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Inhibition of transglutaminase 2 reduces efferocytosis in human macrophages: Role of CD14 and SR-AI receptors

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Scavenger receptor SR-AI

Abstract *Background and aims:* Transglutaminase 2 (TGM2), a member of the transglutaminase family of enzymes, is a multifunctional protein involved in numerous events spanning from cell differentiation, to signal transduction, apoptosis, and wound healing. It is expressed in a variety of cells, macrophages included. Macrophage TGM2 promotes the clearance of apoptotic cells (efferocytosis) and emerging evidence suggests that defective efferocytosis contributes to the consequences of inflammation-associated diseases, including atherosclerotic lesion progression and its sequelae. Of interest, active TGM2 identified in human atherosclerotic lesions plays critical roles in plaque stability through effects on matrix cross-linking and TGFβ activity. This study explores the mechanisms by which TGM2 controls efferocytosis in human macrophages.
Methods and results: Herein we show that TGM2 increases progressively during monocyte differentiation towards macrophages and controls their efferocytic potential as well as morphology and viability. Two experimental approaches that took advantage of the inhibition of TGM2 activity and protein silencing give proof that TGM2 reduction significantly impairs macrophage efferocytosis. Among the mechanisms involved we highlighted a role of the receptors CD14 and SR-AI whose levels were markedly reduced by TGM2 inhibition. Conversely, CD36 receptor and $\alpha_v\beta_3$ integrin levels were not influenced. Of note, lipid accumulation and IL-10 secretion were reduced in macrophages displaying defective efferocytosis.
Conclusion: Overall, our data define a crucial role of TGM2 activity during macrophage differentiation via mechanisms involving CD14 and SR-AI receptors and show that TGM2 inhibition triggers a pro-inflammatory phenotype.

Introduction

Transglutaminases are a superfamily of isoenzymes found in cells and plasma (Factor XIII) that catalyze the post-translational modification of proteins by transamidation of available glutamine residues. In particular, transglutaminase 2 (TGM2), besides the transamidation or deamination activity, binds and hydrolyses GTP, possesses protein disulfide isomerase and protein kinase activity and may irreversibly cross-link proteins of the extracellular matrix (ECM). TGM2, due to its widespread localisation within the cell is implicated in a variety of events spanning from cell proliferation/differentiation, signal transduction and apoptosis, to wound healing [1]. It is widely expressed in vascular cells and macrophages [2] and emerging

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evidence has documented diverse roles in cardiovascular physiopathology, including vascular remodeling, atherosclerosis and arterial stiffening [3,4]. Interestingly enough, increased active TGM2 has been evidenced in human carotid and coronary artery atherosclerosis with potential implications for plaque stability [5,6].

Macrophages play key roles in many aspects of human physiology and pathology. They are heterogeneous both in term of morphology and function and can exert beneficial or detrimental effects depending on activation status determined from external cues received from microenvironment and ECM. Macrophage heterogeneity has been extensively reported within the atherosclerotic lesions where distinct subsets exert opposite functions driving plaque progression or stability [7]. In particular, a severe macrophage defect in the clearance of apoptotic cells has been linked to progression of advanced atherosclerotic lesions [8].

TGM2 is among the molecules that orchestrates the engulfment of apoptotic cells (efferocytosis), a role well evidenced by studies carried out in TGM2$^{-/-}$ mice that display abnormal inflammatory responses [9,10].

Prior studies from our group showed that human blood-derived monocytes spontaneously differentiated *in vitro* (MDMs) generate two dominant macrophage subsets, distinct for morphology (spindle and round), transcriptional profiles and proteome. TGM2 was more abundant in round MDMs that are lipid-enriched and possess the functional traits reminiscent of a non-inflammatory and reparative phenotype [11,12].

Herein, taking advantage of this model that proved suitable to address macrophage heterogeneity, we focus on defining the role of TGM2 in efferocytosis and related events. We report that TGM2 inhibition during spontaneous differentiation of blood-derived monocytes toward macrophages decreases efferocytosis turning on a pro-inflammatory phenotype.

Methods

Macrophage culture

The study was approved by the Local Research Ethics Committee and conducted according to the principles expressed in the Declaration of Helsinki. Blood from healthy donors who did not receive any medication for at least 2 weeks before the sampling was collected, after informed consent. Mononuclear cells were isolated and plated as described [13]. Adherent monocytes were cultured for 7 days in medium M199 (Lonza, EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine (Lonza, EuroClone, Milan, Italy), 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza, EuroClone, Milan, Italy), and 10% freshly obtained autologous serum. Medium was not replaced during culture. Differentiation was carried out with or without the transglutaminase competitive inhibitors cystamine (Sigma–Aldrich, Milan, Italy), monodansylcadaverine (Sigma–Aldrich, Milan, Italy) or Z-DON (Zedira, Darmstadt, Germany), and with or without the nonlipoprotein ligand for SR-A fucoidan (Sigma–Aldrich, Milan, Italy).

THP-1 culture and differentiation to macrophage-like cells

THP-1 cells (ATCC, LGC Standards S.r.l., Milan, Italy) were cultured in RPMI-1640 medium (Lonza, EuroClone, Milan, Italy) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (EuroClone, Milan, Italy). After seeding, macrophage-like cells were obtained by THP-1 exposure to 250 nM phorbol ester (PMA, Sigma–Aldrich, Milan, Italy) for 72 h.

Morphology and cytotoxicity

MDM morphology was examined by phase contrast microscopy (AxioVert 200M, Zeiss, Milan, Italy) at 20× or 40× magnification and cell viability, measured by neutral red, was calculated as follows: relative viability = [(Ae − Ab)/(Ac − Ab)] × 100. Ab: background absorbance, Ae: experimental absorbance, Ac: absorbance of cells differentiated in the absence of cystamine.

Western blot

Western analysis was carried out as described [13]. Membranes were incubated with primary mAbs against TGM2 (Cell Signaling, EuroClone, Milan, Italy) and factor XIII (Abcam, Prodotti Gianni S.r.l., Milan, Italy), and subsequently with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, Li-StarFISH, Milan, Italy). β-Actin was used for control of protein loading.

Immunofluorescence staining

Adherent MDMs were fixed in 2% para-formaldehyde and non-specific reactive sites were blocked (30 min, RT) with 5% bovine serum albumin solution containing 0.1% saponin. MDMs were stained over-night at 4 °C with mAb anti-CD68 (Abcam, Prodotti Gianni S.r.l., Milan, Italy) or mAb anti-TGM2 (Cell Signaling, EuroClone, Milan, Italy). Alexa Fluor488-conjugated goat IgG (Life Technologies, Monza, Italy) was used as secondary antibody. Nuclei were stained (10 min at RT) with 1 μg/ml Hoechst 33258 (Sigma Aldrich, Milan, Italy). Fluorescent images were captured by Apotome fluorescence microscope (Zeiss S.p.A., Milan, Italy) and data expressed as mean fluorescence intensity (Arbitrary Fluorescent Units, AFU)/μm^2, subtracted of the negative control value obtained in the absence of primary antibody. Multiple fields of view (at least three fields, 400× magnification) were captured for each culture.

Flow cytometry

MDMs were detached with trypsin and stained for 15 min at RT with mAbs against CD34 (FITC), CD14 (APC), $\alpha_v\beta_3$ (PE), CD36 (FITC), SR-AI (PE). Isotype-matched irrelevant

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