Amyloid- β Increases Metallo- and Cysteine Protease Activities in Human Macrophages

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Key Words

Macrophages · Metalloproteinases · Cathepsin S · Transforming growth factor- β 1 · Atherosclerosis

Abstract

Background/Aims: Amyloid- β (A β) plays a crucial role in the onset and progression of atherosclerosis. Macrophages are a source of matrix metalloproteinases (MMPs), cysteine proteases and transforming growth factor (TGF)- β 1 in the vascular wall. The aims of this study were to analyze the capacity of A β peptide (1–40) (A β 40), A β peptide (1–42) (A β 42) and fibrillar A β 42 (fA β 42) to modulate the expression and activity of MMP-9, MMP-2 and tissue inhibitor of MMP-1 (TIMP-1) in human monocyte-derived macrophages (HMDM). Additionally, we analyzed whether A β internalization alters the secretion of cathepsin S (CatS) and TGF- β 1 by macrophages. **Methods:** HMDM were exposed to native and fibrillar A β . MMPs and TIMP-1 expression was analyzed by real-time PCR, and MMP abundance by zymography. Protein levels of precursor and active forms of CatS were analyzed by Western blot and TGF- β 1 levels by ELISA. **Results:** A β 40, A β 42 and especially fA β 42 strongly induced MMP-9/MMP-2 levels. Moreover, we showed enhanced active CatS and reduced TGF- β 1 protein levels in the secretome of A β 42 and fA β 42-

exposed macrophages. **Conclusions:** A β can regulate the proinflammatory state of human macrophages by inducing metallo- and cysteine protease levels and by reducing TGF- β 1 secretion. These effects may be crucial in atherosclerosis progression.

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Introduction

Amyloid- β (A β) peptides refer to amyloid precursor protein (APP)-derived peptides. APP is a transmembrane protein expressed by neurons and astrocytes that is rapidly metabolized by two pathways, namely a nonpathogenic nonamyloidogenic pathway and an A β -synthesizing amyloidogenic pathway [1]. This amyloidogenic pathway is initiated by sequential cleavage of APP through β - and γ -secretases [2]. β -Secretase cleavage occurs 16 amino acid (aa) residues toward the amino terminal of the α -secretase cleavage site, between residues APP671 and APP672, generating soluble APP (sAPP) sAPP β [3]. γ -Secretase cleavage at positions APP712 and APP713 generates 40 aa-long peptides (A β 40), while cleavage at APP714 results in 42 aa-long peptides (A β 42). A β peptides are amphiphilic 38–43 aa peptides as the first 28 aa

residues are polar while the remaining residues are non-polar [4]. These chemical characteristics make these peptides highly prone to aggregation [5, 6]. The accumulation of amyloid fibrils leads to amyloid plaque formation in Alzheimer's disease. However, fibrillar amyloid species are also present in aortic atherosclerotic lesions [7]. In atherosclerotic plaques, amyloid can derive from diverse proteins that lose their native conformation, acquire predominantly β -sheet structure, and increase their propensity to aggregate. These proteins include several members of the apolipoprotein family such as apolipoprotein AI, apoA-I, apolipoprotein AII, apoA-II, apolipoprotein A-IV, ApoA-IV, apolipoprotein E, apoE, and serum amyloid A, as well as α_1 -antitrypsine. The amyloid form localized in the arterial wall derives from an integral fragment of smooth muscle cell-produced lactadherin, termed medin [8], and from Apo-AI derived from the plasma high-density lipoprotein [9]. Besides these proteins, tropoelastin can also give rise to amyloidogenic elastin-derived peptides when it breaks down [10–12]. A β 40 peptides are predominant species in atherosclerotic aortas [13] while both A β 40 and A β 42 are abundant in the brain. A β 42 is less soluble and is present in all types of senile plaques, whereas A β 40 is the major species deposited in the cerebral blood vessels [14–17]. A β interacts with a wide variety of proteins including APP, NMDA receptors, integrins, α_7 nicotinic acetylcholine receptors, α_7 nAChR, p75 neurotrophin receptors, p75NTR, collagen-like Alzheimer amyloid plaque component precursor/collagen type XXV, CLAC-P/ColXXV, the receptor for advanced glycosylation end products, RAGE, serpin-enzyme complex receptor, SEC-R, insulin receptors, scavenger receptors and heparan sulfate proteoglycans [18]. Most of these interactions are pathological for the cells. It has been previously reported that macrophages have the ability to internalize and degrade A β in an apoE-facilitated manner [19]. These authors suggested that macrophage-mediated A β degradation is, in part, mediated by secretion of matrix metalloproteinase-9 (MMP-9). The degradation of A β by macrophages is an essential physiological mechanism to limit A β accumulation within brain parenchyma and blood vessels [20]. However, an excess of metalloproteinase activity may contribute to extracellular matrix remodeling and the onset of acute thrombotic complications [21–24]. Among the proteases, MMP-9 and MMP-2 have the capacity to cleave integral membrane APP to form A β peptides [20]. In addition, other proteases that are abundant in atherosclerotic lesions, including cathepsins B, K, L and S may cleave apolipoproteins to generate intermediate-sized fragments

that form amyloid [25]. Macrophages express cathepsin S (CatS) [26, 27] and intracellular CatS activity increases in response to proinflammatory cytokines such as interferon- γ -modulating MHC class II maturation [28, 29]. Remarkably, CatS levels are reportedly increased in atherosclerosis [30]. Reduced transforming growth factor (TGF)- β 1 activity/signaling is also a feature of atherosclerosis, as evidenced by low TGF- β levels in vessel walls [31, 32] and in the plasma of patients with coronary artery disease [33, 34]. On the basis that amyloid is present in atherosclerotic plaques and that macrophage-derived elastases in combination with MMPs play a crucial role in vascular extracellular matrix remodeling and atherosclerotic plaque stability, the aim of this study was to analyze the effect of A β peptides – A β (1–40) (A β 40), A β (1–42) (A β 42) and fibrillar A β 42 (fA β 42) – on the expression and activity of MMP-9, MMP-2 and tissue inhibitor of MMP-1 (TIMP-1) in human monocyte-derived macrophages (HMDM). Additionally, we analyzed the effect of A β peptides on the levels of secreted active CatS and TGF- β 1.

Methods

A β 40, A β 42 and fA β 42 Preparation and Characterization

A β 40 and A β 42 were purchased from rPeptide (Bogart, Ga., USA). For the solubilization of A β 40 and A β 42, lyophilized A β peptides were dissolved at 10 mg/ml concentration in DMSO and then directly diluted into cell culture medium to the final treatment concentration. fA β 42 was prepared by incubating freshly solubilized A β 42 peptide at 10 μ M in cell culture medium at 37°C for 7 days [35, 36]. For transmission electron microscopy, A β preparations were negatively stained with 2% uranyl acetate for 1 min and were observed under a Hitachi 600-AB transmission electron microscope (Hitachi High Technologies America, Dallas, Tex., USA). Images were digitalized with a Gatan BioScan camera (Gatan, Pleasanton, Calif., USA).

Isolation and Differentiation of HMDM

Monocytes were obtained by standard protocols from buffy-coats (35–40 ml) of healthy donors procured from the Blood and Tissue Bank of Barcelona. Cells were applied on 15 ml of Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Little Chalfont, UK) and centrifuged for 45 min at 400 g and 22°C with no break. In each experiment, macrophages were isolated from 2 different individuals and mixed. The same experimental setting was repeated three times (a total of 6 independent healthy subjects) with duplicates for each condition. Mononuclear cells were obtained from the central white band of the gradient, exhaustively washed in phosphate-buffered saline, and resuspended in RPMI medium (Gibco, Life Technologies, Carlsbad, Calif., USA) supplemented with 10% human serum AB (Lonza Group, Basel, Switzerland), 1% HEPES (Gibco) and antibiotics (Invitrogen, Life Technologies, Carlsbad, Calif., USA). Human serum AB was obtained from hu-

man plasma blood type AB collected from healthy donors at FDA-licensed facilities located entirely within the USA. The fraction of mononuclear cells isolated by this procedure from fresh blood has been described to be more than 99% pure [37]. Monocytes were allowed to differentiate into macrophages by addition of complete medium with 10% human serum AB for 5 days. HMDM were serum deprived several times, depending on the experimental set, before the incubation with DMSO alone (control) or with different concentrations of native or fibrillar A β 40 or A β 42. HMDM were then exhaustively washed and collected for mRNA/protein extraction. Cell culture media samples were stored at -20°C for determination of TGF- β 1 concentrations.

Immunocytochemistry

HMDM were seeded onto glass coverslips, serum deprived, exposed to fluorescently-labeled fA β 42 (N-terminal FAM-labeled A β 42; AnaSpec, Fremont, Calif., USA) $5\text{ }\mu\text{M}$ for 4 h, and finally fixed with 4% paraformaldehyde to analyze macrophages fA β 42 internalization. Solubilization of lyophilized FAM-labeled A β 42 and the obtainment of fibrillar forms were assessed as described above with unlabeled A β peptides. Images of immunostained cells were analyzed on a Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany) with a 63×1.4 oil immersion objective and processed with the Leica Standard Software TCS-AOBS.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from cultured HMDM cells using TriPureTM isolation reagent (Roche Applied Science, Rotkreuz, Switzerland) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was eluted in $15\text{ }\mu\text{l}$ of nucleases-free water. RNA yield and quality were assessed by agarose gel electrophoresis and spectrophotometry, and then stored at -80°C until used. RNA was digested with DNase I (Invitrogen). For cDNA synthesis, $1.5\text{ }\mu\text{g}$ of total RNA was used according to the protocol provided with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad, Calif., USA). Recombinant RNasin Ribonuclease Inhibitor (Applied Biosystems) was added to prevent RNase-mediated degradation. The cDNA was stored at -20°C .

Determination of Gene Expression by Real-Time PCR

Gene expression analyses of MMP-9, MMP-2, TIMP-1 and CatS mRNA were performed at mRNA level by quantitative real-time reverse transcriptase PCR. We used the following assays-on-demand (Applied Biosystems): MMP-9 (Hs00234597_m1), MMP-2 (Hs00234422_m1), TIMP-1 (Hs01092511_m1) and CatS (Hs00175407_m1). 18S rRNA (4319413E) was used as a housekeeping gene. We mixed $2\text{ }\mu\text{l}$ of single-stranded cDNA with $2\text{ }\mu\text{l}$ of $20\times$ TaqMan Gene Expression Assays for each assay-on-demand, $25\text{ }\mu\text{l}$ of TaqMan Universal PCR Master Mix (Applied Biosystems), and $21\text{ }\mu\text{l}$ of nucleases-free water. After gentle mixing, the mixture was transferred into a real-time PCR microplate. The real-time PCR microplate was sealed, centrifuged, and then placed in the sample block of an Applied Biosystems 7900 HT real-time PCR System. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Expression levels were measured in triplicate. The threshold cycle (Ct) values were normalized to the housekeeping gene.

Western Blot Analysis

Blots were incubated with monoclonal antibodies against human CatS (sc-271619; Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA). CatS (E-3) is a mouse monoclonal antibody specific for an epitope mapping between aa 302–331 at the C-terminus of CatS of human origin and recognizes precursor (37 kDa) and active (24 kDa) forms. Phosphorylation state of signaling proteins including Pyk2 and ERK1,2 was analyzed by incubating blots with monoclonal antibodies against phospho-Pyk2 (p-Pyk2, No. 3291; Cell Signaling Technology, Danvers, Mass., USA) or against phospho-ERK1,2 (p-ERK1,2, No. 3480; Cell Signaling Technology). Blots were also exposed to total Pyk2 (No. 9101; Cell Signaling Technology) and total ERK1,2 (Assay Designs, KAP-MA001).

Zymography

MMP abundance was measured by gelatinolytic zymography of the cell culture medium. The cell medium was dialyzed against $0.01\text{ M NH}_4\text{HCO}_3$ at 4°C , lyophilized, and dissolved in RIPA buffer (150 mM NaCl , 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA , $50\text{ mM Tris-HCl pH }8$) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science). Equal amounts of supernatants were subjected to SDS-PAGE in 10% polyacrylamide (Bio-Rad, Berkeley, Calif., USA) gels containing 1 mg/ml porcine skin type A gelatin (Sigma-Aldrich, St. Louis, Mo., USA) at 4°C . After electrophoresis, the substrate gels were soaked twice with 2.5% Triton X-100 (Sigma-Aldrich) for 15 min each at room temperature to remove SDS. The gels were then incubated in 50 mM Tris-HCl , 10 mM CaCl_2 , and 0.02% N_3Na for 20 h at 37°C . Gel staining was performed with 0.5% Coomassie Brilliant Blue R-250 (Thermo Scientific Pierce, Rockford, Ill., USA) and then destained in 50% 2-propanol, 1% acetic acid (Panreac, Barcelona, Spain). Clear bands against the blue background indicated the presence of MMP abundance measured by densitometric scanning. Bands were quantified with a GS-800 calibrated imaging densitometer (Bio-Rad) using the Quantity-One 1-D Analysis Software (Bio-Rad). Results are expressed as arbitrary units that refer to units of intensity $\times$ millimeters.

Determination of TGF- β 1 Levels in Cell Culture Media Samples from Human Macrophages

Cell culture media samples were thawed, centrifuged and used for determination of TGF- β 1 levels by ELISA (DGR Diagnostics, Marburg, Germany) according to the manufacturer's instructions. In brief, the neutralized standards and samples were added to the antibody-coated (polyclonal) microtiter wells. After the first incubation, the unbound sample material was removed by washing. Then a monoclonal mouse anti-TGF- β 1 antibody, a biotinylated anti-mouse IgG antibody and the Streptavidin-HRP enzyme complex were incubated in succession. An immuno-enzyme sandwich complex was formed. After incubation, the unbound conjugate was washed off. Having added the substrate solution, the intensity of color developed was proportional to the concentration of TGF- β 1 in the sample.

Data Analysis

Data were expressed as mean $\pm$ SEM. A StatView (Abacus Concepts, Piscataway, N.J., USA) statistical package for the Macintosh computer system was used for all the analyses. Multiple groups were compared by ANOVA or Wilcoxon test as needed. Statistical significance was considered when $p < 0.05$.

Fig. 1. Negative staining electron microscopy images of fibrillar A β 42 (fA β 42, 5 μ M; **a**) and freshly dissolved A β 42 (5 μ M; **b**). **c** Confocal microscopy image of HMDM exposed to FAM-fA β 42 (5 μ M) for 18 h. Cytoplasmatic fA β 42 is shown in green and nuclei are shown in blue (colors refer to the online version only).

Results

Effects of A β on MMP-9 and MMP-2 Expression and Activity in Human Macrophages

Uranyl acetate-negative images from electron microscopy revealed that fresh A β 42 was transformed into insoluble amyloid fibrils after 7 days at 37°C (fig. 1a). In native preparations, filaments were rarely found and they were extremely small and thin (fig. 1b). Confocal microscopy experiments showed that FAM-labeled fA β 42 (5 μ M, time 4 h) was avidly taken up by human macrophages (fig. 1c). Real-time PCR experiments showed that A β 40 and A β 42 (5 μ M, 48 h) increased MMP-9 mRNA to 1.99- and 2.06-fold, respectively (fig. 2a). Zymography experiments (fig. 2b) showed that A β 40 and A β 42 (5 μ M, 48 h) strongly increased MMP-9 abundance to 2.31-fold and to 2.76-fold, respectively. MMP-9 levels were not altered by exposure of HMDM to A β 40 or A β 42 for 12 h (data not shown).

A β 40 increased MMP-2 mRNA expression and abundance by approximately 2-fold independently of the tested dose (fig. 2c, d). In contrast, A β 42 increased MMP-2 mRNA expression in a dose-dependent manner, from 1.64-fold at 0.01 μ M to 3.13-fold at 5 μ M (fig. 2c). In agreement with real-time PCR results, zymography experiments (fig. 2d) showed that MMP-2 abundance was strongly increased by 4.73-fold in the presence of the highest A β 42 dose (5 μ M). MMP-2 mRNA expression and abundance was not altered by exposure of HMDM to A β 40 or A β 42 for 12 h (data not shown). TIMP-1

mRNA expression was significantly decreased by the highest doses (1 and 5 μ M, 48 h) of A β 40 and A β 42 (fig. 2e) without differences between the effect of A β 40 and A β 42.

Since insoluble amyloid fibrils are crucial in the pathogenesis of Alzheimer's disease, we compared the effects of soluble and insoluble A β peptides on MMP expression and activation. Our results show that fA β 42 exerted a stronger upregulatory effect than A β 42 on both MMP-9 mRNA expression (2.9- vs. 2.3-fold, $p < 0.01$) and MMP-9 abundance (10.3- vs. 6.7-fold, $p < 0.01$; fig. 3a, b). There were no significant differences between the effect of A β 42 and fA β 42 on MMP-2 mRNA expression or abundance (fig. 3c, d). A β 42 and fA β 42 also exerted a similar down-regulatory effect on TIMP-1 mRNA expression in human macrophages (fig. 3e).

Effect of A β on Macrophage-Secreted Active CatS

We explored the distribution of CatS in control and A β -exposed macrophages. The precursor CatS form (37 kDa) was detected in the cell-culture media but not in the whole macrophage lysates (fig. 4a). This secreted CatS precursor form was not altered by A β . In contrast, the 24-kDa active CatS form, detected in both cell lysates and cell culture media, was significantly altered by A β (fig. 4b). While A β 42 and fA β 42 (5 μ M, 48 h) significantly decreased the active cellular CatS, both A β 42 and fA β 42 significantly increased the active secreted CatS. CatS mRNA levels were not significantly altered by macrophage exposure to A β (data not shown).

Fig. 2. Effect of Aβ peptides on MMP-9 and MMP-2 expression and abundance in human macrophages. HMDM were treated with increasing doses (0.01, 1 and 5 μM) of Aβ40 and Aβ42 for 48 h. MMP-9 (a), MMP-2 (c) and TIMP-1 (e) mRNA expression, and MMP-9 (b) and MMP-2 (d) abundance was analyzed by real-time PCR and zymography, respectively. Real-time PCR data were processed with a specially designed software program based on Ct values of each sample and normalized to 18S rRNA. Zymography bands were quantified and expressed as arbitrary units. Results are shown as mean ± SEM of three independent experiments performed in duplicate. * p < 0.05 versus control cells (incubated in absence of Aβ); # p < 0.05 versus Aβ40-exposed HMDM.

Fig. 3. Comparative effect of Aβ42 and fAβ42 on MMP-9 and MMP-2 expression and abundance in human macrophages. HMDM were treated with Aβ42 and fAβ42 (5 μM) for 48 h. MMP-9 (a), MMP-2 (c) and TIMP-1 (e) mRNA expression, and MMP-9 (b) and MMP-2 (d) abundance was analyzed by real-time PCR and zymography, respectively. Real-time PCR data were processed

with a specially designed software program based on Ct values of each sample and normalized to 18S rRNA. Zymography bands were quantified and expressed as arbitrary units. Results are shown as mean ± SEM of three independent experiments performed in duplicate. * p < 0.05 versus control cells (incubated in absence of Aβ42); # p < 0.05 versus Aβ42-exposed HMDM.

Effect of Aβ on Pyk2 and ERK1,2 Phosphorylation

Western blot analysis showed that Aβ induced a sustained phosphorylation of Pyk2 at 48 h (fig. 5a) in human macrophages. fAβ42 exerted a stronger upregulatory effect than Aβ42 on Pyk2 phosphorylation (3- vs. 2-fold, p < 0.01). Total Pyk2 was not significantly altered by Aβ42 or fAβ42 in human macrophages. ERK1,2 phosphorylation was also sustainably upregulated by Aβ at 24 h (fig. 5b) and the effect of fAβ42 was higher than that of

Aβ42 (3.25- vs. 2.05-fold, p < 0.01). Total ERK1,2 protein levels were not affected by Aβ42 or fAβ42.

Effect of Aβ on Secreted TGF-β1 Levels

Both native and fibrillar forms of Aβ40 and Aβ42 at 5 μM significantly reduced TGFβ1 levels in the secretome of human HMDM at 12 h. At 48 h, fAβ42 exerted much higher downregulatory effect on secreted TGF-β1 levels than the other Aβ forms (fig. 6).

Fig. 4. Distribution of CatS in intracellular and extracellular fractions from control and HMDM exposed to Aβ42 or fAβ42. HMDM were exposed to Aβ42 or fAβ42 (5 μM, 48 h) and collected for protein analysis. Representative Western blot analysis showing the precursor (a) and mature CatS (b) protein bands in HMDM and HMDM culture medium. Bar graphs show the quantification of

precursor (a) and mature CatS (b) bands in control and Aβ-exposed HMDM (grey bars) and HMDM culture medium (black bars). Data are expressed as mean ± SEM of three independent experiments performed in duplicate. * $p < 0.05$ versus control HMDM.

Fig. 5. Comparative effect of Aβ42 and fAβ42 on Pyk2 (a) and ERK1,2 (b) phosphorylation in human macrophages. HMDM were treated with Aβ42 and fAβ42 (5 μM) for 48 h. Representative Western blot analysis showing LRP1, p-Pyk2, total Pyk2, p-ERK1,2 and total ERK1,2. Bar graphs represent quantification of specific

p-Pyk2 and p-ERK1,2 bands normalized by total Pyk2 and total ERK1,2, respectively. Results from two experiments performed in triplicate are expressed as mean ± SEM. * $p < 0.05$ versus control cells (incubated in absence of Aβ42); # $p < 0.05$ versus Aβ42-exposed HMDM.

Fig. 6. Effect of A β peptides on macrophage-secreted TGF- β 1 levels. HMDM were exposed to native and fibrillar forms of A β (5 μ M) for 12 and 48 h. Cell culture medium was collected and extracellular TGF- β 1 levels were determined by ELISA. Results are expressed as pg/ μ g cell protein and shown as mean $\pm$ SEM of four independent experiments performed in duplicate. * $p < 0.05$ versus control cells (incubated in absence of A β); # $p < 0.05$ versus A β 42-exposed HMDM.

Discussion

The main finding of the present study is that A β strongly impacts macrophage metallo- and cysteine proteolytic activities and macrophage-TGF- β 1 secretion. Our study shows that A β contributes to increase the macrophage-proinflammatory phenotype by increasing the levels of MMP-9/MMP-2 and CatS, and by reducing the levels of TGF- β 1 in the secretome of macrophages.

Results from the present study show that human macrophages can avidly internalize A β and that this process has an effect on MMP-9 activation, in agreement with previous results in mice peritoneal macrophages [19]. However, our study shows that in human macrophages, in contrast to the results in mouse peritoneal macrophages, A β peptides exerted an upregulatory effect not only on MMP-9, but also on MMP-2 expression and abundance. In fact, A β peptides reportedly induce MMP-9/MMP-2 activation in mixed rat hippocampal and enriched astrocyte cultures [38], but at higher dose (20 μ M) than that used in our study (5 μ M). In fact, we observed very little dose dependence and a strong effect at the low-

est tested dose (10 nM) in our time-course experiments. These results suggest that human macrophages may have a limited capacity to internalize and degrade A β . In line with this hypothesis, it has been previously reported that adult microglia has lower capacity to take up and phagocytose A β [39, 40]. In the present study, we also showed that A β negatively modulated TIMP-1 mRNA expression in HMDM. These results differ to those showing a positive effect of A β peptides on TIMP levels in rat astrocytes [38]. Interestingly, the imbalance of MMPs over their tissue inhibitors in HMDM exposed to A β has been previously described in vascular cells exposed to other proatherosclerotic stimuli such as aggregated low-density lipoprotein [41] and also in tumoral cells [42]. Our data revealed that fA β 42 was more efficient than A β 42, and A β 42 more efficient than A β 40 at increasing both MMP-9 and MMP-2 abundance, suggesting that less soluble A β forms had higher ability to induce MMPs in human macrophages. In the present study we also showed that A β activates Pyk2 and ERK1,2 phosphorylation in macrophages, as previously described in THP-1 cells [43], and human cortical neuronal cultures [44]. Previous studies have shown that Pyk2 phosphorylation may be involved in ERK1,2 activation [45, 46] and that the Pyk2-ERK1,2 signaling axis is involved in crucial processes such as cell proliferation and migration [45–47]. In relation to migration, previous studies reported the involvement of Pyk2/ERK1,2 on MMP-3 [48] and MMP-13 [49] activation by fibronectin fragments in chondrocytes. Further studies are required to know whether Pyk2/ERK1,2 axis are also involved in the upregulatory effect of A β on MMP-9/MMP-2 expression and activity. The induction of metalloproteinase activity in macrophages reportedly induces atherosclerotic plaque disruption in different animal models [22]. Moreover, several studies in humans showed that MMP-9 overexpression strongly correlates with plaque instability and clinical manifestations of atherosclerosis [23–24]. Our study points to A β as a critical inducer of both MMP-9 and MMP-2 expression and activation in human macrophages. Amyloid-like structures which accumulate in atherosclerotic lesions [7] induced protease activity in macrophages. Therefore, amyloid may be a potent contributor to extracellular matrix degradation linked to plaque rupture.

Cathepsins or lysosomal cysteine proteases, like other extracellular proteases, participate in extracellular matrix degradation in atherosclerosis. In atherosclerotic plaques, CatS and K are overexpressed [50]. Moreover, CatS has been previously localized in intimal infiltrated macrophages [51]. CatS is localized on cell membranes but can

also be secreted to the extracellular space. Results from the present study show that A β enhanced active CatS levels in the secretome of human macrophages. However, A β did not alter CatS mRNA expression, at least at 48 h of HMDM exposure to A β . These results may be explained by time-course differences in the A β modulation of CatS mRNA expression and CatS release in human macrophages. Previous studies have reported that macrophage-derived CatS in collaboration with MMP-9 degrade medial elastin [52]. Consistent with the elastolytic properties of CatS, CatS-deficient animals showed reduced fragmentation of elastic lamina in aortas of atherosclerotic lesions [30]. Elastin fragmentation reportedly induces fibrillar A β formation [10–12]. Our results thus highlight the crucial role of A β on extracellular matrix degradation and support the notion that the capacity of A β to induce metallo- and CatS activation in macrophages can create a positive feedback loop for A β generation in the vascular wall, continuing directly in extracellular matrix degradation, plaque rupture and evolution of atherosclerosis to thrombosis. Results from the present

study also highlight the crucial role of A β peptides in the secretion of TGF- β 1 by human macrophages. Since macrophages are one of the main cellular sources of TGF- β 1, the exposure of macrophages to A β may be, at least in part, the cause of TGF- β 1 reduction in advanced atherosclerotic plaques [31, 32]. In summary, our results highlight the crucial role of A β on the modulation of macrophage protease activation and TGF- β 1 secretion, two key processes in plaque stability.

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