Immunology Letters 126 (2009) 22–28

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Over-expression of integrin β3 can partially overcome the defect of integrin β3 signaling in transglutaminase 2 null macrophages

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ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 10 July 2009
Accepted 20 July 2009
Available online 28 July 2009

Keywords:
Macrophages
Apoptotic cells
Transglutaminase 2
Integrin signaling

ABSTRACT

Transglutaminase 2 (TG2) is a protein crosslinking enzyme with many additional biological functions. We have previously shown that in $TG2^{-/-}$ mice the *in vivo* clearance of apoptotic cells is defective leading to autoimmunity. TG2 contributes to the formation of phagocytic portals by binding to both integrin β_3, a known phagocytic receptor, and its bridging molecule, MFG-E8. In TG2 null macrophages integrin β_3 cannot accumulate around the apoptotic cells and its signaling is impaired. In the present study we describe a subline of TG2 null mice, in which a compensatory increase in integrin β_3 expression, which resulted alone in a high receptor concentration around the apoptotic cells without the requirement for accumulation, partially corrected the defect in integrin β_3 signaling. Our data provide a proof for the concept that the function of TG2 is to stabilize accumulated integrin β_3 concentration in the phagocytic cup.

1. Introduction

Apoptotic cells are generated by diverse physiological processes, ranging from the elimination of damaged (or precancerous) cells to deletion of cells during developmental morphogenesis [1]. The culmination of the apoptotic program *in vivo* is the phagocytosis of the apoptotic cell. In mammals prompt removal of apoptotic cells is required to prevent the release of potential self-antigens and the onset of autoimmune-like syndromes [2].

A number of receptors have been identified on macrophages that either directly or indirectly facilitate apoptotic cell recognition and uptake [3]. These receptors converge on two evolutionally conserved pathways upstream to the activation of the low molecular weight GTPase Rac1, which is obligatorily required for the uptake [4]. One of these receptors is the integrin $\alpha_v\beta_3$ (vitronectin receptor), which is bridged via the milk fat globulin EGF factor 8 (MFG-E8) molecule to the phosphatidylserine appearing on apoptotic cells [5]. The integrin β_3 pathway regulates Rac1 activity via the 180 kDa protein downstream of chicken tumor virus no. 10 (CT10) regulator kinase II (Dock180) and the engulfment and migration protein (ELMO), which form together an unconventional two-part guanine nucleotide exchange factor for Rac1 [6]. The formation of the DOCK180/ELMO complex is induced in the integrin β_3 pathway by the Trio-controlled RhoG-GTP [7]. Rac activation then leads to the formation of an actin-rich phagocytic cup, followed by the internalization of the target [8].

In addition to its role in the phagocytosis of apoptotic cells, the RhoG-mediated pathway also participates in cell migration, and is transiently activated wherever lamellopodias are formed [9] resulting in the formation of a basal Rac-GTP level in the continually migrating macrophages.

Transglutaminases are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in target proteins [10]. Eight distinct enzymatically active transglutaminases have so far been described [11]. TG2 is very unique among the TG family members, because besides catalyzing the formation of protein crosslinks, it is also a G protein, and possesses protein disulfide isomerase and protein kinase activities [12]. In addition, TG2 also interacts with integrins of the β_1 and β_3 subfamilies, and integrin/TG2 complexes are detected inside the cell during biosynthesis and accumulate as coreceptors on the cell surface [13,14]. TG2 can also bind to the major extracellular protein

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doi:10.1016/j.imlet.2009.07.009

fibronectin [15], and cooperate with integrins in cell adhesion and motility through either a direct noncovalent interaction with the β_1 and β_3 integrin subunits or formation of stable ternary complexes with integrins and fibronectin [13,14]. These interactions induce integrin clustering and modify integrin signaling [16].

We have previously reported that TG2$^{-/-}$ mice develop an age-dependent autoimmunity due to defective *in vivo* clearance of apoptotic cells [17]. Recently we found that TG2 expressed on the cell surface of macrophages promotes the engulfment of apoptotic cells by forming a complex with both integrin β_3 and MFG-E8 [18]. In the absence of TG2 the apoptotic cell-induced integrin β_3 signaling leading to RhoG and Rac1 activation is impaired. Additionally, we also described that the uptake of apoptotic cells by wild-type macrophages occurs via one or two phagocyte portals that are characterized by the accumulation of phagocytic receptors in the phagocytic cup and concentration of Rac molecules to this pole of the macrophages. In the absence of TG2 both the formation and the uptake rate of these portals are less efficient than that of wild-type macrophages. TG2 null macrophages try to compensate the loss of TG2 by increasing the expression of both integrin β_3 and RhoG [18]. Here we describe a subline of TG2 null mice, in which a strong compensatory increase in integrin β_3 expression was able to correct partially the defect in integrin β_3 signaling.

2. Materials and methods

2.1. Antibodies and reagents

Purified mouse anti-Rac1 monoclonal (clone 102), and phycoerythrine (PE)-conjugated anti-mouse β_3 integrin (clone 2C9.G2) antibodies were purchased from BD Pharmingen (San Diego, CA). 6-Carboxy-3′,6′-diacetylfluorescein (CFDA), 5-(and 6-)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR), Alexa 647-conjugated goat anti-mouse IgG, and Prolong antifade reagent were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse IgGs, anti-mouse IgG-FITC, and vitronectin were purchased from Sigma–Aldrich (Budapest, Hungary). Anti-RhoG (clone C-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence for immunoblots analysis was purchased from Millipore (Millipore, Billerica, MA).

2.2. Cell culture

TG2$^{+/+}$ and TG2$^{-/-}$ [19] mice were injected with 2 ml 4% thioglycollate and 4 days later macrophages were obtained by peritoneal lavage. Macrophages were allowed to adhere in 24-well plates and non-adherent cells were washed away. For phagocytosis assays, macrophages were stained overnight with CMTMR (10 μM). Thymocytes from 4 weeks old WT mice were used as apoptotic target cells. Thymi were mechanically disrupted, and isolated thymocytes were labeled overnight with CFDA (6 μM). To induce apoptosis cells were treated with 4 μM ionomycin (Sigma–Aldrich) for 6 h, at which time, 40–50% of thymocytes were Annexin V positive (*i.e.* apoptotic), and less than 5% of Annexin V positive cells were propidium iodide positive (*i.e.* necrotic). Thymocytes and macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin and streptomycin (Gibco, Grand Island, NY). The study protocol was approved by Animal Care Committee of University of Debrecen.

2.3. Phagocytosis assay

CMTMR-stained macrophages were incubated with apoptotic thymocytes labeled with CFDA in 40:1 target/macrophage ratio for 1 h. Cells incubated with apoptotic thymocytes incubated at 4 °C were used as controls. After washing, the cells on the plate were trypsinized, resuspended in cold medium with 0.5% sodium azide, and 10,000–20,000 cells were analyzed for each point by two-color flow cytometry (Epix Coulter) to determine the percentage of double labeled (engulfing) macrophages.

2.4. Immunofluorescence staining and confocal microscopy

Peritoneal macrophages isolated from WT and TG2$^{-/-}$ mice were plated in two-well chamber-slides (5×10^5/well) and cultured for 48 h before use. After co-culturing macrophages with apoptotic cells for 30 min, cells were washed, fixed in ethanol/ acetone 1:1 for 10 min at −20 °C. For integrin β_3 staining macrophages were blocked with 50% FBS for 30 min at 37 °C, then washed with ice cold HEPES buffer and stained with PE-conjugated anti-mouse β_3 integrin antibody for 15 min on ice. For intracellular staining, cells were permeabilized with 0.1% Triton X after fixation and blocked with 1% BSA. For detecting Rac1 cells were labeled with purified mouse anti-Rac1 monoclonal antibody for 30 min at room temperature. After washing, Alexa 647-conjugated goat anti-mouse IgG was used as a secondary antibody. Images were taken with a Zeiss LSM 510 or Olympus FV1000 confocal laser scanning microscope. For visualizing the distribution of integrin β_3 and Rac1, overview images and 3D stacks were acquired at 1 μm optical thickness. 3D reconstructions and XYZ projections were created with the LSM 4.0 software.

2.5. Q-PCR analysis for detecting the expression of various phagocytosis receptors

Total RNA was isolated by TRI reagent (Sigma–Aldrich). Total RNA concentrations were determined by spectrometry after DNAse treatment (Sigma–Aldrich). TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA) was used for generating cDNA according to manufacturer's instructions. 200 ng total RNA was used in a reaction volume of 50 μl. ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used to determine the relative gene expression. Gene primers and probes were designed and supplied by Applied Biosystems. 18S ribosomal RNA was used as an endogenous control to normalize the amount of the sample cDNA added to the reaction. The 18S primers were labeled with VIC and sample primer with FAM. All samples were run in triplicate. Relative mRNA expression was quantified by comparing the cycle threshold (CT) between control and knockout cell samples.

2.6. Detection of active Rac1 and RhoG

Macrophages plated overnight were exposed to 2 μm carboxylated latex beads for 40 min, or left untreated as controls, to detect GTP-bound forms of Rac1 and RhoG. Pull-down assay was performed with the EZ-Detect Rac1 Activation Kit (Pierce, Rockford, IL) according to manufacturer's instruction. For the RhoG pull-down assay PAK-GST was replaced with an ELMO-GST protein. Active RhoG was detected by Western blot analysis using anti-RhoG antibody. For these assays 1–1.5 mg of total cell proteins (determined by the Bradford method) was used in the pull-downs.

2.7. Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ and the murine wild-type or constitutively active Rac1 [20] gene were produced using the AdEasy XL system (Stratagene) according to the manufacturer's instruction. Virus titers were determined by plaque assay in 293 cells after exposing them to