EXPERIMENTAL PAPERS

Phagocytic Activity of Rat Primary Astrocytes Is Regulated by Insulin and Ganglioside GM1

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Abstract—A timely and efficient removal of apoptotic cells and their fragments is essential to maintain tissue homeostasis in normal and pathological conditions. Since the removal of apoptotic substrates is executed by the cells endowed with phagocytic activity, the issue of its regulation is of particular interest. In this work, we studied the effect of two biologically active substances, insulin and ganglioside GM1, on phagocytic activity of rat primary astrocytes. We showed that cell incubation with 1 μ M insulin significantly decreased the phagocytic activity of astrocytes (58.5% vs. control), whereas the incubation with 10 μ M GM1 caused an increase in phagocytic activity (133.4% vs. control). Preincubation of brain astrocytes with GM1 completely blocked the inhibitory effect of insulin. These results can be instrumental in developing novel therapeutic strategies for the treatment of neurodegenerative diseases accompanied by the emergence of apoptotic substrates.

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Apoptosis is a vital process that occurs in all tissues in normal and pathological conditions [1]. Apoptotic substrates, formed due to normal vital activity of cells in different organs, are promptly removed by specialized cells endowed with phagocytic activity. A timely and complete removal of apoptotic substrates is necessary to maintain tissue homeostasis, as well as to exclude the development of autoimmune reactions in response to intracellular antigens released from damaged and/or apoptotic cells [2].

Phagocytosis of apoptotic substrates is a complex receptor-mediated process in which the coordinated interaction of specific receptors on the plasma membrane of a phagocyte triggers signaling mechanisms that ensure the ingestion and further cleavage of substrates by the intracellular lysosomal apparatus [3]. Thus, substrate recognition (binding), ingestion (engulfment), and cleavage are the main stages of phagocytosis that will be hereinafter referred to as stages 1, 2 and 3, respectively. Moreover, the implementation of one of

these stages of phagocytosis does not entail the automatic implementation of the next one.

Phagocytic function in the central nervous system (CNS) is implemented by two cell types: microglial and astroglial cells. The former are cells of hematopoietic origin and refer to professional phagocytes, while the latter are non-professional phagocytes [4]. Astrocytes are the most numerous neuroglial cells that account for up to 40% of the total population of brain cells [5]. These cells provide conditions for the generation and transmission of nerve impulses, thus contributing to the maintenance and implementation of synaptic function; they are involved in the formation of the blood–brain barrier and implementation of supporting, trophic, secretory and phagocytic functions. Astrocytes control not only the formation and function of synapses, but also their elimination in the developing and mature brain [6]. In pathological conditions of various etiologies (neurodegenerative diseases, ischemia, aging, traumatic and radiation damage to the brain), the removal of apoptotic substrates and degenerative myelin by astrocytes takes on special significance [5].

As is currently known, the phagocytic activity of astrocytes is mediated by two receptor proteins of the plasma membrane: MEGF10 (Multiple EGF-Like Domains 10) and the receptor tyrosine kinase MERTK (MER proto-oncogene Tyrosine Kinase) [7]. The MEGF10 and MERTK proteins are involved in the removal of synapses, as well as damaged and/or apoptotic neurons formed during aging and/or under pathological conditions of the CNS [5]. In addition, the MERTK receptor is assigned a key role in phagocytosis of myelin debris generated during natural processes of myelin renewal [8].

In tentative studies conducted in our laboratory, the process of phagocytosis of various apoptotic substrates by rat brain primary astrocytes, as well as its kinetic characteristics, were thoroughly studied, and it was shown that phagocytosis of apoptotic substrates leads to an increase in the proliferative activity of brain astrocytes.

Astroglia proliferation is known to be an aggravating factor in the development of pathological conditions of the CNS, accompanied by degenerative changes and death of nerve cells. The accumu-

lation of astrocytes at the sites of local damage contributes to the development of astrogliosis, i.e. the process of replacing dead neurons with astrocytes. In this regard, the problem of optimization of astrocyte phagocytic activity (toward its decrease or increase) is fundamentally important for developing strategies to correct the pathological process, depending on the stage of its development.

In our laboratory, during the recent years, the neuroprotective effects of some natural biomolecules have been studied *in vivo* and *in vitro*, namely those of gangliosides and insulin during oxidative stress and a number of adverse effects on the organism [9]. Since the removal of apoptotic substrates is a vitally important factor that influences the survival of the CNS under unfavorable conditions, it appeared significant to find out whether these agents are able to regulate the phagocytic function of astrocytes.

Gangliosides, complex glycosphingolipids of cell membranes, are an integral component of the brain, and it has been shown in our laboratory that in mammals, their relative amount reaches 2.5 mg per 1 g of brain tissue (GM1) [10]. At the same time, it is well known that in degenerative and traumatic brain injuries, GM1 is released into the extracellular space, which suggests the possibility of its interaction with various brain cells, including astrocytes. In addition, in our laboratory it was established that GM1 causes multiple activation of the phagocytic activity of professional phagocytes (macrophages) [11].

The brain is an insulin-dependent organ, and various methods of insulin administration (intracerebroventricular, intranasal, systemic) contribute to changes in eating behavior, as well as to weight loss, in experimental animals and humans [12]. Insulin receptors have been found on both neuronal and glial cells, including astrocytes [13, 14]. Astrocytes are active components involved in insulin metabolism in the CNS. At the same time, astrocytes not only express the insulin receptor and insulin-degrading enzyme [13, 15], but are themselves insulin-secreting brain cells [16], which indicates the importance of insulin signaling for the normal functioning of these cells.

Nevertheless, in the available literature, we failed to find any data on the effect of insulin on the phagocytic activity of brain astrocytes. There is also

no information on the possible modulating effect of gangliosides. Therefore, the goal of this study was to elucidate how insulin and GM1 affect the phagocytic activity of rat primary brain astrocytes.

MATERIALS AND METHODS

Primary culture of astroglia

The studies were carried out on a primary culture of astroglia cells, which were isolated from the brain of newborn rats as described elsewhere [17]. The cells were cultured in a DMEM medium (Biolot, Russia) containing 10% inactivated fetal calf serum (FCS; Biolot, Russia) and antibiotics (50 U/mL penicillin G and 50 µg/mL streptomycin; Biolot, Russia), on Petri dishes (Orange Scientific Ø3.5 cm), for 7–9 days at 37°C and 5% CO₂ until 75% confluence is reached. Cell viability was assessed by staining with Trypan Blue (Biolot, Russia), which is able to penetrate through damaged cell membranes.

Modeling of induced phagocytosis

As an apoptotic substrate of phagocytosis, we used the fraction of the rod outer segments (ROS) of the rat retina, which was isolated using a modified method described previously [18]. ROS preparations were conjugated to fluorescein isothiocyanate (FITC; Merck, Germany) as reported in our previous publication [18]. The cells were washed with PBS (pH 7.4) and incubated in a DMEM medium containing 2% inactivated FCS. Phagocytosis substrates were then added at a cell/substrate ratio of 1:10. The number of cells and ROS fragments was counted using a Fuchs–Rosenthal chamber.

Cell treatment with modulators of phagocytic activity

When analyzing the effect of insulin on the process of phagocytosis, cells were incubated with 1 µM insulin (Sigma, USA) for 1 h, after which the FITC-ROS conjugate was added. When studying the effect of GM1 on phagocytosis, cells were incubated for 24 h with the GM1 preparation at final concentrations of 0.1, 1 and 10 µM, after which the FITC-NSP conjugate was added. GM1 was isolated from the bovine brain lipid extract obtained by the Folch method [19] as

described previously [20].

When studying the combined effect of insulin and GM1, cells were incubated for 24 h with the GM1 preparation at final concentrations of 0.1, 1 and 10 µM, after which insulin was added at a concentration of 1 µM, and the cells were kept for 1 h. Next, the FITC-ROS conjugate was added.

Analysis of phagocytic activity

After the end of the incubation with retinal ROS, the excess substrate was washed out with cooled PBS, and the cells were fixed for 3–5 min in 4% paraformaldehyde/PBS. After washing the preparations with PBS, the nuclei were stained with 0.001% Hoechst 33258/PBS (Serva, Germany). The experimental results were evaluated using a Zeiss Axio Imager A1 fluorescence microscope (Germany) equipped with an AxioCamMRc digital camera (Zeiss, Germany). ImageJ software (NIH, USA) was used to assess phagocytic activity; in doing this, we counted the number of cell nuclei (blue staining) and determined the relative area occupied by a phagocytosis substrate (green staining). The phagocytic activity of cells was assessed based on determining the implementation effectiveness of phagocytosis stages 1 and 2. The quantitative assessment of the process of substrate recognition and binding to astrocytes (stage 1) was carried out in arbitrary units by calculating the ratio of the fluorescent substrate area to the number of nuclei in the preparation. The morphological criterion for assessing the substrate absorption by astrocytes (stage 2) was the presence of a fluorescent FITC label in the perinuclear region, where the stained ROS fragments interact with lysosomes (the ratio of the number of cells containing a FITC-labeled substrate in the perinuclear region to the total number of cells in the preparation was calculated). For quantitative analysis, cells were photographed, and two parallel slides were used for each point in the experiment. Fifteen randomly selected fields containing up to 1500 cells were photographed in each slide. Three independent experiments were carried out. The results were statistically processed using a one-way analysis of variance (ANOVA), followed by Bonferroni's test for multiple comparisons, and GraphPad Prism software (San Diego, USA). Data are presented as $M \pm SEM$.

Fig. 1. Phagocytic activity of cultured primary rat brain astrocytes under different conditions of incubation with insulin and/or GM1 ganglioside. (a, a')—Control preparations of primary rat brain astrocytes after incubation with retinal rod outer segment (ROS)-FITC conjugate for 3 h. (a') — image obtained by merging (a) with differential interference contrast (DIC) projection for better visualization of cell contours. (b, b')—Primary rat brain astrocytes pretreated with 10 μ M GM1 for 24 h and then incubated with ROS-FITC for 3 h. (b') is a merge of (b) with differential interference contrast (DIC) projection for better visualization of cell contours. (c, c')—Primary rat brain astrocytes pretreated with 1 μ M insulin for 1 h and then incubated with ROS-FITC for 3 h. (c') is a merge of (c) with differential interference contrast (DIC) projection for better visualization of cell contours. (d, d')—Primary rat brain astrocytes pretreated with 10 μ M GM1 for 24 h, then incubated with 1 μ M insulin for 1 h, and lastly incubated with ROS-FITC for 3 h. (d') is a merge of (d) with differential interference contrast (DIC) projection for better visualization of cell contours. Arrows indicate fluorescent substrates located in the perinuclear region (phagocytosis stage 2), asterisks point to the substrates associated with cell membrane (phagocytosis stage 1).

RESULTS

Incubation with GM1 increases phagocytic activity of primary rat brain astrocytes

In the first series of experiments, we assessed the phagocytic activity of astrocytes in the presence of GM1 ganglioside. As a substrate of phagocytosis, we used rat ROS preparations, which represent a substrate of the apoptotic type [21]. In our previous studies, we have shown that ROS preparations are effectively recognized and ingested by rat brain astrocytes, with the kinetic parameters of ROS phagocytosis being comparable to those characteristic of the capture and ingestion of apoptotic neurons. The results of the experiments are shown in Fig. 1 and Table 1. As

follows from the data in Figs. 1a, 1a', in the control samples, the fluorescent signal was present both at the periphery and in the perinuclear region of cells, indicating the implementation of both phagocytosis stages (1 and 2).

Figures 1b, 1b' and Table 2 show the results of experiments on assessing the phagocytic activity of rat brain astrocytes in the presence of GM1. We analyzed the effect of different GM1 concentrations ranging from 0.1 to 10 μ M. It was found that due to incubation of astrocytes with GM1, the total amount of fluorescent substrate significantly increased compared to the control at GM1 concentrations ranging from 1 to 10 μ M (Table 1). A quantitative assessment of the number of astrocytes containing a fluorescent substrate in the perinu-

Table 1. Effectiveness assessment of phagocytosis stage 1 (substrate binding) by primary rat brain astrocytes under various conditions of incubation with insulin and ganglioside GM1

Sample	Effectiveness of phagocytosis stage 1 (arb. units)	% vs. control
Control	198.2 ± 18.4	100
Insulin 1 µM	207.4 ± 25.6	104.7
GM1 0.1 µM	242.4 ± 33.9	122.3
GM1 1 µM	334.3 ± 49.4 (*)	168.7
GM1 10 µM	328.8 ± 34.7 (*)	165.9
GM1 0.1 µM + insulin 1 µM	234.3 ± 30.2	118.3
GM1 1 µM + insulin 1 µM	327.0 ± 53.6 (*)	165.0
GM1 10 µM + insulin 1 µM	339.9 ± 51.4 (*)	171.6

Phagocytosis stage 1 effectiveness is expressed in arb. units; for the calculation method see Materials and Methods. (*)—Differences are significant at $p \leq 0.05$.

Table 2. Effectiveness assessment of phagocytosis stage 2 (substrate ingestion) by primary rat brain astrocytes under various conditions of incubation with insulin and ganglioside GM1

Sample	Effectiveness of phagocytosis stage 2	% vs. control
Control	14.7 ± 1.5	100
insulin 1 µM	8.6 ± 1.2 (**)	58.5
GM1 0.1 µM	12.6 ± 1.8	86.2
GM1 1 µM	19.2 ± 2.7 (**)	131.1
GM1 10 µM	19.5 ± 2.4(**)	133.4
GM1 0.1 µM + insulin 1 µM	13.8 ± 2.0	93.9
GM1 1 µM + insulin 1 µM	15.4 ± 1.7	105.1
GM1 10 µM + insulin 1 µM	26.6 ± 2.4 (**)	181.3

Effectiveness of phagocytosis stage 2 is assessed as a ratio of the relative number of cells containing ROS-FITC conjugate in the perinuclear area to the total number of cells per preparation (%). (**)—Differences are significant at $p \leq 0.01$.

clear area also revealed a significant increase compared to the control (19.2 ± 2.7 and 19.5 ± 2.4 vs. 14.6 ± 1.5 , or 131.5% and 133.6% of the control, respectively) (Fig. 1b, b'; Table 2). These data indicate that GM1 stimulates the processes of both substrate binding (phagocytosis stage 1) and ingestion (phagocytosis stage 2) by astrocytes.

Incubation with insulin decreases phagocytic activity of primary rat brain astrocytes

After the incubation of astrocytes with insulin, the total amount of the FITC fluorescent label was statistically indistinguishable from the control (Figs. 1c, 1c'; Tables 1, 2). However, the amount

of fluorescent label in the perinuclear region of cells preincubated with insulin was significantly reduced (to 58.5% of control), indicating a slowdown in the implementation of phagocytosis stage 2 (Fig. 1; Table 2). Indeed, the fluorescent substrate was predominantly localized at the periphery of astrocytes, associated with the plasma membrane. Thus, insulin turned out to be a potent inhibitor of the phagocytic activity of non-professional brain phagocytes.

Since our laboratory is investigating a coaction of insulin and GM1 ganglioside, in the next series of experiments, we analyzed the joint effect of these natural biologically active molecules on the

phagocytic activity of astrocytes.

Coincubation of rat brain astrocytes with both GM1 and insulin increases their phagocytic activity

Figures 1d, 1d' show the results of a representative experiment, from which it follows that GM1 application led to a sharp increase in the fluorescent signal in the samples. Indeed, with GM1 concentrations of 1 and 10 μM , the amount not only of the bound substrate (Table 1), but also the absorbed substrate localized in the perinuclear region of astrocytes (Fig. 1d; Table 2) significantly increased. The calculation of phagocytic activity showed that coincubation of astrocytes with GM1 blocks the inhibitory effect of insulin on the phagocytic activity of cells. At the same time, with a GM1 concentration of 1 μM , the inhibitory effect of insulin did not manifest itself, while the matching value of the amount of ingested substrate reached the values characteristic of the control samples (15.4 ± 1.78 vs. 14.6 ± 1.5). Remarkably, with a GM1 concentration of 10 μM , the amount of substrate ingested by brain astrocytes (phagocytosis stage 2) was twice as high as the control values (26.6 ± 2.4 vs. 14.6 ± 1.5).

DISCUSSION

According to the recent data obtained in our laboratory on in vivo experimental ischemia in rats, the coadministration of insulin and GM1 ganglioside produced a unidirectional neuroprotective effect. These data are in good agreement with the results of the present in vitro study, in which the combined effect of these biologically active agents exceeded the stimulating effect of GM1 on the phagocytic activity of astrocytes.

According to the data obtained in the present work, GM1 applied at concentrations of 1 and 10 μM has a well-pronounced ability to significantly increase the phagocytic activity of astrocytes when retinal ROS are used as an apoptotic substrate. At the same time, GM1 increased the effectiveness of both phagocytosis stages 1 and 2 (binding and ingestion, respectively). We are the first to demonstrate here such effects of GM1 ganglioside. It is noteworthy that GM1 ganglioside is the most stable one of the 4 major brain gangliosides, which share similar neuroprotective effects and, as a rule, simi-

lar functional activity [22]. Therefore, it can be assumed that the main brain gangliosides may have the ability to activate the astroglia-mediated processes of phagocytosis, which is shown here using the effect of GM1 as an example.

The ability of gangliosides to activate phagocytosis of apoptotic substrates by brain astrocytes, which we have established in this work, can be very valuable for developing novel strategies to treat various pathological conditions associated with the destruction of neurons and, probably, other brain cells. The destruction of cells in the central nervous system is characteristic of ischemic, traumatic and other brain lesions, including neurodegenerative diseases. It is well known that when brain neurons are destroyed, gangliosides, which are abundant in the brain, are released into the intercellular space [10]. Previously, we found that substances from destroyed brain cells first accumulate in the choroid plexus, and then are gradually excreted therefrom through the cerebrospinal fluid. Indeed, it has been demonstrated that in children with meningoencephalitis (postmortem studies), the level of gangliosides in the choroid plexus is higher than in the brain tissue [23]. Thus, if the level of gangliosides in the cerebrospinal fluid significantly increases during the destruction of neurons and other brain cells, then they could activate phagocytosis of apoptotic neurons by brain astrocytes, thus exerting a neuroprotective effect on the remaining viable brain neurons.

Although the mechanism behind the GM1 effect on the phagocytic activity of brain astrocytes is still unclear, the data on the effect of gangliosides on receptor tyrosine kinases, which also include the phagocytic receptor MERTK, are of special interest. Furthermore, it is known that gangliosides can act through the tyrosine kinase Trk-A receptor, thus activating the PI3K-Akt1-mTOR signaling pathway [24, 25]. As is known, mTOR activation leads to inhibition of autophagy [26]. In the case of non-professional phagocytes, there is an inverse relationship between the processes of autophagy and phagocytosis, i.e. activation of autophagy may entail a decrease in phagocytic activity. It is believed that this effect is due to the competition of both processes for the use of lysosomes, which finalize the cleavage of phagocytosis substrates and/or the process of

autophagy in cells [27–29].

Besides, the *in vitro* beneficial effect of gangliosides on the phagocytic activity of astrocytes can be enhanced by their coaction with insulin, which supports the data obtained in our laboratory *in vivo*. In our studies, insulin had an inhibitory effect on phagocytosis of apoptotic substrates by brain astrocytes. The molecular mechanism underlying this effect requires further research, while the data we have found in the available literature are scarce. For instance, similar results were obtained on the example of non-professional phagocytes of bronchoalveolar epithelial cells [30]. To explain this phenomenon, the authors pay special attention to AKT kinase in the regulation of phagocytic activity of non-professional phagocytes of bronchoalveolar epithelial cells [30].

On the other hand, gangliosides are mainly localized in the membrane microdomains known as lipid rafts, being combined with other sphingolipids and cholesterol [31]. The interaction of lipid rafts with membrane proteins plays an important role in cellular processes, such as signal transduction of cytokines, adhesion, intracellular transport, etc. [32]. Recently, it has been shown that on the plasma membrane of aortic endothelial cells, which can also be considered as non-professional phagocytes [33], GM1 ganglioside is co-localized with the insulin receptor, while the disruption of the normal stoichiometric insulin receptor–GM1 relationship (towards an increase in GM1) leads to a blockade of insulin signaling [34]. It is noteworthy that in brain astrocytes, GM1 ganglioside is detected in insignificant amounts [35]; however, exogenously added GM1 can easily incorporate into the plasma membrane of astrocytes, leading to a modification of membrane microdomains [36] and a change in the cellular response to some stimuli [37]. In this context, our data on the abolition of the inhibitory effect of insulin in astrocytes that have undergone preliminary incubation with GM1 ganglioside are well consistent with the literature data.

CONCLUSION

In tissues delimited from the general circulation by histo-hematic barriers, the removal of apoptotic substrates produced during their normal life is

implemented by tissue-resident cells exhibiting phagocytic activity. These cells promote the maintenance of tissue homeostasis and prevent the development of autoimmune reactions that may arise in response to the release of intracellular components from damaged or dying cells. In this work, we report for the first time data on two natural modulators exhibiting a cooperative stimulating effect on the phagocytic activity of non-professional brain phagocytes, such as astroglial cells.

AUTHORS' CONTRIBUTION

T.V.S., M.P.R., D.S.V.: conducting experiments; T.V.S., D.S.V., N.E.B., M.G.E.: experimental data analysis, statistical data processing, preparing graphical material, writing a manuscript; M.G.E. and N.F.A.: writing and editing a manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest, both evident and potential, that would be associated with the publication of this article.

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