International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 44–48

DOI 10.11603/ijmmr.2413-6077.2016.1.6382

MOLECULAR APOPTOSIS MECHANISMS WITH UNDERLYING EXPERIMENTAL ACUTE LUNG INJURY

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Background. *Current data suggest systemic autoimmune activation in the pathogenesis of bronchopulmonary diseases. The imbalance in the system of pro- and anti-inflammatory cytokines is very important in immunopathogenesis.*

Objective. *The aim of our research was to determine the caspase-3 rate in the dynamics of experimental acute lung injury and to study the relationship between their level and the number of cells carrying membrane binding TNF receptor type 1 to define the main mechanisms of cell death.*

Results. *The analysis of the results of caspase-3 rate in lung homogenate showed that this cysteine proteinase was uniformly increasing in all experimental groups during simulating of ALI induced by administration of hydrochloric acid ($p<0.001$). When comparing the results of caspase course of apoptosis it was defined that, despite the progressive increase in caspase-3 rate in lung homogenate, cysteine proteinase rate in plasma did not change.*

The receptor mechanism of apoptosis was studied by establishing correlation relationships with the number of cells carrying membrane binding TNF type 1 (TNF-R1) receptor. A strong positive correlation relationship between the number of neutrophils with TNF-R1 and caspase-3 rate in lungs of all research groups was determined.

Conclusions. *The implementation of neutrophils death by apoptosis is caused by change of activity of caspase cascade effector components, such as caspase-3, in cases of ALI induced by intratracheal administration of hydrochloric acid. One of the potential mechanisms responsible for the activation of caspase course is excessive generation of active forms of oxygen and increase in the number of neutrophils carrying membrane binding TNF receptor type 1.*

KEY WORDS: **caspase-3, tumour necrosis factor alpha receptor 1, acute lung injury**

BIOMEDICAL SCIENCES

Introduction

Current data suggest systemic autoimmune activation in the pathogenesis of bronchopulmonary diseases. The imbalance in the system of pro- and anti-inflammatory cytokines is very important in immunopathogenesis. Acute lung injury is manifested by acute inflammatory response in the lung parenchyma that is associated with the severity of damage to the epithelial and endothelial barriers [1]. The latest researches have proved that cytokines, such as tumour necrosis factor alpha (TNF), are the signal molecules of the beginning, development and progression of inflammatory response at local and systemic levels. TNF antagonists are soluble forms sTNF-RI and sTNF-RII, which are formed by separation of active receptor extracellular part from cell membrane [2, 3]. Currently some rather contradictory evidence was published on the effect of cytokines on the programmed cell death. Cytokine rate can be determined by its dose, type of target cells, their functional state and lesions [4].

Two courses of apoptosis are: internal or mitochondrial by Bcl-2 protein family, cytochrome C and caspase-9; and external by caspase-8 activation upon binding of specific Fas cells receptor – and soluble receptors of tumour necrosis factor on the cell surface [5]. Caspases, or cysteine asparagine-protease can be considered a critical effector molecules of programmed cell death, in this case caspase-3 is important for the implementation of both mitochondrial and receptor apoptosis activating [6].

The aim of our research was to determine the caspase-3 rate in the dynamics of experimental acute lung injury and to study the

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relationship between their level and the number of cells carrying membrane binding TNF receptor type 1 to define the main mechanisms of cell death.

Materials and Methods

The experiments were performed on 54 white nonlinear mature male rats 200–220g in weight, which were kept on a standard diet at the vivarium of Ternopil State Medical University. The animals were kept and experiments were conducted in accordance with the "European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" [7]. The animals were divided into 5 groups: the 1st – control group (n=6), the 2nd – animals affected by hydrochloric acid for 2 hours (n=12), the 3rd – animals affected by hydrochloric acid for 6 hours (n=12), the 4th – animals affected by hydrochloric acid for 12 hours (n=12), the 5th – animals affected by hydrochloric acid for 24 hours (n=12).

Anaesthesia for the rats was administrated intraperitoneally with sodium thiopental, 40 mg/kg of animal weight. The ventral side of the neck was treated with chlorhexidine and a 0.5 cm medisection was made to visualize the trachea. Animals were placed in horizontal position at an angle of 45°, HCl, pH 1.2, 1.0 ml/kg was injected by insulin syringe into the trachea at inhale. Physiologic saline, 1.0 ml/kg was administered to the animals of the control group.

In 2, 6, 12 and 24 hours euthanasia was performed for rats by administration of sodium thiopental, 90 mg/kg of the animal weight, following the principles of humane treatment of animals. After their death chest was prosected and cardiopulmonary complex was separated. Heparinized whole blood, lung homogenates and bronchoalveolar lavage (BAL) was used for the research. The standard technique was performed to obtain BAL from lungs [8].

To determine caspase rate in lung homogenate supernatant and leukocyte-lymphocyte blood fractions, 0.25 ml of buffer and 50 mcl of 2 mM DEVD-p-NA was added to 0.7 ml of the test liquid and it was incubated for 2 hours at 37°C; the intensity of light absorbance was measured at 405 Nm, which is directly proportional to the product of hydrolysis of acetyl-Asp-Glu-Val-Asp n-nitroanilide caspase – 3-n-nitroanilide [9].

The number of BAL neutrophils that keep membrane binding TNF receptor type 1 (TNF-R1) was evaluated by the method of flow laser cytometry by means on flow cytometer Epics XL (Beckman Coulter, USA) using radio-labeled monoclonal antibodies to TNF-R1 (CD120a) (Hycult biotech, Netherlands) [10].

Statistical analysis was conducted using the software STATISTICA 6.0. To compare the differences between groups we used t-test in cases of a parametric distribution of alternatives, for calculating other data – one-way ANOVA (Fisher LCD post-hoc test), nonparametric analysis (Mann-Whitney test). The values are presented as Mean±SD, where Mean denotes the mean rate, SD – standard mean error. $P<0.05$ was considered statistically significant.

Correlation analysis was performed between all the studied rates. Coefficient of linear correlation (r) and its fidelity (p) was calculated that was accordingly denoted in the tables (correlation matrices). The correlation coefficient was significant at $p<0.05$.

Results

Caspase-3, which cleaves proteins important for maintaining of cellular homeostasis, is considered to be the main effector molecule of the 'executive' stage in many models of apoptosis. So it was reasonable to determine its rate during apoptosis induced by hydrochloric acid when simulating ALI. Our research on caspase-3 rate showed that the content of this proteinase in blood of the experimental animals suffering from ALI did not change if compared with the data of the control and experimental groups ($p>0.05$) (Table 1).

The analysis of the results of caspase-3 rate in lung homogenate showed that this cysteine proteinase was uniformly increasing in all experimental groups during simulating of ALI induced by administration of hydrochloric acid ($p<0.001$). So, in 2 hours after the beginning of the experiment the caspase-3 rate increased by 49.33% in comparison with the control, in 6 hours – by 26.94% if compared to the second experimental group, in 12 hours – by 23.67% if compared to the third experimental group and in 24 hours – by 28.66% if compared to the previous group (Table 1).

When comparing the results of caspase course of apoptosis it was defined that, despite the progressive increase in caspase-3 rate in lung homogenate, cysteine proteinase rate in plasma did not change. This evidenced the difference in the implementation of programmed cell death, which could be caused by: 1. the varying levels pro-apoptotic signals in blood and lungs; 2. different amount of cells bearing apoptogenic receptors.

Table 1. Rates of caspase-3 in blood plasma and lung homogenate of rats with underlying experimental acute lung injury (M±m)

Rate	Control group n=6	Experimental groups			
		2 n=6	3 n=6	4 n=6	5 n=6
caspase-3, pmol/mg of protein (blood)	19,43±0,88	18,50±1,45	16,65±1,64	15,98±1,41	16,23±1,36
p		p_1>0,05	$p_{1,2}$>0,05	$p_{1,2}$>0,05	$p_{1,2}$>0,05
caspase-3, pmol/mg of protein (BAL)	23,96±4,40	35,78±2,54	45,42±2,72	56,17±3,42	72,27±4,71
p		p_1<0,001	$p_{1,2}$<0,001	$p_{1,2}$<0,001	$p_{1,2}$<0,001

Legends: p_1 – significant difference if compared to the control animals; p_2 – significant difference if compared to the affected animals.

It was established that all populations of white blood cells, which are involved in the inflammatory process of ALI such as neutrophils, secrete cytokines, and vascular endothelium is their main target [11]. The recent researches have proved that cytokines, such as tumour necrosis factor alpha (TNF), are signal molecules of the beginning, development and progression of inflammatory response at local and systemic levels. The bioactivity of TNF depends on the content of cytokine corresponding receptors on the surface of target cells and the number of circulating antagonists [12]. So, the receptor mechanism of apoptosis was studied by establishing correlation relationships with the number of cells carrying membrane binding TNF type 1 (TNF-R1) receptor. A strong positive correlation relationship between the number of neutrophils with TNF-R1 and caspase-3 rate in lungs of all research groups (Table. 2) was determined.

Table 2. Possible relationships between caspase-3 rate and the number of cells carrying membrane binding TNF type 1 receptor in cases of acute lung injury

Rate		Experimental groups	Correlation coefficient, rxy	Correlation relationship probability, p
TNF-R1 rate in ronchoalveolar lavage, %	Caspase-3 rate in lung homogenate, pmol/mg of protein	2	0,88	<0,01
		3	0,90	<0,001
		4	0,95	<0,001
		5	0,81	<0,01

Discussion

A significant increase in caspase-3 rate could be caused by involvement of mitochondrial course of apoptosis, which was associated with the pro-apoptotic signals from inside the cells, such as active forms of oxygen. Previously we proved that intensification of free radical peroxidation processes happened in cases of ALI, and active forms of oxygen were the main cause of that [13]. The generation of oxygen radicals stimulated apoptosis by decrease in mitochondrial membrane potential that verified the mitochondria cell membrane poration and depolarization [14].

Caspase-8, which is activated by the interaction of tumour necrosis factor-α and membrane binding receptor of this interleukin, contribute to pores formation. As a result, mitochondrial matrix swelling developed; internal mitochondrial membrane ruptured; and cytochrome c, AIF (apoptosis inducing factor), which stimulated caspase-3, secondary activator of caspases of mitochondrial origin and other pro-apoptotic proteins released from the intermembranous space into cytosol [15, 16] (Figure 1).

Caspase-3 rate is regulated by both external and internal TNF receptor mediated mechanisms of apoptosis. Currently, it is established that most of the cytotoxic effects of TNF are mediated by TNF-R1 due to its interaction with TRADD (death domains caused by TNF-R1) [17]. Our research also proved it. We evidenced a significant increase in caspase-3 rate with increase in percentage of neutrophils carrying TNF-R1 in cases of ALI induced by intratracheal administration of hydrochloric acid.

Conclusions

The implementation of neutrophils death by apoptosis is caused by change of activity of caspase cascade effector components, such as

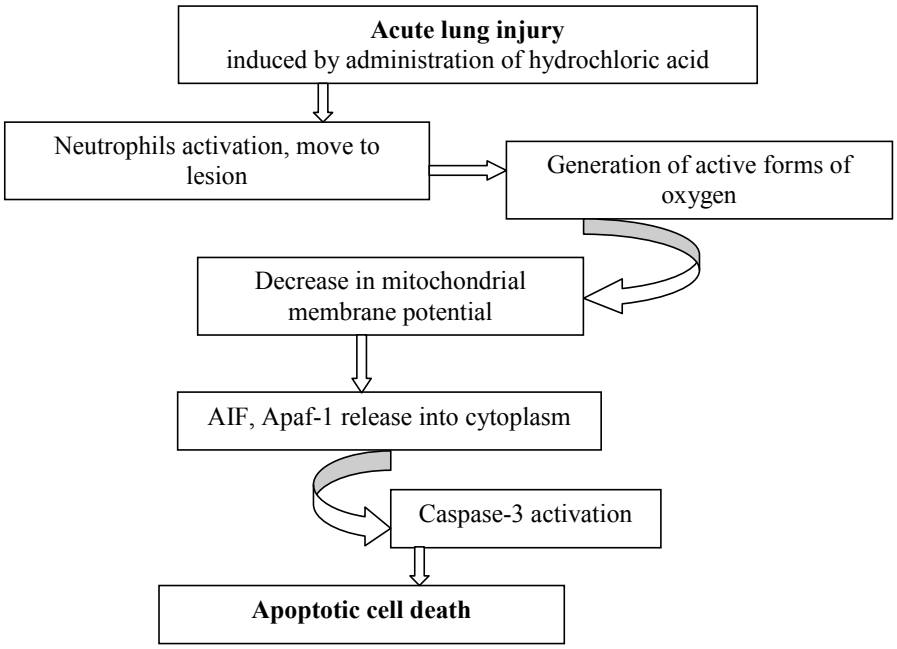

Figure 1. Pathogenic justification of mitochondrial course of apoptosis in cases of acute lung injury.

caspase-3, in cases of ALI induced by intratracheal administration of hydrochloric acid. One of the potential mechanisms responsible for the activation of caspase course is excessive generation of active forms of oxygen and increase in the number of neutrophils carrying membrane binding TNF receptor type 1.

Future Prospects of the Research

In the further research, for pathogenetic study of programmed cell death course we plan to conduct a comparative analysis of the correlation relationships between the early apoptosis level and mitochondrial transmembrane potential rates, active forms of oxygen and caspase rate in blood and bronchoalveolar lavage in rats to detect additional pathogenetic mechanisms of acute lung injury development.

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Received: 2016-02-02