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ORIGINAL ARTICLE

Transglutaminase 2 silencing reduced the beta-amyloid-effects on the activation of human THP-1 cells

Monica Currò · Nadia Ferlazzo · Salvatore Condello · Daniela Caccamo · Riccardo Ientile

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Abstract The aberrant expression and activation of transglutaminase 2 (TG2), the ubiquitous enzyme which catalyzes calcium-dependent protein cross-linking reactions, has been reported in many inflammatory diseases. Chronic inflammation, mediated by prolonged activation of brain-resident immunocompetent cells, appears to be involved in the pathogenesis of several age-related diseases, such as Alzheimer's disease. Given that increased TG2 expression has been observed in AD brains, this study was aimed to characterize the role of TG2 in THP-1 monocytes stimulated with amyloid-beta (Aβ). A$\beta_{1–42}$ treatment dose-dependently increased TG2 expression in THP-1 cells. In particular, a fivefold up-regulation of TG2, compared with control cells, was observed in the presence of 0.5 μM A$\beta_{1–42}$. At the same concentration, A$\beta_{1–42}$ was able to promote monocyte maturation as suggested by increased expression of the cell surface antigen CD14 as well as the adhesion-promoting factor fibronectin. The stimulation of THP-1 cells with A$\beta_{1–42}$ also led to a significant up-regulation of tumor necrosis factor α (TNF-α) and matrix metalloproteinase 9 (MMP-9). Interestingly, THP-1 cell transfection with small interfering RNA directed against TG2 was able to reduce A$\beta_{1–42}$ increased levels of all the examined markers of monocyte maturation (CD14, fibronectin), and activation (TNF-α, MMP-9). These results indicate that TG2 up-regulation is required for the functional THP-1 monocyte activation induced by A$\beta_{1–42}$. This work suggests that TG2 inhibition may represent a therapeutic target to ameliorate the inflammation and progression in Alzheimer's disease.

Keywords Transglutaminase 2 · Amyloid-beta · THP-1 monocyte activation · Inflammation · Transglutaminase 2 silencing

Abbreviation

Aβ	Amyloid-beta
AD	Alzheimer's disease
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
MMP-9	Matrix metalloproteinase 9
MMPs	Matrix metalloproteases
pro-MMP-9	Pro-matrix metalloproteinase 9
TG2	Transglutaminase 2
TNF-α	Tumor necrosis factor α

Introduction

Tissue transglutaminase or transglutaminase 2 (TG2) belongs to the multigene transglutaminase family of Ca^{2+}-dependent enzymes, which catalyze the formation of intra-molecular or inter-molecular gamma-glutamyl-(epsilon)-lysine isopeptide bonds (cross-links) between proteins or the incorporation of polyamines into proteins (Griffin et al. 2002).

Several physiological roles for TG2 have been reported, including differentiation, wound healing, apoptosis, and

M. Currò · N. Ferlazzo · S. Condello · D. Caccamo · R. Ientile (✉)
Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Policlinico Universitario, 98125 Messina, Italy
e-mail: ientile@unime.it

extracellular matrix stabilization (Fesus and Piacentini 2002).

TG2 aberrant expression and activation may be induced by various toxic stimuli (Ientile et al. 2007), and have been associated with a variety of diseases including neurodegenerative disorders, autoimmune diseases, and cancer (Alaedini et al. 2007; Kotsakis and Griffin 2007; Caccamo et al. 2010).

Furthermore, increased TG2 expression has been reported in many inflammatory diseases, such as Crohn's disease, renal fibrosis, and sporadic inclusion-body myositis (Kim 2006).

However, TG2 role in the inflammation process is not yet clear.

Perivascular macrophages and microglia are brain-resident immunocompetent cells which play an important role in neurodegenerative diseases, as they are activated by different stimuli, including vascular alterations as well as accumulation of toxic protein aggregates (Liu and Hong 2003).

The deposition of extracellular aggregates of β-amyloid (Aβ) within senile plaques, as well as the intracellular accumulation of neurofibrillary tangles, is the main hallmarks of Alzheimer's disease (Selkoe 2004).

Aggregated Aβ stimulates cell signaling involved in the modulation of inflammatory response. Indeed, the presence of Aβ appears to encourage monocyte/macrophage infiltration to sites in close proximity of parenchymal Aβ senile plaques, as well as monocyte adhesion and differentiation into macrophages (Fiala et al. 1998; Giri et al. 2000; Simard et al. 2006). Aβ has also been shown to induce the release of pro-inflammatory products by activated monocytes/macrophages and microglia, and the increase in ROS/NO production leading to neuronal apoptosis (Salminen et al. 2009; Sondag et al. 2009).

Given that TG2 expression and activity have been shown to be elevated in AD brains (Kim et al. 1999; Jeitner et al. 2009), this study was aimed to characterize the role of TG2 in the inflammatory mechanisms associated with cell response to Aβ. To this purpose, we used the human monocytic THP-1 cell line that has been widely accepted as a good model of monocytes/macrophages (Hsu et al. 1996).

Materials and methods

Materials

The human pre-monocytic cell line, THP-1, was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen-Braunschweig, Germany).

RPMI-1640, L-glutamine, HEPES, sodium pyruvate, glucose, aprotinin, leupeptin, pepstatin, beta-amyloid$_{1-42}$, and other chemicals of analytical grade were from Sigma (Milan, Italy). Fetal bovine serum (FBS), as well as TRIzol for RNA extraction, and Lipofectamine 2000 for cell transfection were from Invitrogen Life Technologies (Milan, Italy).

RT-PCR-specific primers for human CD14, and β-actin, were synthesised by MWG Biotech (Monza, Italy).

RT-PCR reagents were from Pierce (Celbio, Milan, Italy).

Monoclonal antibodies for TG2, fibronectin, pro-matrix metalloproteinase-9 (pro-MMP-9) and β-actin were from Sigma (Milan, Italy). Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG, and ECL Chemiluminescence detection kit were from Amersham Pharmacia Biotech (Milan, Italy). Developer, fixer and Kodak X-ray film were from Kodak (Milan, Italy).

High-capacity cDNA archive kit, *Taq*Man Gene Expression Mastermix, siRNA directed against TG2 (ID: 111472, s14088 and s14087), *Taq*Man Gene Expression assays (Assays-on-Demand) for human β-actin (ID: Hs99999903_m1), TGM2 (ID: Hs00190278_m1) and TNF-α (ID: Hs00162752_m1) were from Applied Biosystems (Applera Corp., Milan, Italy). Assay location (mid-position of fluorogenic probe), reference sequences and other relevant information are published online by Applied Biosystems (Foster City, CA) at the web page http://myscience.appliedbiosystems.com/common/search.jsp?/assayType=geneExpression.

Cell culture and treatment

THP-1 cells were maintained in RPMI 1640 supplemented with L-glutamine (2 mM), HEPES (10 mM), sodium pyruvate (1 mM), glucose (2.5 g/l), and 10% fetal bovine serum, at 37°C in a 5% CO_2/95% air humidified atmosphere. Medium was renewed every 2 days, and split performed when cells reached maximum density (1×10^6 cells/ml).

Prior to stimulation with Aβ_{1-42}, THP-1 cells were seeded at a density of 5×10^5/well into 24-well culture plates in RPMI plus 5% fetal bovine serum, and incubated for 24 h at 37°C.

Before use, Aβ_{1-42} dissolved in endotoxin-free water was kept at 37°C for 7 days to allow fibril formation as previously described by Giri et al. (2003).

To evaluate the dose-dependent effect of Aβ_{1-42}, cells were first stimulated by the addition of Aβ_{1-42} with concentration ranging from 0.1 to 2 μM for 24 h. Further experiments were carried out with 0.5 μM Aβ_{1-42}.

Cell transfection using small interfering RNA

Transfection with duplex siRNA designed to silence TGM2 was carried out using Lipofectamine 2000 according to the

manufacturer's instructions. Three different siRNAs were tested in this work, and the most effective (ID 111472) was used for subsequent experiments.

Transfection complexes were added, at a 30 nM siRNA final concentration, to suspended THP-1 cells, that were seeded at a density of 5×10^5/well into 24-well culture plates in RPMI plus 5% fetal bovine serum. After 24 h of incubation with siRNA, cells were further incubated until 24 h with Aβ_{1-42}, at a concentration of 0.5 μM in RPMI plus 5% fetal bovine serum.

Cells transfected either with 30 nM scrambled siRNA or lipofectamine only were used as internal negative control for transfection.

Western blot

After treatment, THP-1 cells were homogenized in a Dounce homogenizer. Proteins (30 μg) were separated by 8.5% SDS-PAGE, and then transferred to nitrocellulose membranes. The blots were blocked by incubation with 5% non-fat dry milk in Tris-buffered saline containing 0.15% Tween 20 for 1 h at room temperature. Then, membranes were incubated overnight at 4°C with specific antibodies against TG2, fibronectin, pro-MMP-9 (diluted 1:1,000 in TBS-T) or β-actin (diluted 1:5,000 in TBS-T) followed by incubation for 2 h with horseradish peroxidase-conjugated anti-mouse, and anti-rabbit, secondary antibodies (diluted 1:3,000 in TBS-T). Immunoblots were developed with ECL chemiluminescent system; then, bands were scanned and quantified by densitometric analysis with AlphaImager 1200 System (Alpha Innotech; San Leandro, CA).

RT-PCR

After RNA isolation with TRIzol reagent from suspended cells, RNA (1 μg) was reverse transcribed with High-Capacity cDNA Archive kit according to the manufacturer's instructions. Then, 25 ng of cDNA was amplified in a total volume of 50 μl also containing 1× PCR buffer, 1.7 mM $MgCl_2$, 0.2 mM dNTP, 1.0 U of *Taq* DNA polymerase, and 0.2 μM each of specific primers for human CD14 and β-actin (as internal control). Primer sequences were the following: 5′-ACTCCCTCAATCTGTCGTTC GCTG-3′ (sense) and 5′-CTGAAGCCAAGGCAGTTTGA GTCC-3′ (anti-sense) for CD14; 5′-ATCTGGCACCA CACCTT CTACAATGAGCTGCG-3′ (sense) and 5′-C GTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (anti-sense) for β-actin.

Each PCR reaction mixture was heated at 95°C for 5 min for denaturation, followed by 35 cycles of 1 min of denaturation at 95°C, 1 min of primer annealing at 58°C, and 1 min of extension at 72°C, and finally one cycle of 7 min at 72°C in a Hybaid PCR sprint thermocycler.

RT-PCR products were separated on 2% agarose gel, and the band intensity was quantified by densitometric analysis using an AlphaImager 1200 System.

Real-time PCR

After RNA isolation and reverse transcription as above described, mRNA levels of TG2 and tumor necrosis factor-α (TNF-α) were analyzed by real-time PCR using *Taq*Man gene expression assays according to the manufacturer's instructions. β-actin was used as endogenous controls.

Quantitative PCR reactions were set up in triplicate in a 96-well plate and were carried out in 20 μl reactions containing 1× TaqMan® Gene Expression Mastermix, 1× *Taq*Man-specific assay, and 25 ng RNA converted into cDNA.

RT-PCR was performed in a 7900HT Fast Real-Time PCR System with the following profile: one cycle at 50°C for 2 min, then 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Data were collected and analyzed using SDS 2.3 and RQ manager 1.2 software (Applied Biosystems, Foster City, CA) using the $2^{(-\Delta\Delta C_t)}$ relative quantification method. Values are presented as fold change relative to unstimulated cells.

Statistical analysis

Data obtained from three separate experiments were expressed as mean ± SD, and analyzed by two-tailed Student's *t* test using GraphPad Prism (version 4.03) software (San Diego, CA). Statistical significance was assessed based on a P value <0.05.

Results

Several reports have previously shown that the treatment of THP-1 monocytes with Aβ concentrations in the micromolar range (10–25 μM) is able to trigger the release of pro-inflammatory factors (Fiala et al. 1998; Szczepanik et al. 2001). However, other in vitro studies demonstrated that nanomolar concentrations of Aβ promote monocyte trans-migration across an endothelial cell monolayer (Giri et al. 2000). Given the reported Aβ plasma concentrations in a sub-micromolar range in Alzheimer patients (Kuo et al. 1999), in this study we used THP-1 monocytes to test the effects of nanomolar concentrations of Aβ_{1-42}, since this is the primary component of senile plaques (Selkoe et al. 2004). Moreover, Aβ_{1-42} in vitro forms fibrillar species with pro-inflammatory activity which more effectively activate THP-1 cells compared to Aβ_{1-40} (Ajit et al. 2009).

We first show that Aβ_{1-42} was able to affect the expression of TG2 in THP-1 cells. Indeed, a 24 h

stimulation with A$\beta_{1–42}$ (0.1–2 μM) increased the amount of TG2 mRNA transcripts in a dose-dependent manner, as shown by real-time PCR analysis (Fig. 1). The lowest A$\beta_{1–42}$ concentration able to significantly affect TG2 expression was 0.5 μM, that achieved a fivefold increase ($P < 0.05$) of TG2 mRNA levels in comparison to low amounts found in basal conditions.

Since a 0.5 μM concentration of A$\beta_{1–42}$ is closer to the observed one in Alzheimer patients, further experiments were carried out using this A$\beta_{1–42}$ concentration.

In order to evaluate the role of TG2 in THP-1 monocyte response to A$\beta_{1–42}$, cell cultures were transiently transfected with siRNA directed against TG2 prior to stimulation with A$\beta_{1–42}$.

Real-time PCR analysis showed that the near fivefold TG2 up-regulation, induced by 24 h treatment with A$\beta_{1–42}$ (0.5 μM), was reduced by 56% in cells transfected with TG2 siRNA compared with non-transfected cells (Fig. 2a). Cell transfection with either scrambled TG2 siRNA or lipofectamine only in the presence of A$\beta_{1–42}$ (data not shown), as well as transfection with siRNA in control cells, did not display any significant effect on TG2 expression.

These results were confirmed by Western blotting and immunoblot densitometric analysis that showed a dramatic reduction of TG2 protein amounts in A$\beta_{1–42}$-treated cells which were transfected with TG2 siRNA compared with non-transfected ones (Fig. 2b).

TG2 involvement in THP-1 monocyte activation induced by A$\beta_{1–42}$ was further evaluated by analysis of the expression of CD14, a typical macrophage cell surface antigen, or fibronectin, a key protein in monocyte adhesion.

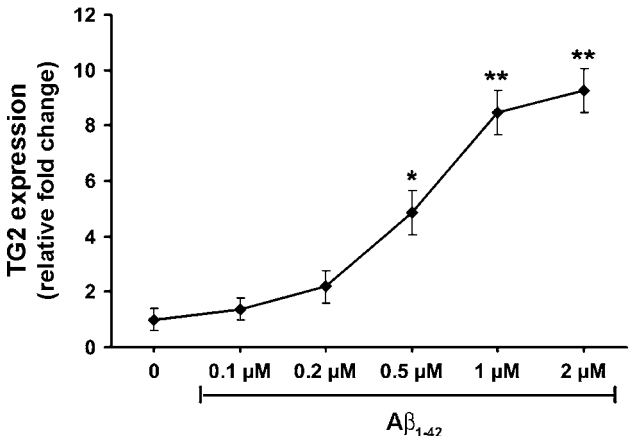

Fig. 1 Dose-dependent effects of A$\beta_{1–42}$ on TG2 expression in THP-1 monocytic cells. Cells were stimulated for 24 h with A$\beta_{1–42}$ at the indicated concentrations. TG2 mRNA levels were evaluated by quantitative real-time PCR, after normalization against beta-actin as endogenous control, as described in "Materials and methods". The data represent the mean ± SD from three experiments. *$P < 0.05$, **$P \leq 0.005$, significant values in comparison with controls

RT-PCR analysis of CD14 mRNA levels showed that the stimulation of THP-1 cells with A$\beta_{1–42}$ was able to increase CD14 expression, which was strongly down-regulated upon THP-1 cell transient transfection with TG2 siRNA (Fig. 3a).

Western blot analysis showed that A$\beta_{1–42}$ significantly increased (60%; $P < 0.05$) fibronectin amounts in comparison with control monocytes, while TG2 silencing resulted in a significant reduction (30%; $P < 0.05$) of A$\beta_{1–42}$-induced fibronectin increase (Fig. 3b).

Given the reported secretion of TNF-α as well as matrix metalloproteases (MMPs) by THP-1 monocytes stimulated with Aβ (Chong et al. 2001; Szczepanik et al. 2001), we investigated the possible effects of the siRNA-mediated TG2 silencing on the expression of TNF-α and pro-MMP-9.

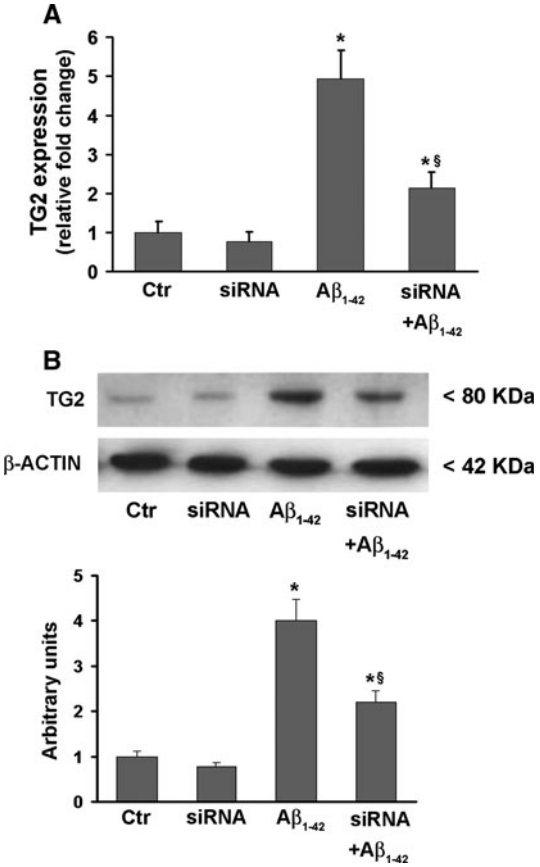

Fig. 2 Effects of siRNA-directed TG2 silencing in THP-1 cells stimulated with A$\beta_{1–42}$. After 24 h of incubation in the presence or absence of siRNA targeting TG2, cells were further incubated until 24 h with 0.5 μM A$\beta_{1–42}$. Then, TG2 expression was evaluated by real-time PCR (**a**) as well as Western blotting and immunoblot densitometric analysis (**b**), after normalization against beta-actin levels. *Columns* and *bars* represent means and standard deviations from triplicate experiments. *$P < 0.05$, significant values in comparison with control cells; $^{\S}P \leq 0.05$, significant values in comparison with A$\beta_{1–42}$ treated cells

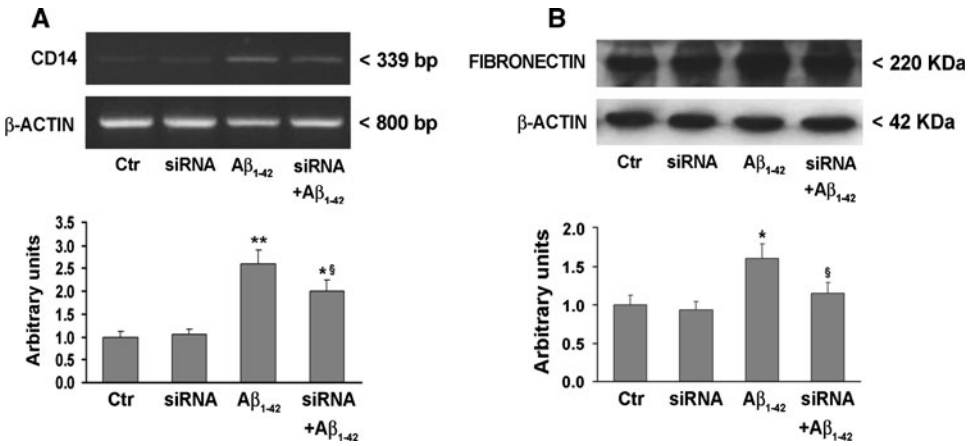

Fig. 3 Effects of TG2 silencing on CD14 and fibronectin expression in THP-1 cells stimulated with A$\beta_{1–42}$. After 24 h of incubation in the presence or absence of siRNA targeting TG2, cells were further incubated until 24 h with 0.5 μM A$\beta_{1–42}$. Then, the presence of CD14 mRNA transcripts was detected by RT-PCR (**a**), while fibronectin protein amounts were examined by Western blotting and immunoblot densitometric analysis (**b**), after normalization against beta-actin levels. Data are representative of three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.005$, significant values in comparison with control cells; $^{\S}P \leq 0.05$, significant values in comparison to A$\beta_{1–42}$ treated cells

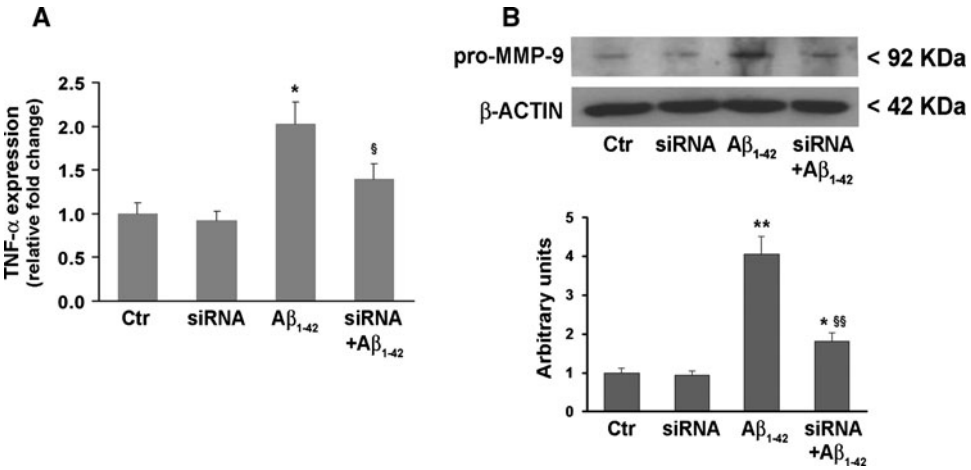

Fig. 4 Effects of TG2 silencing on TNF-α and MMP-9 expression in THP-1 cells stimulated with A$\beta_{1–42}$. After 24 h of incubation in the presence or absence of siRNA targeting TG2, cells were further incubated until 24 h with 0.5 μM A$\beta_{1–42}$. Then, the presence of TNF-α mRNA transcripts was detected by real-time PCR (**a**), while pro-MMP-9 protein amounts were examined by Western blotting and immunoblot densitometric analysis (**b**), after normalization against beta-actin levels. Data are representative of three separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.005$, significant values in comparison with control cells; $^{\S}P \leq 0.05$ and $^{\S\S}P \leq 0.005$ significant values in comparison with A$\beta_{1–42}$ treated cells

TNF-α mRNA levels in A$\beta_{1–42}$-treated cells were two-fold higher than those found in untreated cells (Fig. 4a). The A$\beta_{1–42}$-induced TNF-α up-regulation was reduced ($\sim$30%; $P < 0.05$) after cell transfection with TG2 siRNA (Fig. 4a). In parallel, a fourfold elevation in pro-MMP-9 protein amounts was observed in A$\beta_{1–42}$-stimulated cells compared with control ones (Fig. 4b). A$\beta_{1–42}$-mediated increase in MMP-9 expression was significantly reduced (55%; $P < 0.01$) upon siRNA-directed transcriptional TG2 gene silencing (Fig. 4b).

Discussion

Several in vitro studies as well as clinical trials and epidemiological data have indicated the importance of inflammation in the pathogenesis of AD (Maccioni et al. 2009).

It has been reported that Aβ deposition triggers a pathological inflammatory response, as evidenced by clusters of activated microglial cells and pro-inflammatory cytokines within brain parenchyma. Microglial-mediated inflammatory processes culminate in neuronal loss and cognitive

decline which are a hallmark of AD (Van Eldik et al. 2007).

Activated human THP-1 monocytes are known to release inflammatory factors in response to various stimuli, such as LPS, IFN-γ, and amyloid peptides (Giri et al. 2003).

We first demonstrated that stimulation of THP-1 monocytes with Aβ_{1-42} concentrations (0.1–2 μM) not far from those observed in the plasma of AD patients is able to induce TG2 up-regulation.

This is interesting given that increased TG2 expression and activity have been reported in AD brains (Kim et al. 1999). Moreover, TG2 induction is a specific response of monocytes strictly correlated with the extent of their morphological and functional differentiation (Mehta et al. 1987).

Monocyte maturation plays a relevant role in AD. It has been suggested that Aβ-induced activation and migration of monocytes across the blood–brain barrier, followed by their differentiation into macrophages/microglial cells within the brain parenchyma, initiate the inflammatory process leading to disease progression (Fiala et al. 1998).

Here, we show that Aβ_{1-42} increased the expression of proteins involved in monocyte maturation into macrophages, such as the cell surface antigen CD14 and the adhesion-promoting fibronectin. This was in agreement with previous reports showing that oligomeric Aβ_{1-42} was effective as phorbol myristate acetate at differentiating either cultured peripheral monocytes or THP-1 monocytes, based on cell adhesion and cell surface antigen expression (Fiala et al. 1998; Crouse et al. 2009). However, previously reported effects of Aβ_{1-42} were recorded in different experimental conditions, i.e. after monocyte stimulation up to 7 days (Fiala et al. 1998), or with Aβ_{1-42} concentrations of 15 μM (Crouse et al. 2009).

Cell surface expression of CD14, acting as receptor for the phagocytosis of the Alzheimer's Aβ peptides, is a well established marker of Aβ_{1-42}-induced monocyte activation (Liu et al. 2005). This is interesting given that TG activity has been suggested to participate in Fc receptor-mediated phagocytosis in macrophages (Fesus et al. 1981).

Fibronectin has been reported to mediate cell adhesion through the interaction with TG2 (Akimov and Belkin 2001). Because endothelial cells express fibronectin (Daramola et al. 1997), the up-regulation of TG2 by monocytes is required for adhesion to endothelial cells. Indeed, cell surface-bound TG2 serves as an integrin-associated adhesion co-receptor that may be responsible for the extravasation and migration of monocytes into tissues containing fibronectin matrices during inflammation (Akimov and Belkin 2001).

Under our experimental conditions, siRNA-directed TG2 silencing dramatically reduced Aβ_{1-42}-increased expression of fibronectin as well as CD14, suggesting that TG2 up-regulation is required for Aβ_{1-42}-induced monocyte activation.

Indeed, increased TG2 expression is considered an useful and reliable marker for activation levels in resident and inflammatory macrophages/microglial cells, as it has been shown to contribute to the release of inflammatory factors (Park et al. 2004).

Interestingly, it has recently been reported that TG2 plays a key role in the initiation of inflammation by sequestering PPARγ, a nuclear hormone receptor expressed in monocytes, macrophages, and epithelial cells, which negatively regulates inflammatory gene expression (Maiuri et al. 2008).

In agreement with these observations, we found that TG2 silencing was able to reduce the Aβ_{1-42}-induced TNF-α up-regulation in THP-1 monocytes, indicating a role for TG2 in the regulation of the inflammatory response evoked by Aβ_{1-42} stimulation.

The release of inflammatory mediators, including TNF-α, by immune cells has been shown to greatly modulate the expression of MMPs. In particular, TNF-α has been demonstrated to be one of the strongest physiological inducers of MMP-9 expression as its interaction with fibronectin induces the MMP-9 secretion in peripheral blood monocytes (Vaday et al. 2000; Heidinger et al. 2006). Accordingly, our results indicate that TG2 silencing also reduced the Aβ_{1-42}-evoked increase of pro-MMP-9 in THP-1 monocytes.

Taken together, these results suggest that TG2 might be required for the functional activation of monocytes by Aβ_{1-42}, given that the expression of cell surface markers and adhesion molecules, such as CD14 and fibronectin, as well as pro-inflammatory mediators, such as TNF-α and MMP-9, was found to depend on Aβ_{1-42}-induced TG2 up-regulation.

In conclusion, it is possible to hypothesize that in AD pathogenesis, TG2 expression and activity increase in response to stimulating factors such as Aβ accumulation. In this regard, TG2 inhibition could become a therapeutic target to control inflammation and consequent progression of the disease.

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