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Proinflammatory effect of AbetaPP induced ST6GAL1 secretion from C2C12 myogenic cell line

[C2C12 miyojenik hücre hattında AbetaPP tarafından indüklenen ST6GAL1 salgılanmasının proinflamatuar etkisi]

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ABSTRACT

Objective: ST6 beta-galactosamide α-2,6-sialyltranferase 1 (ST6GAL1), the major α2,6-sialyltransferase responsible for the broad synthesis of glycoproteins and glycolipids, is another physiological substrate of Beta site APP-cleaving enzyme 1 (BACE1) other than amyloid-beta precursor protein (AbetaPP). We have previously shown that AbetaPP overexpression in C2C12 mouse myoblast cell line increased the expression and secretion of ST6GAL1 enzyme under *in vitro* conditions. Since the secretion of ST6GAL1 is known to be enhanced during inflammation, we investigated whether AbetaPP induced ST6GAL1 secretion from C2C12 cells affected proinflammatory cytokine production by J774 mouse macrophage cell line.

Methods: J774 macrophage cells were cultured with conditioned medium derived from either AbetaPP or ST6GAL1 overexpressing C2C12 cells and analyzed for proinflammatory cytokine (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6) expression by quantitative real-time PCR (qRT-PCR). Also, culture supernatants were analyzed for IL-1β release by ELISA.

Results: The results of our study demonstrated that secretion of ST6GAL1 enzyme from either AbetaPP or ST6GAL1 overexpressing C2C12 cells induces the expression of TNF-α, IL-1β, IL-6 and the secretion of IL-1β by J774 macrophages.

Conclusion: In our study, results were obtained pointing that secreted ST6GAL1 might have a potential role in the inflammation process observed in the muscle tissue. Our results should be confirmed under *in vivo* system.

Key Words: Skeletal muscle, AbetaPP, Abeta, ST6GAL1, inflammation, proinflammatory cytokine

Conflict of Interest: Authors have no conflict of interest.

ÖZET

Amaç: ST6 Beta galaktozamid alfa-2,6-siyaliltransferaz (ST6GAL1), glikolipitlerin ve glikoproteinlerin sentezinde görev alan temel α-2,6 siyaliltransferaz olmasının yanısıra, *Beta site APP-cleaving enzyme 1* (BACE1) enziminin amiloid-beta öncül proteini (AbetaPP) dışındaki fizyolojik substratlarından biridir. Daha önce yapmış olduğumuz *in vitro* çalışmada; AbetaPP'nin yüksek düzeyde ifadesinin sağlandığı C2C12 hücrelerinde, ST6GAL1'in ifade düzeyinin ve salgılanma seviyesinin arttığı saptanmıştır. ST6GAL1 salgılanma seviyesinin inflamasyon sürecine bağlı olarak arttığı bilinmektedir. Bu nedenle, C2C12 hücrelerinde AbetaPP'nin yüksek düzeyde ifadesine bağlı olarak artan ST6GAL1 salgılanmasının, J774 fare makrofaj hücrelerinde proinflamatuar sitokinlerin ifade ve salgılanması üzerindeki etkileri araştırılmıştır.

Metod: AbetaPP veya ST6GAL1 enziminin yüksek düzeyde ifadesinin sağlandığı C2C12 hücrelerinin süpernatanı ile kültüre edilen J774 makrofaj hücrelerinde, kantitatif gerçek zamanlı PCR (qRT-PCR) ile proinflamatuar sitokinlerin (tümör nekroz faktör (TNF)-α, interlökin (IL)-1β ve IL-6) ifadesi analiz edilmiştir. Ayrıca, kültür süpernatanlarında ELISA yöntemi ile IL-1β salgılanma analizi gerçekleştirilmiştir.

Bulgular: AbetaPP veya ST6GAL1 enziminin yüksek düzeyde ifadesinin sağlandığı C2C12 hücrelerinden salgılanan ST6GAL1'in, J774 hücrelerinde TNF-α, IL-1β, IL-6 ifadesini ve IL-1β salgılanmasını arttırdığı sonucuna ulaşılmıştır.

Sonuç: Çalışmamızda, salgılanan ST6GAL1'in kas dokusunda gözlenen inflamasyon sürecinde potansiyel işlevi olabileceği yönünde bulgular elde edilmiş olup, sonuçlarımızın *in vivo* sistemde doğrulanması gerekmektedir.

Anahtar Kelimeler: İskelet kası, AbetaPP, Abeta, ST6GAL1, inflamasyon, proinflamatuar sitokin

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Skeletal muscle is one of the known tissues marked by the intracellular accumulation of the amyloid-beta (Abeta) peptide [1]. The accumulation of this amyloidogenic product has been proposed to underlie the muscle pathology observed in sporadic inclusion body myositis (s-IBM), the most common muscle disease of persons aged 50 years and older [2]. Although the etiology and pathogenesis are still unknown, skeletal muscle tissues of the s-IBM patients show profound inflammation with an increased expression of various chemokines and cytokines [3,4]. Also, the muscle phenotype shows several similarities with Alzheimer's disease brain such as accumulation of several proteins including amyloid-beta precursor protein (AbetaPP) and Abeta [5]. The amyloidogenic Abeta is generated by a series of sequential cleavages of AbetaPP by β- and γ-secretases. Beta secretase-1 (BACE1) also known as Beta site APP-cleaving enzyme 1 is one of the glycosylated transmembrane β-secretases that mediates the primary amyloidogenic cleavage of AbetaPP [6,7]. It has also been involved in the proteolytic processing of a Golgi-resident sialyltransferase, namely, ST6 beta-galactosamide α-2,6-sialyltranferase 1 (ST6GAL1) which is probably the most extensively studied sialyltransferase among the eukaryotic sialyltransferases [8,9].

ST6GAL1 creates α2,6 attachment of sialic acid residues to Gal(β1,4)GlcNAc termini of glycoproteins. It is a type II membrane protein and composed of short NH2 terminal cytoplasmic domain, a single pass transmembrane domain, a stem region, and a large COOH terminal catalytic domain. The enzyme has been shown to be localized in the trans-cisternae and the trans-Golgi network of the Golgi but can be proteolytically processed by BACE1 to generate a catalytically active soluble form [8-10]. The soluble form of ST6GAL1 has been detected in body fluids and cell culture media and has been suggested to be implicated in α-2,6 sialylation of soluble glycoprotein substrates [11]. There have been studies strongly correlating expression and secretion of ST6GAL1 with systemic inflammatory response as an immune regulator [12-14]. However, the physiological significance of soluble ST6GAL1 in skeletal muscle system remains enigmatic.

We recently analyzed the effect of overexpression of AbetaPP on expression and secretion of ST6GAL1 in skeletal muscle cells by inducing overexpression of wild-type full-length 751- AbetaPP in the mouse myogenic cell line C2C12 and demonstrated that AbetaPP overexpression in C2C12 cells increased the expression and the secretion of ST6GAL1 enzyme under *in vitro* conditions [15]. In this study we investigated whether AbetaPP induced ST6GAL1 secretion from C2C12 cells affect proinflammatory cytokine production by J774 mouse macrophage cell line.

Materials and Methods

Cell culture and transfection

C2C12 mouse myoblast cells [American Type Culture Collection (ATCC) product number CRL-1772] were plated at a density of 1×10^5 cells in 35mm cell culture dishes 24 h before transfection, and expanded in growth medium that consisted of RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine. Cells were then transfected either with AbetaPP751-pCMVSport6 (Invitrogen), STtyr-pSVL*, SP(sol)STtyr-pSVL [16,17]*, empty pCMVSPORT6 vector, empty pSVL vector* (*generous gift from Dr. K.J. Colley) or PEGFP-C1 vector for 24 h using Fugene HD Transfection Reagent (Roche Applied Science) at a DNA:Fugene HD ratio of 3:10. Following transfection for 24 h, the medium was replaced with serum-free medium for an additional 24 h to induce ST-6GAL1 secretion [15]. Conditioned media of transfected C2C12 cells were collected for subsequent experiments. Also, conditioned medium of non-transfected cells (control medium) was generated at the same time.

J774 mouse macrophage cells (ATCC product number TIB-67) were then plated at a density of 1×10^6 cells in 35mm cell culture dishes in growth medium that contains RPMI 1640 with 10% fetal bovine serum and 2 mM L-glutamine. After 24 h, the cultured medium was changed with conditioned medium derived from transfected/non-transfected C2C12 cells. J774 cells and supernatants were harvested after 6 h and stored for quantitative real-time PCR (qRT-PCR) and ELISA, respectively. Unstimulated J774 cells were used as experimental negative controls, whereas lipopolysaccharide (LPS; *Escherichia coli*; Sigma) (1 μg/mL) stimulated cells served as positive controls.

Quantitative real-time PCR (qRT-PCR)

In order to assess the expression of proinflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6), total RNA was extracted from conditioned media treated J774 cells using RNeasy fibrous tissue mini kit (Qiagen) and cDNA was synthesized from 1000 ng RNA using QuantiTect Reverse Transcription kit (Qiagen) according to manufacturer's guidelines. qRT-PCR was then performed using SYBR Green JumpStart TaqReadyMix kit (Sigma) on Rotor-Gene 6000™ (Corbett Research) with the following thermocycler conditions: 2 min at 94°C, followed by 40 cycles of 1 s at 94°C and 15 s at 60°C. Reactions were performed in a total volume of 10 μL, including 5 μL SYBR Green JumpStart TaqReadyMix kit (Sigma), 1.2 μl of 25 mM MgCl2 (Sigma), 0.4 μl each of 10 mM forward and reverse primers and 1.5μl of template cDNA. The relative amount of mRNA, normalized to an internal control mouse β-actin and relative to a calibrator (control), was calculated by $2^{-\Delta\Delta CT}$. Primers used were as follows: mouse TNF-α(F): 5'-CGCTCTTCTGTCTACTGAACTTCG-3'; mouse TNF-α(R): 5'-GATGATCTGAGTGTGAGGGTCTGG-3'; mouse IL-6(F): 5'-ATTTCCTCTGGTCTTCTTCTGGAGTAC-3';

mouse IL-6(R): 5'- GCTTATCTGTTAGGAGAG-CATTGG-3'; mouse IL-1β(F): 5'-CAACCAACAAGT-GATATTCTCCAT-3'; mouse IL-1β(R): 5'-ATT-GCTTGGGATCCACACTCT-3'; mouse β-actin(F): 5'-GTGCTATGTTGCCCTAGACTTCG-3' and mouse β-actin(R): R: 5'-GATGCCACAGGATTCCATACCC-3'. All reactions were performed in triplicate and optimal threshold in 95–100% efficiency. All data was analyzed using Rotor-Gene 6000 Series Software 1.7 (Corbett Research).

ELISA assay

IL-1β release into the culture media of J774 cells was evaluated by ELISA assay. The medium of each culture was collected in a sterile cryotube and stored at –20°C until assay. The culture media were thawed, centrifuged briefly, and processed with a mouse IL-1β Instant ELISA kit (eBioscience) according to the manufacturer's instructions. The sensitivity of this assay was 3.0 pg/ml and each sample was analyzed in duplicate.

Statistical analysis

Mean and SD were calculated from data obtained in three separate experiments performed in triplicate. The significance of differences between experimental groups was determined using Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

Our previous studies revealed that AbetaPP overexpression in C2C12 cells increased the expression and secretion of ST6GAL1 enzyme, another physiological substrate of BACE1 enzyme other than AbetaPP, under *in vitro* conditions [15]. To investigate the effect of AbetaPP induced ST6GAL1 secretion on proinflammatory cytokine production by macrophage cells, ST6GAL1 secretion was stimulated by transient transfection of C2C12 cells with wild-type human full-length AbetaPP751 (AbetaPP751-pCMVSport6). In our previous studies, we have already demonstrated the transfection efficiency of C2C12 cells as 60% by measuring the percentage of transfected cells that were immunofluorescently labelled with an antibody against AbetaPP [15]. In order to show a direct relationship between ST6GAL1 secretion and proinflammatory cytokine production, C2C12 cells were also transfected either with STtyr-pSVL and SP(sol)STtyr-PSVL vectors encoding soluble isoform of rat ST6GAL1 (STtyr) and a novel fusion protein harboring signal peptide of interferon-γ and STtyr, respectively [16-18]. At 24 h after transfection, culture media was replaced and ST6GAL1 secretion into the culture media of transfected C2C12 cells was confirmed by Western blot analysis (data not shown). J774 macrophage cells were then cultured in conditioned media obtained from cultures of C2C12 cell lines for 6 h and analyzed for TNF-α, IL-1β and IL-6 expression by qRT-PCR. An untransfected C2C12 culture, a C2C12 culture transfected with an unrelated vector encoding GFP

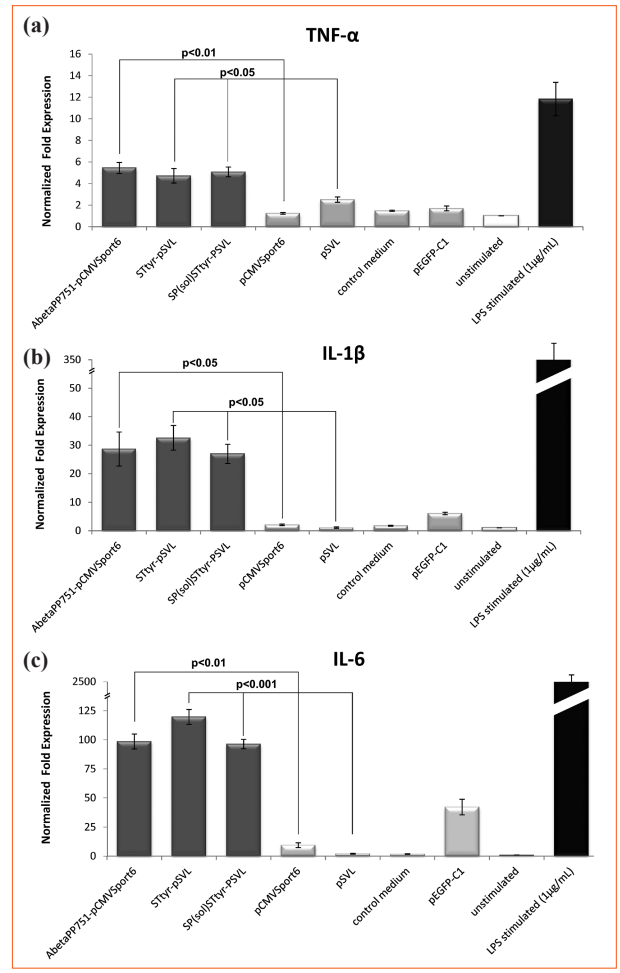

Figure 1. Induction of proinflammatory cytokine expression in J774 cells in response to conditioned medium including soluble ST6GAL1. The levels of (a) TNF-α, (b) IL-1β and (c) IL-6 mRNA were determined by using qRT-PCR. Data are expressed as fold change over the level in unstimulated J774 cells and represent means of three independent experiments performed in triplicate. A statistically significant increase in the expression levels of (a) TNF-α, (b) IL-1β and (c) IL-6 were determined by Student's *t*-test. Shown *P*-values represent statistical comparisons of gene expression levels observed in J774 cells incubated with conditioned medium from C2C12 cells transfected with either AbetaPP751-pCMVSport6, STtyr-pSVL or SP(sol)STtyr-pSVL vs appropriate empty vector controls. *Error bars* indicate standard deviations.

(pEGFP-C1) or the appropriate empty vectors (pCMV-Sport6 and pSVL) were used as experimental controls. In addition, unstimulated and LPS stimulated J774 cells served as negative and positive controls, respectively.

As shown in Figure 1, the conditioned medium of AbetaPP or ST6GAL1 overexpressing myogenic C2C12 cells significantly increases the expression of TNF-α, IL-1β and IL-6 by J774 macrophages. We observed very similar expression patterns with AbetaPP751-pCMVSport6, STtyr-pSVL and SP(sol)STtyr-PSVL. No signal was detected in the negative control (unstimulated J774 cells), whereas the expression of proinflammatory cytokines were strongly induced by LPS. There was no significant increase in

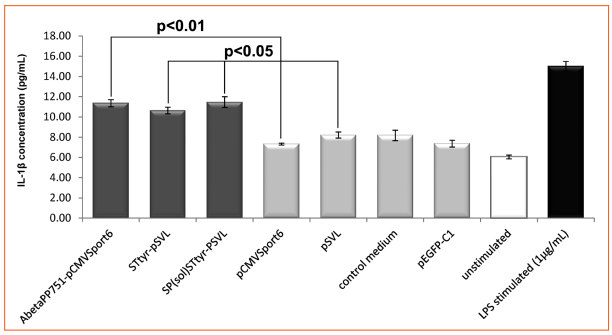

Figure 2. Induction of IL-1β secretion from J774 cells in response to conditioned medium including soluble ST6GAL1. ELISA was used to measure IL-1β concentrations in the final supernatants of J774 cells. Results are representative of 3 independent experiments. A statistically significant increase in the secretion of IL-1β was determined by Student's *t*-test. Shown *P*-values represent statistical comparisons of secretion levels observed in J774 cells incubated with conditioned medium from C2C12 cells transfected with either AbetaPP751-pCMVSport6, STtyr-pSVL or SP(sol)STtyr-pSVL vs appropriate empty vector controls. *Error bars* indicate standard deviations.

the expression of these genes when J774 cells were incubated in the control medium and conditioned medium of C2C12 cells transfected with either empty vectors or PEGFP-C1. Since the secretion of proinflammatory cytokines is an important stage of a proinflammatory signal [19], we also investigated whether enhanced ST6GAL1 secretion from C2C12 cells induces the release of an important mediator of the inflammatory response, IL-1β, from J774 cells. The levels of mouse IL-1β in the culture supernatant of J774 cells were determined by ELISA. The results of the ELISA were consistent with the qRT-PCR analysis and showed that the secretion of IL-1β from J774 cells was significantly increased in the presence of soluble form of ST6GAL1 (Figure 2). Notably, ~15-30 fold increase in mRNA resulted in a ~1.2-1.6 fold increase in secreted IL-1β.

Discussion

We concluded that the secretion of ST6GAL1 enzyme from either AbetaPP or ST6GAL1 overexpressing myogenic C2C12 cells significantly induces the expression of TNF-α, IL-1β, IL-6 and the secretion of IL-1β by J774 macrophages. Proinflammatory cytokines are important molecules in the inflammatory response and in immune regulation. The production of these molecules by activated macrophages plays a fundamental role in the pathogenesis of several muscular dystrophies and primary inflammatory myopathies [20-22]. In this study, stimulation of J774 cells with soluble ST6GAL1 resulted in clearly enhanced expression of the major proinflammatory cytokines that are responsible for early responses (Figure 1). In all experimental sets, as expected, the effect of LPS was significantly more potent than that of J774 cells treated with conditioned medium of AbetaPP or ST6GAL1 overexpressing myogenic C2C12 cells. LPS is a major component of the outer membranes of Gram-negative bacteria and known to be the main toxic element that induces proinflammatory cytokine production by various signal transduction events which lead to the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways [23]. Therefore, J774 cells were also treated with LPS to serve as a positive control in our experiments. In addition, LPS-mediated dramatic increase in the expression of inflammatory mediators have also been observed in some published studies even at low concentrations of LPS (30 ng/mL) [24]. Although the proinflammatory cytokine response to LPS alone was greater than AbetaPP751-pCMVSport6, STtyr-pSVL and SP(sol)STtyr-PSVL, the response of J774 cells to soluble ST6GAL1 were statistically significant when compared with their counterparts transfected with empty vector. ST6GAL1 might be stimulated macrophages through distinct signalling pathways. Furthermore, IL-1β which is a prototypic multifunctional cytokine is an important mediator of the inflammatory response [25]. It is involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis. Among other proinflammatory cytokines, the release of IL-1β appears to play a special role in inflammatory modulation through the induction of other inflammatory cytokines [26]. It has been indicated that the induced release of IL-1β modulates the production of other inflammatory cytokines such as IL-8, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α in human primary gingival epithelial cells (HGECs) [27]. Therefore, we also tested whether increase in the mRNA expression of IL-1β results in the increase of secretion from J774 cells. Using ELISA, enhanced secretion of IL-1β was detected in culture supernatants of J774 cells, compatible with the results in qRT–PCR analysis. Taken together, these results reveal a critical role for ST6GAL1 in the regulation of inflammation under *in vitro* conditions.

It has been known that soluble form of ST6GAL1 is detected in systemic circulation where secretion is enhanced during acute-phase hepatic reactions, in case of inflammation [12-14]. Also, there have been reports suggesting a novel function for ST6GAL1 in attenuating acute neutrophilic inflammation and in regulating myelopoiesis [28]. However, the contribution of soluble ST6GAL1 to the development of muscle inflammation has remained unproven. Our results point to a potential proinflammatory role for soluble ST6GAL1 in promoting muscle inflammation. We have previously shown that AbetaPP overexpression in C2C12 cells increased the expression and secretion of ST6GAL1 enzyme with an unknown mechanism [15]. In this study, we proved that enhanced secretion from myogenic cells triggers inflammation by inducing the proinflammatory cytokines by macrophages. These findings suggest a possible relationship between ST6GAL1 secretion and inflammatory process induced by overexpression of AbetaPP.

s-IBM is the most common myopathy associated with aging but may occasionally also affects younger individuals [29]. It is classified as an inflammatory myopathy but also has myodegenerative features [30]. The degenerative process results in atrophic muscle fibers, vacuolar degeneration and abnormal protein aggregations in vacuolated and/or non-vacuolated muscle fibers including AbetaPP and Abeta [31]. However, the inflammatory process is characterized by cytotoxic CD8+ T cells and macrophages invading non-necrotic muscle fibers expressing major histocompatibility complex class I [32]. The network of interactions between inflammatory and degenerative processes in the pathogenesis of s-IBM remains uncertain. It has been established in our study that AbetaPP induced ST6GAL1 secretion from myogenic cells triggers inflammation by enhancing the production of proinflammatory cytokines by macrophages under *in vitro* conditions. Therefore, accumulation of AbetaPP and of its proteolytic fragment Abeta may appear to be early upstream events that initiate the inflammatory response, as suggested by several researchers [2,33]. Although further *in vivo* studies are necessary to confirm our results, these findings may provide an alternate way to understand the causes of muscle inflammation in other types of inflammatory myopathies since AbetaPP and other IBM related proteins are also known to be overexpressed in polymyositis, hereditary inclusion-body myopathy (hIBM) and other myopathies [34,35]. The elucidation of the causes of muscle fiber degeneration will be possible with the identification of triggering events and pathogenic pathways that contribute to disease pathogenesis.

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Conflict of Interest

There are no conflicts of interest among the authors.

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