NK-КЛЕТКИ ЧЕЛОВЕКА ИНТЕРНАЛИЗУЮТ РЕКОМБИНАНТНЫЙ ОСНОВНОЙ СТРЕСС-ИНДУЦИРУЕМЫЙ БЕЛОК HSP70

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Резюме. Одной из основных функций белков теплового шока семейства 70 кДа (HSP70) является защита внутриклеточных протеинов от повреждающих воздействий стрессирующих факторов различной природы. Наряду с этим HSP7070 играют важную роль в жизнедеятельности клеток и в нормальных физиологических условиях, выполняя вспомогательные, так называемые шаперонные функции. Эти упомянутые функции реализуются во внутриклеточном пространстве; однако в некоторых случаях эти белки также обнаруживаются на клеточной поверхности и во внеклеточной среде. Причины и механизмы такой транслокации на клеточную поверхность и секреции HSP70 во внеклеточное пространство еще недостаточно изучены. Вместе с тем показано, что такая необычная внеклеточная локализация HSP70 активирует клетки иммунной системы. Поверхностные HSP70 и их внеклеточный пул стимулируют цитотоксическую активность, в том числе NK-клеток. Однако прямых экспериментальных доказательств возможности интернализации белков HSP70 NK-клетками еще не было продемонстрировано. В данной работе представлены результаты взаимодействия внеклеточного пула HSP70 с NK-клетками периферической крови здоровых доноров. Результаты исследования подтвердили возможность интернализации экзогенных молекул HSP70 NK-клетками. С этой целью нами были получены флуоресцентно меченые молекулы рекомбинантного стресс-индуцируемого HSP70 человека. О связывании HSP70 с флуорохромом свидетельствовала флуоресценция полученного конъюгата при воздействии освещения с длиной волны 488 нм. Данные электрофореза свидетельствовали об отсутствии деградации белка в процессе мечения, чистоте и стабильности модифицированного белка. Для оценки взаимодействия HSP70 с NK-клетками флуоресцентно меченый HSP70 добавляли к культуре NK-клеток *in vitro*, выделенных методом магнитной сепарации из мононуклеарной фракции периферической крови, и анализировали с помощью конфокальной микро-

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скопии. Этот анализ показал, что живые NK-клетки интернализуют внеклеточные HSP70, которые локализуются как в лизосомах, так и в фагосомах. Наши эксперименты впервые иллюстрируют процесс проникновения внеклеточных HSP70 в эти клетки. Полученные результаты позволяют предположить, что активация NK-клеток под действием экзогенного HSP70 может быть связана в том числе и с интернализацией этих белков.

Ключевые слова: HSP70 – белок теплового шока 70 кДа, NK-клетки, интернализация, флуоресцентное мечение, флуоресцентная визуализация, конфокальная микроскопия

HUMAN NK CELLS INTERNALIZE RECOMBINANT MAJOR STRESS PROTEIN HSP70

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Abstract. Heat shock proteins 70 kDa (HSP70) protect intracellular proteins from the damaging effects of stress factors of various natures. Moreover, HSP70 play an important role in the vital activity of cells under normal physiological conditions, performing chaperone functions. These functions are realized in the intracellular space; however, in some cases, these proteins are also found on the cell surface and in the extracellular environment. The causes and mechanisms of HSP70 translocation to the cell surface and secretion into the extracellular space have not yet been well understood, but such an unusual localization of HSP70 activates the immune system. The surface HSP70 and their extracellular pool stimulate the cytotoxic activity of NK cells. However, direct experimental evidence for the internalization of HSP70 molecules by NK cells has not yet been demonstrated. This paper presents the results of the interaction of the extracellular HSP70 pool with NK cells from the peripheral blood. The results demonstrated the confirmation of the internalization of exogenous HSP70 molecules by NK cells. To this end, fluorescently labeled recombinant stress-inducible human HSP70 were obtained. The electrophoretic data indicated the absence of protein degradation during the labeling process, the purity and stability of the modified protein. To assess the interaction of HSP70 with NK cells, the fluorescently labeled HSP70 was added to an *in vitro* culture of NK cells isolated by magnetic separation from the peripheral blood mononuclear fraction and analyzed by confocal microscopy. This analysis indicated that living NK cells internalize extracellular HSP70 with localization both in lysosomes and in phagosomes. Our experiments illustrated for the first time the process of penetration of the extracellular form of HSP70 into these cells. The results suggest that the activation of NK cells under the action of exogenous HSP70 could be associated with the internalization of these protein molecules.

Keywords: HSP70 – heat shock protein 70 kDa, NK cells, internalization, fluorescent labeling, fluorescent imaging, laser confocal microscopy

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Introduction

The 70 kDa heat shock proteins (HSP70) belong to the large family of heat shock proteins, HSPs. One of the main functions of these molecules is the protection of intracellular proteins from the damaging effects of stress factors of various nature. Along with this, HSPs play an important role in the cells under normal physiological conditions, interacting with a wide range of intracellular proteins and performing auxiliary, so-called chaperone functions. These HSP functions are realized in the intracellular space, however, in some cases, these proteins are also found on the cell surface and in the extracellular environment and behave as cytokines ("chaperokines"). This unusual localization is most characteristic of members of the HSP70 family, which are found on the surface of tumor, virus-infected, and stressed cells [8]. It has been established that transport of HSP70 to the cell surface is carried out with the help of a non-classical,

independent mechanism of the Golgi apparatus. It was also found that the translocation of HSP70 to the cell surface is enhanced at the final stages of apoptosis and is probably aimed at stabilizing the membrane of dying cells.

The phenomenon of HSP70 secretion in cell cultures of different tissues was demonstrated [11], in particular, in the culture of human peripheral blood mononuclear cells [6]. The possibility of HSP70 exocytosis in vivo is indicated by data on the presence of HSP70 in blood serum in normal subjects and in various pathologies [14]. The causes and mechanisms of HSP70 translocation to the cell surface and secretion of these proteins into the extracellular space have not yet been studied, but it has been shown that such an unusual localization of HSP70 is a "danger signal" and activates the immune system. Thus, it is now well known that surface HSP70 stimulates the cytotoxic activity of NK cells [9], while extracellular HSP70 enhances cytokine production and accelerates the maturation of antigen presenting dendritic cells [13]. At the organism level, extracellular HSP70 also perform immunoregulatory functions. In particular, these proteins are considered by many authors as an evolutionarily formed alarm signal for the immune system, indicating the emergence and localization of the focus of stress development in the body [7].

Previously, we demonstrated that recombinant human HSP70 after intranasal administration can penetrate various brain regions of mice in its native form and subsequently undergo rapid degradation. It was also shown that labeled HSP70 added to culture medium of different human and mouse cell lines enters the cells with strikingly different kinetics, which positively correlates with the basic levels of membrane bound Toll-like receptors (TLR) that are characteristic of these cell lines [15]. Our preliminary data also point to the possibility of receptor-independent internalization of extracellular HSP70 by lymphoid cells. It can be assumed that this pathway of exogenous HSP70 internalization is associated with the demonstrated ability of these proteins to interact with plasma membrane phospholipids [1].

There are currently several hypotheses regarding the physiological functions of the extracellular pool of HSP70. In cell culture models, it was shown that these proteins are adsorbed on the cell surface and internalized fairly quickly [5]. Moreover, absorbed HSP70 retain their protective activity and prevent the development of apoptosis in cell cultures [4]. Perhaps, at the cellular level, one of the functions of extracellular HSP70 produced during stress is aimed at protecting the entire population of cells from stress-mediated damage. Obviously, circulating serum HSP70 can have similar activity at the organism level, aimed at preventing cell damage [10].

Characteristically, using RNA-Seq, we identified a lot of differentially expressed genes in the hippocampus of a late-onset model of hereditary Alzheimer's disease compared with those of nTg mice. Most importantly, we observed that recombinant human HSP70-induced upregulation of multiple genes participating in antigen processing and presentation especially the members of major histocompatibility complex (class I and II) in the brains of old Tg animals, suggesting that recombinant human HSP70 executes its beneficial role via activation of adaptive immunity [2]. The totality of these data indicates a significant, but poorly understood, role of extracellular HSP70 in the functioning of the immune system.

This paper presents the results of our study of the interaction of the HSP70 extracellular pool with NK cells, in particular, the experimental confirmation of the internalization of exogenous HSP70 molecules by these cells.

Materials and methods

In this study, we used NK cells, isolated from human periphery blood. Blood samples were collected from healthy donors, who gave their informed consent (approved by the local ethics committee of the Pirogov Russian National Medical University). The fraction of peripheral mononuclear blood cells was obtained by gradient centrifugation in 1.077 density Ficoll solution (PanEco, Russia), then NK cells were isolated by negative magnetic separation using the NK cell isolation kit (MiltenyiBiotec, Germany) in accordance with the manufacturer recommendations. NK cells were incubated overnight at 37 °C and 5% CO_2, transferred to 18 well polymer μ-Slide (Ibidi, Germany), and underwent a vital staining with CellMasck Deep Red and LysoTracker Red DND-99 (all from ThermoFisher, USA) in accordance with the manufacturer recommendations. Nuclei were counterstained with Hoechst33342 (PanEco, Russia).

To investigate HSP70-NK cell interactions, we obtained recombinant HSP70 using expression construct of human HSP70 (HSPA1A, P0DMV8 HS71A_HUMAN) and pET14a (Novagen, USA) as described previously [3]. The obtained protein contained 6-His-Tag on the N-terminus. HSP70 was purified using Ni-NTA-Sepharose (Amersham Biosciences, UK) and subjected for dialysis against DPBS (PanEco). Endotoxin was removed using High Capacity Endotoxin Removal Resin (ThermoFisher) in accordance with the manufacturer recommendations. The protein was characterized quantitatively using Coomassie Plus (Bradford) Assay Kit (ThermoFisher). SDS-PAGE and Western-blot with monoclonal antibodies HSC70/HSP70 monoclonal antibody (BB70) (Enzo Life Sciences) were used to estimate the purity of HSP70.

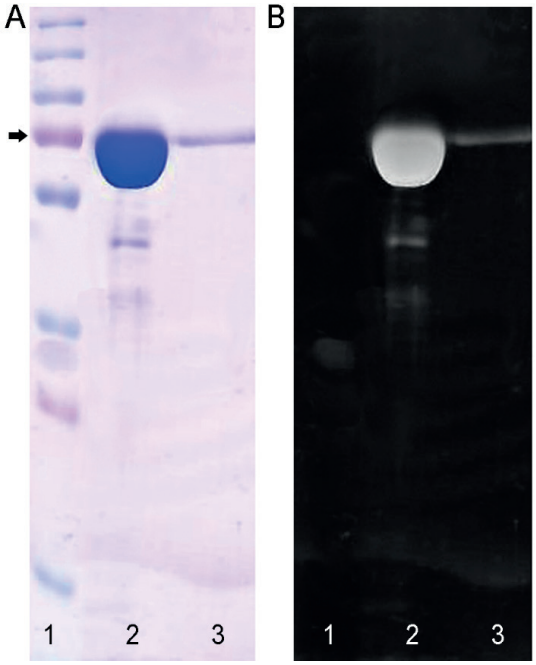

Figure 1. Labelling of Hsp70 with AlexaFluor488

Note. (A) Electrophoretic separation of Hsp70 proteins in SDS-PAGE. Sample explanation: 1, molecular weight marker (70 kDa band is indicated with arrow); 2, Hsp70-AlexaFluor488 in concentration 10 μg; 3, Hsp70-AlexaFluor488 in concentration 100 ng. (B) 488 nm laser-excited image of the gel presented in A.

HSP70 was labelled with AlexaFluor488 NHS Ester (ThermoFisher) in accordance with manufacturer recommendations. Unreacted labelling reagent was removed using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-50 regenerated cellulose membrane (Merck, Germany). The concentration of labelled HSP70 was tested with Protein assay (Bio-Rad, Germany) using BSA as a referent protein.

SDS-PAGE was made to prove the absence of HSP70 degradation during the labelling. Fluorescent imaging of the SDS-PAGE gel was made to confirm the HSP70-AlexaFluor488 conjugation.

AlexaFluor488-labelled HSP70 was added in excessive concentration of 10 μg/mL to NK cell culture. Cells were incubated in the presence of HSP70 at 37 °C and 5% CO_2. 30 min after, cells were washed three times with DPBS (PanEco) and subjected to imaging using Zeiss LSM980 (Carl Zeiss, Germany) equipped with 100 oil objective and 405, 488, and 633 nm lasers. Image analysis was performed using Imaris 9.8 (Oxford Instruments, UK).

Results and discussion

For labelling, HSP70 in concentration of 10 mg/mL was incubated with AlexaFluor488 NHS, and 0.65 mg of HSP70-Alexa488 was yielded from each

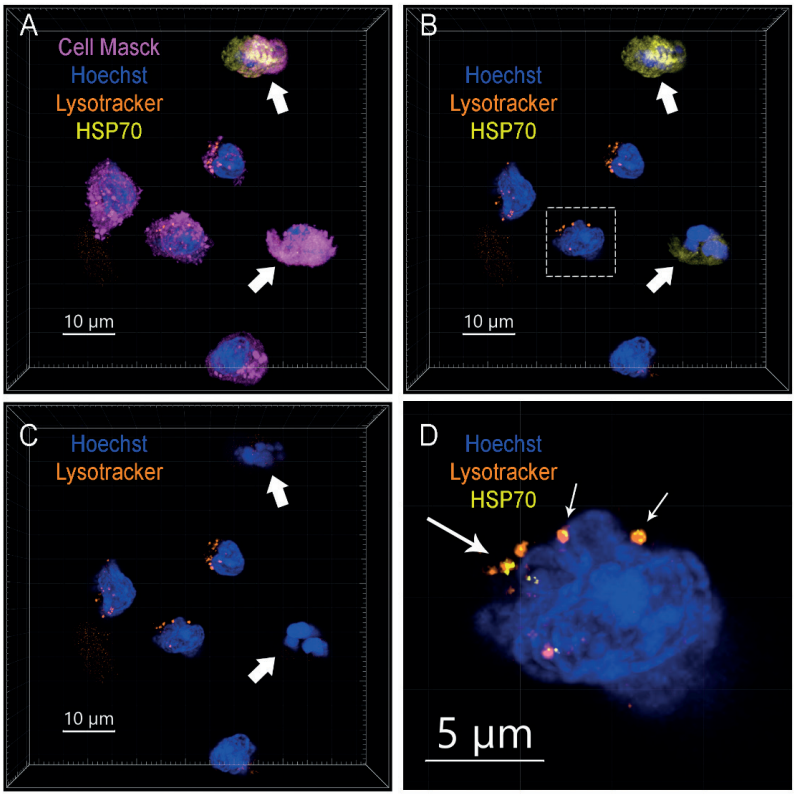

Figure 2. Hsp70-NK cell interactions

Note. (A-C) Representative image of NK cells that were stained with CellMask (magenta), LysoTracker (orange), and Hoechst (blue) and cultivated for 30 min in presence of Hsp70 (yellow). Cells covered with Hsp70 are indicated with bold arrows. Scale Bar 10 μm. (D) Enlarged image of the cell framed in B. Lysosomes with Hsp70 are indicated with small arrows, Hsp70 outside lysosome is indicated with a large arrow. Scale bar 5 μm.

1 mg of HSP70. The purity and stability of the protein was confirmed by electrophoresis (Figure 1A). The AlexaFluor488-conjugated state of the protein was confirmed by the fluorescent imaging of the protein (Figure 1B).

Freshly isolated NK cells were not adhesive; however, on a specifically treated surface of slide they attached to the slide bottom. Thus, within 30 min at 37 °C and 5% CO_2 NK cells were mostly detected at the slide bottom (Figure 2A). Upon supplying to the NK cell culture, HSP70 dissolved and steadily sedimented, and 30 min after the administration, large amount of AlexaFluor488-labelled HSP70 can be identified in the slide bottom (data not shown).

Thirty minutes after the supplementation, we detected HSP70-AlexaFluor488 both covering NK cells (Figure 2A-C, bold arrows) and internalized by NK cells (Figure 2A-D). Precise analysis indicated that in the cases when HSP70 covered NK cells, the cells were mostly dying. The morbid status of these cells was confirmed by the chromatin disaggregation in the nuclei (Figure 2C, bold arrows).

In the cases of the internalization of HSP70 by NK cells, HSP70 was detected both inside (Figure 2D, small arrows) and outside the lysosomes (Figure 2D, large arrow). LysoTracker dye allows the visualization of acidic cell compartments, such as lysosomes or phagolysosomes, but not phagosomes. Thus, we can assume that internalized AlexaFluor488-labelled HSP70 located outside the lysosomes was in the phagosomes that at the investigated time point were still not fused with the lysosomes.

Thus, to test the possibility of internalization of HSP70 by human NK cells, we obtained fluorescently labeled molecules of recombinant protein HSP70, the inducible member of the human HSP70 family. The electrophoretic data indicated the absence of protein degradation during the labeling process and the purity and stability of the resulting fluorescent protein. The binding of HSP70 to fluorochrome was evidenced by the fluorescence of the obtained conjugate when exposed to illumination with a wavelength of 488 nm.

To assess the interaction of HSP70 with NK cells, the resulting fluorescently labeled protein was added to an *in vitro* culture of NK cells isolated by magnetic separation from the peripheral blood mononuclear fraction of healthy donors. Samples of NK cells incubated with HSP70 were analyzed by laser confocal microscopy using additional fluorescent probes. This analysis indicated that living NK cells internalize extracellular HSP70 molecules, and the absorbed proteins can be located both in lysosomes and in phagosomes.

It should be noted that currently there are data in the literature on the internalization of exogenous HSP70 by different cell types [15]. At the same time, as regards the interaction of NK cells with the extracellular pool of this protein, there is much evidence of the activating effect of HSP70 and fragments of this protein on NK cells [12], however, direct experimental evidence of the internalization of HSP70 molecules by NK cells has not yet been demonstrated.

Conclusion

Our experiments illustrated for the first time the process of penetration of the extracellular form of HSP70 into these cells. The results obtained indicate that the well-characterized activation of NK cells under the action of exogenous HSP70 is associated with the internalization of molecules of this protein.

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