

Original Article

HMGB1-induced autophagy in Schwann cells promotes neuroblastoma proliferation

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Abstract: Neuroblastoma inflicts mostly on children, and the pathogenesis remains elusive. Clinical diagnosis and therapeutic approaches are still on the incipient stage, so further understanding of the molecular and cellular mechanisms of the disease is necessary. Inflammation has been commonly regarded as a hallmark in tumorigenesis and development, and we identified a new inflammatory factor, HMGB1, is considerably increased in neuroblastoma. Our study shows that HMGB1 induces autophagy in Schwann cells through activation of TLR4, and knockdown of TLR4 obviates the HMGB1-induced autophagy. The HMGB1-induced autophagy is through classical pathway, as deficiency of Beclin 1 deprived autophagy in Schwann cells. Coculture of neuroblastoma with Schwann cells pre-treated with HMGB1 promoted the proliferation of neuroblastoma cells, and if Beclin 1 is knocked down in Schwann cells, no promotion effects is observed. Taken together, our study demonstrates that HMGB1-induced autophagy in Schwann cells contributes to neuroblastoma cell proliferation, thus providing a potential therapeutic approach on neuroblastoma development.

Keywords: Autophagy, Schwann cells, neuroblastoma, HMGB1

Introduction

Myelin sheath formation provides an electric insulation of axons and thereby accelerates the transmission of electrical signals. Oligodendrocytes and Schwann cells are the myelinating glial cells of the central (CNS) and peripheral nervous system (PNS), respectively [1]. Derived from the embryonic neural crest, the early-stage immature Schwann cells differentiate to mature myelinating cells, which spirally wrap around axon, thus generating the myelin sheath. During peripheral nervous injury, Schwann cells make a rapid response by reverting to an immature phenotype, and by doing so, can favorably contribute to nerve repair and functional recovery [2]. In addition, Schwann cells express and secrete some axonal growth factors, and myelinate regenerated axons, thus eventually leading to substantial functional recovery. Obviously, the cross-talk between Schwann cells and other cells, like neurons, plays essential roles in multiple physiological and pathological processes.

Neuroblastoma is a rare childhood cancer, and inflicts mostly on children under 15 years of

age, especially in young children. It is a complex and heterogeneous disease, and diverse factors, genetically or non-genetically, participate the pathogenesis and development of this disorders [2, 3]. Despite the advances in clinical diagnosis and staging of neuroblastoma, we are still in the incipient stage on the understanding of the molecular and cellular mechanisms of the disease. Recently, Schwann cells have been shown to be involved in neuroblastoma development [3-5]. Studies have demonstrated that Schwann cells can strongly affect neuroblastoma phenotype. Both Schwann cell-conditioned medium or co-cultured Schwann cells increase neuroblast differentiation [6, 7]. This anti-tumor activity of Schwann cells against neuroblastoma was potentially mediated through inhibition of angiogenesis, as Schwann cell-conditioned media harbors the abilities of dampening angiogenesis *in vivo* and *in vitro* [8, 9].

Macroautophagy (hereafter in this work we refer as autophagy) is an evolutionarily conserved approach to maintain cellular homeostasis [10], and has been shown as an essen-

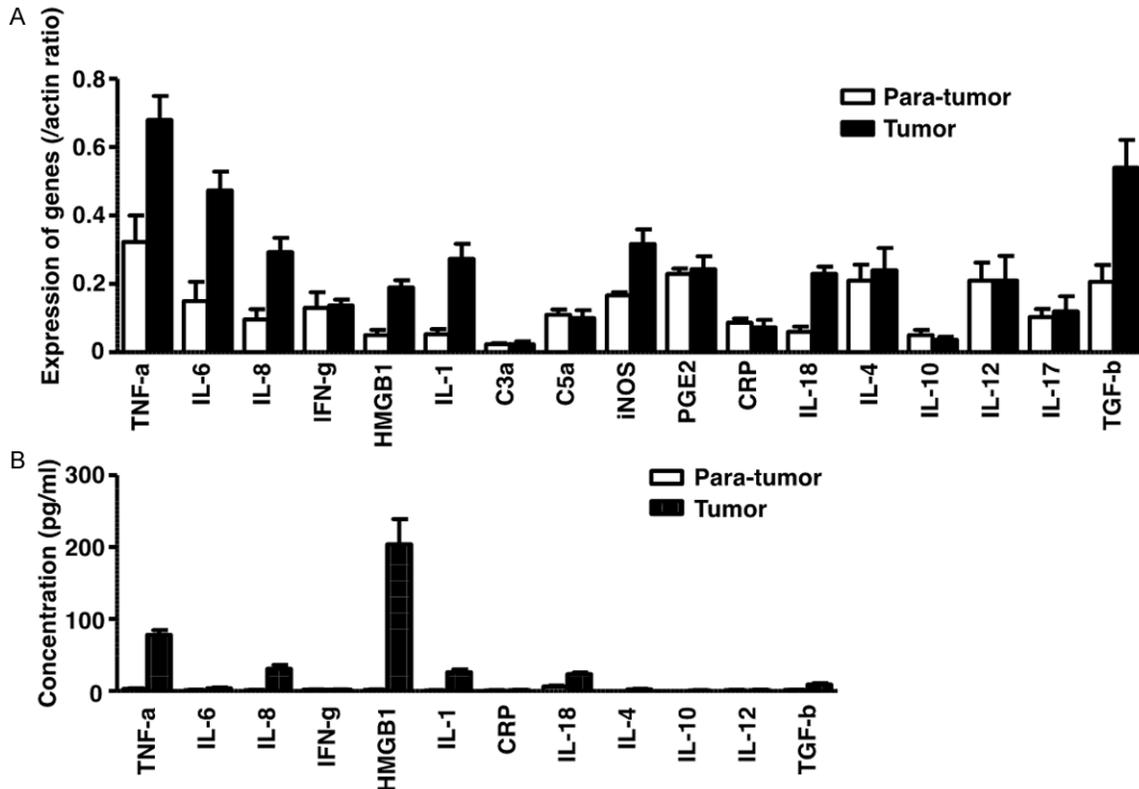


Figure 1. Inflammatory factor profile in neuroblastoma. A. Total RNA was prepared from neuroblastoma tissues or para-tumor tissues using TRIzol reagent, reverse transcribed, and quantitative real-time PCR was carried out with specific primers on an ABI Prism 7900HT. B. Neuroblastoma or para-tumor tissues were homogenized and centrifuged, and the supernatants were collected and subjected to Luminex assay for the concentrations of inflammatory factors. The data represent three independent experiments, and shown as Mean \pm SD. $P < 0.05$ means statistically significant.

tial cellular process involved in fundamental biological events, such as survival, proliferation, migration [11-14]. Increasing evidence revealed the central role of autophagy in the progression of various human diseases, including neurodegenerative diseases, cancer, and an array of other disorders [10, 15-17]. Autophagy in Schwann cells contributes to the clearance of aggresome, and benefit to myelination, suggesting the favorable role in neurodegenerative diseases and peripheral neuropathy [18]. However, the role of Schwann cell autophagy in anti-tumor abilities in neuroblastoma remains to be clarified.

In the present study, we checked the autophagy of Schwann cells in neuroblastoma and identified HMGB-1 as an inducer of autophagy. Additionally, HMGB-1-induced Schwann cell autophagy diminish the anti-tumor activity against neuroblastoma. Thus, our study demonstrated that autophagy counteract the anti-

tumor activity of Schwann cells, potentially providing a novel therapeutical approach on neuroblastoma.

Materials and methods

Reagents and cell lines

JS-1, RSC562 and SH-SY5Y were obtained from ATCC (USA), and maintained in DMEM medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and pen/strep antibiotics according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was prepared from tissues or cells using TRIzol (Invitrogen) and was reverse-transcribed using qRT-PCR kit (Invitrogen). Quantitative real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems). The program conditions as followed, 2-minute incubation at 50°C, then 95°C for 10 minutes, and

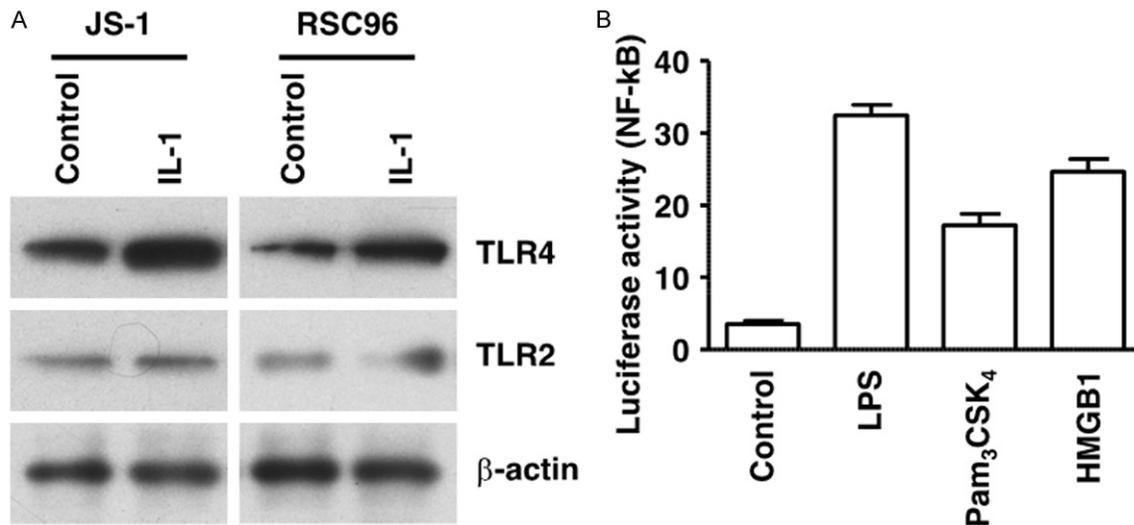


Figure 2. Expression of TLR4 and TLR2 in neuroblastoma cells. A. JS-1 or RSC96 cells pretreated with or without IL-1 were lysed and subjected to SDS-PAGE. After transferred to membrane, the samples were blotted with indicated antibodies. Images shown here are representatives of 3 independent experiments. B. JS-1 or RSC96 cells pretreated with or without HMGB1 were transfected with an ELAM-1 promoter-controlled luciferase reporter gene were lysed, and relative luciferase activities were determined. The data represent three independent experiments, and shown as Mean \pm SD. $P < 0.05$ means statistically significant.

followed with 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. We used β -Actin as an internal control to normalize for differences in the amount of total RNA in each sample.

Immunoblotting

Cells were collected and lysed with lysis buffer containing 1% NP-40. After brief vortexing and rotation, the cell lysates were separated with SDS-PAGE and transferred to nitrocellulose membranes. After 30 min blocking with 5% fat-free milk in PBST, the membrane was incubated with primary antibody, and then with HRP-conjugated secondary antibody. After washes, the protein bands were visualized with ECL plus immunoblotting detection reagents (Pierce).

Lentiviral vectors construction

pLVX-shRNA-zsGreen was used to construct the recombinant lentivirus containing TLR4 or control shRNA sequences from Invivogen (San Diego, CA). TLR4 was cloned into pLVX-IRES-ZsGreen vector to construct the recombinant lentivirus. Recombinant lentivirus were produced by transfecting HEK293T cells with pMD.2G, psPAX2. An MOI of 10 was used to inoculate cells, and the efficiency was checked 48 hours later with fluorescence microscopy.

Autophagy analyses

Autophagy was analyzed by immunoblotting or fluorescence microscopy, as described previously [23]. In the immunoblotting analysis, cells were treated as indicated, and cell lysates were immunoblotted with anti-LC3A antibody to monitor the LC3A-II generated during the formation of autophagosomes. In the fluorescence confocal microscopy analysis, cells were transfected with a GFP-LC3A construct and treated as indicated. These cells were imaged by fluorescence confocal microscopy, with single-line excitation at 488 nm for GFP, for the formation of puncta in autophagic cells. A minimum of 50 cells were analyzed for each treatment, and each experiment was performed at least three independent times.

ELISA assay

Cytokines were detected with ELISA kit from BD Biosciences according to manufacturer's instructions.

MTT cell proliferation assay

Vybrant MTT cell proliferation assay kit (Invitrogen, Eugene, OR) was used to determine the cell proliferation according to manufacturer's instructions.

Autophagy in Schwann cells contributes to neuroblastoma

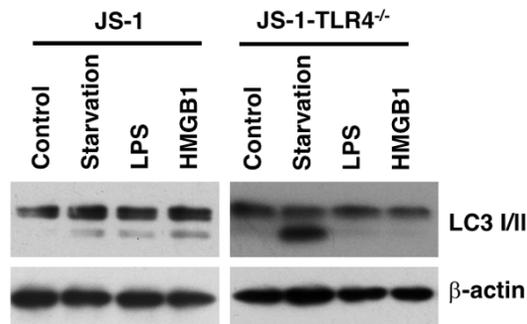


Figure 3. HMGB1 induces autophagy in Schwann cells. JS-1 cells, with or without TLR4 knockdown, pretreated with or without HMGB1 were lysed and subjected to SDS-PAGE. After transfer, the membrane was analyzed by immunoblotting with antibodies against LC3 for the conversion of LC3-I to LC3-II. Images shown here are representatives of 3 independent experiments.

Statistics

The 2-tailed Student's t test or one-way ANOVA was used for statistical analysis in this research. When $P < 0.05$, the difference is defined as statistically significant.

Results

HMGB is markedly increased in neuroblastoma

Inflammation has been commonly considered as a contributing factor to tumor incipient and development [19], and there is inflammation present in neuroblastoma microenvironment [20, 21]. To investigate the inflammatory factor profile in neuroblastoma, we performed a screening using real time-PCR to detect 17 inflammation-related genes, and we discovered that that 8 genes (TNF- α , IL-6, IL-8, HMGB1, IL-1, iNOS, IL-18, and TGF- β) were upregulated in neuroblastoma compared with para-tumor tissue (**Figure 1A**). To further characterize the inflammation microenvironment of neuroblastoma, we then examine the protein levels of these inflammatory factors using luminex system (Millipore, Billerica, MA). Six inflammatory factors (TNF- α , IL-8, HMGB1, IL-1, IL-18, and TGF- β) were markedly increased, with HMGB1 as the most prominent one (> 200 folds) (**Figure 1B**), while no C3a, C5a, iNOS, PGE2, and IL-17 were detected in protein level in both tumor and para-tumor tissues (data not shown). In addition, there are no dedicated reports on the role

of HMGB1 in neuroblastoma tumorigenesis or development, so we determined to explore its implication in these events.

Expression of TLR and its ligands in neuroblastoma

Schwann cells were increasingly displayed to play essential roles in neuroblastoma development [3]. Additionally, TLR activation has been observed in Schwann cell in inflammatory conditions, and in turn leading to inflammation, thus forming a positive-feedback loop [22]. As HMGB1 initiates inflammation through activating TLR4, and probably TLR2, so we assessed the expression of TLR4 and TLR2 in JS-1 and RSC96, two commonly used Schwann cell lines, and our results indicated that TLR4 was expressed in both cell lines, while TLR2 was detected in JS-1 cells, and arguably in RSC96 cells (**Figure 2A**). However, after stimulation with IL-1 β , the expression of TLR4 is increased considerably in both JS-1 and RSC96 cells (**Figure 2A**), suggesting the potential significance of TLR4 in regulation of Schwann cells biological behavior. To test the activating abilities of HMGB1 on Schwann cells, we performed a TLR activation assay. After transfected with a luciferase reporter gene driver by NF- κ B activity, the cells were stimulated with HMGB1 and monitored the luciferase activity. Our results demonstrated that HMGB1 strongly activated TLR4 signaling in Schwann cells, while only marginally activated TLR2 signaling (**Figure 2B**). These data combined show that HMGB1 activates Schwann cells through TLR4 activation.

HMGB1 induces autophagy in Schwann cells

TLR4 activation induces autophagy in diverse cell types, such as macrophages, dendritic cells, and lymphocytes [23, 24], while autophagy has been observed in Schwann cell and potentially played essential roles in its biological behavior. So we determined to analyze if HMGB1 induces autophagy in Schwann cells by monitoring LC3 shift (LC3I and II). LC3 is subject to lipidation with phosphatidylethanolamine (LC3II) during autophagy induction, and usually was used as a common readout for autophagy. After stimulated with HMGB1, autophagy was considerably promoted in Schwann cells (**Figure 3A**), knockdown of TLR4 obviated the autophagy formation (**Figure 3B**),

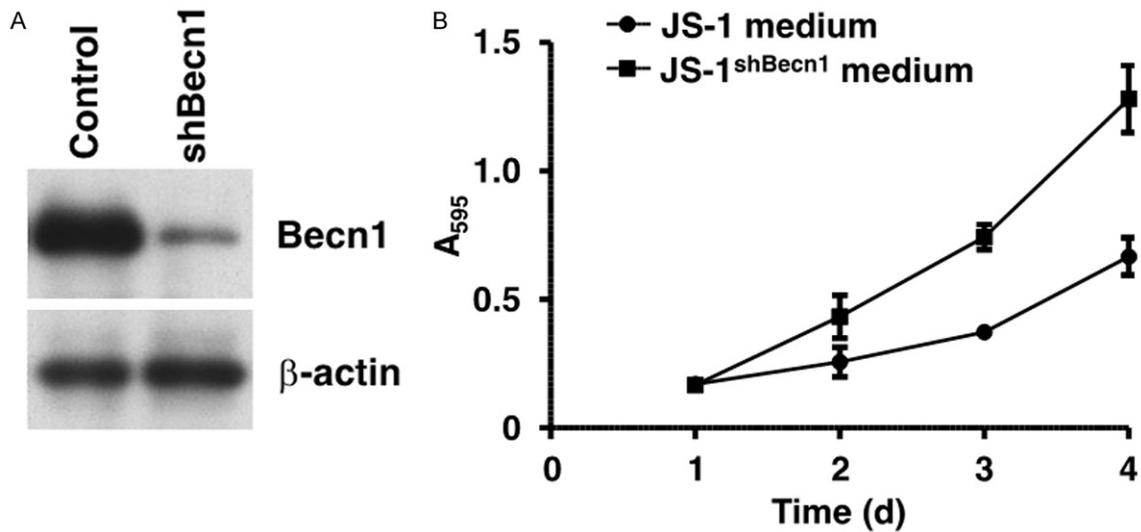


Figure 4. Autophagy in Schwann cell promotes neuroblastoma cell survival. A. JS-1 cells were infected with lentivirus with scramble shRNA (Control) or shRNA against specifically Beclin1 (MOI = 10). After 48 h, the cells were lysed and subject to SDS-PAGE followed by transferred to nitrocellulose membrane. The blot bands were visualized with ECL chemiluminant kit. Images shown here are representatives of 3 independent experiments. B. Medium from JS-1 and JS-1^{shBecn1} cells with or without autophagy were added to cultured SH-SY5Y cells, and MTT assay was used to determine the proliferation of the cells. The data represent three independent experiments, and shown as Mean ± SD. *P* < 0.05 means statistically significant.

implying the HMGB1 induces autophagy in Schwann cells through a TLR4-mediated pathway.

Autophagy in Schwann cell promotes neuroblastoma cell survival

We did observed promoted autophagy in Schwann cells located in neuroblastoma (unpublished data), however, the effects of this event on neuroblastoma remain elusive. To gain insight into the significance of Schwann cell autophagy, we took advantage of a molecular approach to establish a JS-1 cell line with Beclin 1 knockdown (JS-1^{shBecn1}) through a lenti-shRNA system. In JS-1^{shBecn1}, autophagy induction is almost completely deprived. We collected the medium from cultured JS-1 and JS-1^{shBecn1} cells with or without autophagy induction (HMGB-1 treatment or starvation), and then applied the medium to culture of SH-SY5Y, a neuroblastoma cell line. After 24 hours, we found that that medium from JS-1 promoted the proliferation of neuroblastoma cells compared with that of JS-1^{shBecn1} cells (Figure 4), suggesting the contribution of inflammation-induced autophagy of Schwann cells on neuroblastoma development, and potentially providing a novel therapeutic approach against neuroblastoma.

Discussion

In our study, we found that HMGB1, an inflammatory factor, induced autophagy in a TLR4-mediated pathway in Schwann cells, which sequentially promoted neuroblastoma proliferation, potentially providing a novel therapeutic approach for neuroblastoma. Schwann cells have been shown involved in neuroblastoma tumorigenesis and development through several pathways: promoting NB differentiation, inhibiting angiogenesis, and impairing NB growth [6, 8].

Although inflammation has been unanimously acknowledged as a hallmark of tumorigenesis [19], there is no systemic analysis on inflammatory profile in neuroblastoma microenvironment. We performed a screening and found that HMGB1 increased more than 200 folds in tumor tissue compared with para-tumor tissue. HMGB1 is produced upon cellular pyroptosis, a form of cell death, usually resulted from inflammasome activation [25]. We did detect the increase of IL-1β and IL-18 in neuroblastoma, the two readouts of inflammasome activation [26]. Inflammasome has also been shown to involve in tumorigenesis, such as gastrointestinal tumors [25]. However, whether inflammasome participate neuroblastoma remains to

be clarified. Although our study suggests the potential connection of inflammasome and neuroblastoma, further research has to be carried out, for example, which inflammasome (s) is activated during the process.

Although report have shown that stresses such as hypoxia led to significant overexpression of C-MYC and HMGB-1 in SH-SY5Y, a neuroblastoma cell line, with decreased apoptosis, suggesting a negative influence and potentially increased malignancy of tumor cells [27]. As hypoxia, similar to starvation, strongly induces autophagy, so autophagy probably provides a powerful pathway to maintain cellular survival of tumor cells. Differently, in this study, HMGB1-induced autophagy in Schwann cells contributes to tumor cell proliferation, emphasizing the essential role of microenvironment in tumorigenesis and tumor development. On the other hand, neoplastic neuroblasts increase Schwann cell proliferation, both in vivo and in vitro, in an unknown mechanism [6].

Autophagy undoubtedly is of importance to diverse cellular events, such as activation, differentiation, proliferation. In macrophages, autophagy status and extension determine not only the activation and differentiation, but also the production of cytokines, thus guiding the inflammatory function [28]. Similarly, autophagy also controls the release of bioactive factors, manifested by the functional effects of medium collected from autophagy-suppressed cells. The analysis of bioactive components is ongoing, which obviously is interesting given the promoting effects on neuroblastoma proliferation.

In conclusion, our data identified a specific molecule in neuroblastoma through a screening of inflammatory profile with neuroblastoma and para-tumor tissues, HMGB1. HMGB1 stimulates autophagy in Schwann cells through TLR4-mediated pathway, which affects the microenvironment, contributing to neuroblastoma.

Disclosure of conflict of interest

None.

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