A viruses are pneumotropic; they replicate in the pulmonary cells earlier than in other cells and exhibit pronounced cytodestructive effects [6,8,10] implemented via apoptosis and necrosis. Both of them are triggered and realized by different mechanisms, which can be determined by virus machinery on the one hand, and the reaction to it by infected cells and regulatory systems of the body, on the other. However, these mechanisms are little studied, so the novel data are needed to refine pathogenesis of influenza viruses.

This work was designed to study prevalence, specificity of initiation, and implementation peculiarities of the alveolocytic death mechanisms in mice infected with influenza viruses A/H1N1/California/04/2009 (A/H1N1) and A/H5N1/Goose/Krasnoozerskoye/627/05 (A/H5N1).

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MATERIALS AND METHODS

The experiments were carried out on 7-8-week-old CBA mice ($n=40$) obtained from Breeding Center of Vector State Research Center of Virology and Biotechnology (Kol'tsovo, Novosibirsk region). All experimental procedures were carried out in accordance to European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The animals were sacrificed by cervical dislocation under ether narcosis.

The study employed influenza viruses A/H1N1/California/04/2009 (A/H1N1) and A/H5N1/Goose/Krasnoozerskoye/627/05 (A/H5N1), which had been isolated in Novosibirsk region. The mice were intranasally infected with equal viral doses of 10 MLD₅₀. The experiments with influenza viruses were carried out in strict adherence to the sanitary and epidemiological regulations SP 1.2.731 "Safety in Working with Microorganisms of Hazard Groups 3 and 4 and Helminths" (January 25, 2005).

The control groups 1 ($n=10$) and 2 ($n=10$) comprised the intact mice. The groups 3 ($n=10$) and 4

($n=10$) mice were infected with A/H5N1 and A/H1N1 viruses, respectively.

The lung specimens of groups 3 and 4 mice were obtained in 1, 3, 6, and 10 days after infection. The controls were sacrificed in maintenance days 1 and 10. Routine histological processing of the lung specimens included hematoxylin and eosin staining. Immunohistochemical methods [4] with specific primary antibodies were employed to detect the antigen of influenza A virus (InfA; Abcam) and expression of TNF α and TRAIL (DBS) as well as FAS and FADD (DBS) by the pulmonary cells. Expression of caspases 9 and 3 by alveolocytes was also assessed using Abcam assay kits.

Histological examination was performed under an AxioImager A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss). Morphometry of pulmonary structural elements in the histological sections was carried out in a 100-point closed test system with the area of $3.64 \times 10^5 \mu^2$ employing AxioVision (rel. 4.12) software.

The mean values of volume density (Vv) was calculated with Statistica software (StatSoft, Inc.). The data were analyzed statistically using Student's t test at $p < 0.05$. The results are presented as $m \pm SEM$.

RESULTS

In all periods of experiments, the lungs of mice infected with A/H5N1 and A/H1N1 viruses were characterized by the presence of cells in the states of necrosis and apoptosis, interstitial pneumonia with the forma-

tion of inflammatory mononuclear-lymphocytic infiltrates, atelectasis foci, alveolar and interstitial edema, hyaline membranes in alveoli, massive hemorrhages, and thrombosis of blood vessels.

On days 1-6 after infection with A/H5N1, the lungs exhibited a 6.5-fold expansion of the foci with necrotically altered alveolocytes, but on day 10, their volume density decreased by 20%. In A/H1N1-infected mice, the volume density of pulmonary necrotic foci increased by 5.3 times during entire term of the study, and these foci were always larger than in A/H5N1-infected mice (Table 1). In A/H5N1-infected mice, pulmonary foci with apoptotically altered alveolocytes were larger than the foci with necrotically modified cells (Table 1) throughout the observation period; the volume density of pulmonary necrotic foci was higher than that of foci with apoptotically altered alveolocytes (Table 1). However, the foci with inflammatory macrophage-lymphocyte infiltrates were larger in mice, whose alveolocytes were modified mostly by apoptosis than in mice with predominantly necrotic alveolocytes. The scale of the overall apoptotic and necrotic destructive processes was significantly greater in A/H5N1-infected mice than in A/H1N1-infected ones. Hence, the necrotic processes in influenza are implemented via cytodestructive effects of the viruses; they are stimulated by hypoxia, ischemia, and thrombosis of the blood vessels resulting from enhanced tropicity of influenza viruses to endotheliocytes [4,9]. Seemingly, the apoptotic death of the cells is induced by hypoxia; its development depends on the number and activation degree of macrophages in

TABLE 1. Destructive Alterations and Inflammatory Processes in the Lungs of CBA Mice Infected with A/H1N1/California/04/2009 and A/H5N1/Goose/Krasnozerskoye/627/05 Influenza Viruses

Parameter	Postinfection day	Group			
		intact	A/H5N1	intact	A/H1N1
Volume density of necrotic foci, %	1	0.08 $\pm$ 0.02	1.48 $\pm$ 0.54*	0.26 $\pm$ 0.08	3.80 $\pm$ 0.18* ^o
	3		5.76 $\pm$ 0.76**		9.08 $\pm$ 0.16** ^o
	6		9.64 $\pm$ 0.9**		16.48 $\pm$ 0.26** ^o
	10		7.70 $\pm$ 1.26*		17.48 $\pm$ 0.32* ^o
Volume density of apoptotic foci, %	1	0.18 $\pm$ 0.06	6.55 $\pm$ 1.37*	0.08 $\pm$ 0.02	1.1 $\pm$ 0.13* ^o
	3		30.96 $\pm$ 1.91**		2.1 $\pm$ 0.10** ^o
	6		41.96 $\pm$ 4.82**		5.60 $\pm$ 0.13** ^o
	10		21.10 $\pm$ 4.14**		5.80 $\pm$ 0.12* ^o
Volume density of foci with inflammatory infiltrates, %	1	0.12 $\pm$ 0.08	19.26 $\pm$ 2.37*	0.18 $\pm$ 0.06	2.70 $\pm$ 0.16* ^o
	3		23.87 $\pm$ 0.39*		6.98 $\pm$ 0.16** ^o
	6		25.02 $\pm$ 2.27**		10.88 $\pm$ 0.28**
	10		15.12 $\pm$ 2.01**		11.68 $\pm$ 0.29** ^o

Note. $p < 0.05$ in comparison with *the corresponding intact group, *previous postinfection day, ^oA/H5N1-infected mice.

Fig. 1. Effects of A/H5N1 and A/H1N1 influenza viruses on the levels of caspase 9 and caspase 3 in mouse alveocytes.

Fig. 2. Number of TNFα⁺ alveocytes in mice infected with A/H5N1 and A/H1N1 influenza viruses.

Fig. 3. Number of TRAIL⁺ and FADD⁺ alveocytes in mice infected with A/H5N1 and A/H1N1 influenza viruses.

the infiltrates and their up-regulated secretion of nitric oxide, proteases [1,2,4], and apoptosis-inducing factors, which is characteristic of mitochondrial pathway; moreover, apoptosis can be triggered from cell membrane via the plasmalemmal receptor pathway. Probably, the functional status and the number of activated macrophages can select the predominant pathways, which trigger apoptosis during viral influenzas. It is noteworthy that the scales of inflammatory infiltrates correlated with the total volume of the foci of destructive processes (necrosis and apoptosis), but not with that of the necrotic ones (Table 1).

In infected mice, immunohistochemistry visualized the positive staining of alveocytes for viral antigen A [3-5].

On postinfection day 1, numerous apoptotic alveocytes expressing caspase 9 were detected in A/H1N1-infected mice. By postinfection day 10, the number of such cells decreased. In contrast, the number of the alveocytes expressing caspase 3 changed in the reverse order, and it attained maximum to the end of experiments (Fig. 1). In A/H5N1-infected mice, the number of alveocytes expressing caspase 9 was significantly smaller in all terms of experiments than in A/H1N1-infected mice on postinfection days 1 and 3. Overall, the number of alveocytes expressing caspase 3 was significantly higher in A/H1N1-infected mice than in mice infected with A/H5N1 (Fig. 1). Expression of caspases 9 and 3 revealed in apoptotically modified alveocytes at the early terms after infection with A/H1N1 virus indicated that the mitochondrial mechanism of apoptosis initiation takes place after infection with any type of influenza A viruses, although the viral determination of predominant initiation and implementation of apoptotic death probably exists as well.

Probability of realization of the plasmalemmal receptor mechanism of apoptosis in alveocytes was assessed according to expression of TNFα and TRAIL markers (Figs. 2, 3). In A/H5N1-infected mice, the number of apoptotically altered alveocytes expressing TNFα was the greatest on postinfection day 1, thereupon it decreased to postinfection days 6 and 10 (Fig. 2). In contrast, the number of such cells was smaller in the alveocytes of A/H1N1-infected mice at all terms of experiments, although it gradually increased to postinfection day 10 (Fig. 2). It is important that in all terms of experiments, the size of foci of the macrophagal-lymphocytic infiltrates were larger in A/H5N1-infected mice than in A/H1N1-infected ones (Table 1). The number of apoptotically altered TRAIL⁺ and FADD⁺ alveocytes was the greatest on postinfection days 6 and 10 for both viruses, and the number of such alveocytes was significantly higher in A/H5N1-infected mice (Fig. 3). It is a common knowledge that under the effect of primary messengers, TNFα and

TRAIL can form the ligand-receptor complexes thereby activating signal transduction to mitochondria and provoking energy imbalance in the mitochondria of infected cells, which results in damage to DNA and cell death in case of cytotoxic membrane lesions [7].

In addition, the lungs of mice infected with A/H5N1 and A/H1N1 viruses contained the alveolocytes prone to apoptosis via activation of FADD, the apoptotic signal transduction protein. The number of alveolocytes expressing this apoptotic marker increased 9-fold during postinfection days 1-10 in A/H5N1-infected mice by contrast to 2.6-fold increase observed in A/H1N1-infected mice (Fig. 3).

Despite similarities in pathomorphologic alterations in the lungs of CBA mice infected with A/H5N1 and A/H1N1 influenza viruses, the histologic examination revealed a larger scale of destructive apoptotic and necrotic alterations in A/H5N1-infected mice. The immunohistochemical data showed that the leading mechanism of apoptosis in the lungs of A/H1N1-infected mice implicated the mitochondrial pathway of the cell death, although the plasmalemmal receptor pathway also contributed to apoptotic death. In contrast, in A/H5N1-infected mice, the basic mechanism of alveolocytic apoptosis was FAS-mediated pathway realized via activation of FADD.

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