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RGC-32 is expressed in the human atherosclerotic arterial wall: Role in C5b-9-induced cell proliferation and migration

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ABSTRACT

The complement system is an important player in the development of atherosclerosis. Previously reported as a cell cycle regulator, RGC-32 is an essential effector of the terminal complement complex, C5b-9. In this study, our aims were to determine the expression of RGC-32 in the human atherosclerotic arterial wall and to delineate the mechanisms through which RGC-32 affects C5b-9-induced endothelial cell proliferation and migration. We now demonstrate that RGC-32 is expressed in human aortic atherosclerotic wall and that RGC-32 expression increases with the progression of atherosclerosis. Furthermore, silencing of RGC-32 expression abolished C5b-9-induced human aortic endothelial cell (HAEC) proliferation and migration. Of the 279 genes differentially expressed in HAECs after RGC-32 silencing, the genes involved in cell adhesion and cell cycle activation were significantly regulated by RGC-32. RGC-32 silencing caused a significant reduction in the expression of cyclin D1, cyclin D3, Akt, ROCK1, Rho GDP dissociation inhibitor alpha and profilin. These data suggest that RGC-32 mediates HAEC migration through the regulation of RhoA and ROCK1 expression and is involved in actin cytoskeletal organization. Thus, RGC-32 has promising therapeutic potential with regard to angiogenesis and atherosclerosis.

1. Introduction

At present, atherosclerotic cardiovascular disease is still the most pre-eminent cause of mortality and morbidity in the Western world (Mozaffarian et al., 2016). A plethora of evidence has substantiated the general dogma that atherosclerosis is a chronic, immune-mediated inflammatory disease (Hansson and Hermansson, 2011; Vlaicu et al., 2016b), at the cornerstone of which stands endothelial dysfunction (Gimbrone and Garcia-Cardena, 2016). The endothelial lining of the vascular tree makes essential contributions to the maintenance of homeostasis in the cardiovascular system by regulating flow-mediated vasodilation and vascular permeability and by recruiting immune cells in the subendothelial space (Hirase and Node, 2012). Healthy vascular endothelial cells (ECs) exposed to uniform laminar shear stress display an atheroprotective phenotype and are, as such, in a basal anti-inflammatory, quiescent nonproliferative state (Libby et al., 2006). In contrast, the effect of pathological stimuli such as exposure to enzymatically modified LDL, oxidative stress, advanced glycation end products and disturbed shear stress is the activation of the ECs, resulting in a NF-κB signaling-driven atheroprone phenotype that is characterized by an accelerated EC turnover as well as cell-surface expression of adhesion molecules and the production of chemokines and prothrombotic molecules (Gimbrone and Garcia-Cardena, 2016).

Complement activation, through the insertion of the C5b-9 complex into the EC membrane, also elicits the proliferation and migration of ECs (Benzaquen et al., 1994; Fosbrink et al., 2006; Niculescu et al., 1999). When exposed to a limited number of these C5b-9 complexes, nucleated cells survive complement attack by eliminating membrane-inserted complexes (Carney et al., 1985). These sublytic C5b-9 complexes, when inserted into the membrane, are able to exert multiple effects on target cells, the most important of which is the promotion of cellular proliferation and survival (Tegla et al., 2011).

We have previously demonstrated the deposition of C5b-9 complement complexes in the human arterial atherosclerotic wall (Vlaicu et

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al., 1985) and we also found that the levels of C5b-9 in the intimal thickening and fibrous plaques were higher than those in normal and fatty streak intima (Niculescu et al., 1987). Furthermore, we have demonstrated that C5b-9 deposits co-localize with intact cells, cell debris, lipid droplets and cholesterol clefts (Niculescu et al., 2004; Rus et al., 1986; Rus et al., 1989). Induction of human aortic EC cycle activation by sublytic C5b-9 requires the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and simultaneous FOXO1 inactivation (Fosbrink et al., 2006). In addition, experiments on human aortic EC have also revealed that gene transcription regulation by sublytic C5b-9 in these cells involves the activation of the JAK1/STAT3 pathway (Niculescu et al., 1999).

In an effort to decipher the molecular mechanisms that drive cell cycle activation, we previously used differential display to screen for the expression of cell cycle genes induced by complement activation and identified a novel gene, Response Gene to Complement (RGC)-32 (Badea et al., 1998). Sublytic C5b-9 was found to induce the expression of RGC-32 in human aortic smooth muscle cells (SMCs), as well as its nuclear translocation (Badea et al., 2002). Overexpressing RGC-32 stimulated quiescent SMCs to enter S-phase and undergo mitosis. RGC-32 forms a complex with CDC2 and increases its kinase activity both in vivo and in vitro. Therefore, sublytic C5b-9-induced cell cycle activation depends on CDC2 activation through RGC-32 (Badea et al., 2002). With regard to the impact of RGC-32 on EC proliferation, we have shown in primary human aortic ECs that RGC-32 silencing inhibits the ability of C5b-9 to induce the cell cycle and CDC2 activation (Fosbrink et al., 2009).

Although RGC-32 is primarily regarded as a novel cell-cycle regulator involved in cellular proliferation, several bodies of evidence have described a plethora of other roles for this protein: in cellular differentiation, tumorigenesis, immune system regulation, wound healing, scar tissue formation, regulation of lipid and glucose metabolism and atherosclerosis (Cui et al., 2013; Cui et al., 2015; Tegla et al., 2013; Tegla et al., 2015; Vlaicu et al., 2008; Vlaicu et al., 2016a, 2016b).

We investigated the presence and expression of RGC-32 in human atherosclerotic aortic wall. We found RGC-32 to be expressed by endothelial cells and in the media of the atherosclerotic aortic wall, where we show its co-localization with smooth muscle cells (SMCs), immune-inflammatory cells and C5b-9. Of outmost importance, RGC-32 expression increased with the progression of atherosclerosis. Next we set out to explore the mechanisms through which RGC-32 affects the C5b-9-induced proliferation and migration of human aortic endothelial cells. Silencing of RGC-32 expression abolished C5b-9-induced cell cycle activation and migration. We then performed an oligonucleotide expression array analysis in HAECs in which RGC-32 expression was silenced and found that the genes differentially regulated by RGC-32 are involved in actin cytoskeletal organization, cell adhesion, response to stress and cell cycle regulation. The differentially regulated genes included cyclin D1, cyclin D3, Akt, ROCK1, Rho GDP dissociation inhibitor alpha and profilin. Our data indicate that RGC-32 plays an important role in modulation of HAEC proliferation, migration and cytoskeleton reorganization, suggesting that it is a relevant molecular player in both angiogenesis and atherogenesis.

2. Materials and methods

2.1. Immunohistochemical staining of aortic tissue samples

Cross-sections of abdominal aortas prior to bifurcation into the common iliac arteries were obtained from nine autopsy cases. The study was approved by the Committee on Research and Ethics of Iuliu Haţieganu University of Medicine and Pharmacy in Cluj-Napoca, Romania. The specimens were fixed by immersion in neutrally buffered 10% formalin and subsequently dehydrated and embedded in paraffin. The nine human aortic atherosclerotic lesions collected from 9 patients (5 males and 4 females, age 51–84) and consisted of four fibrous plaques and five intimal thickenings (Table 1).

The expression of the RGC-32 protein was examined by indirect immunoperoxidase staining as previously described (Tegla et al., 2015). In brief, paraffin sections were deparaffinized and washed in PBS, and endogenous peroxidase was quenched with 3% H_2O_2. For antigen retrieval, slides were placed in Dako Target Retrieval Solution (Dako, Carpinteria, CA) and boiled for 30 min. Slides were incubated overnight at 4 °C with rabbit IgG anti-RGC-32 (Bioss Inc., Woburn, MA) diluted 1:300 in PBS, then washed three times in PBS and incubated for 1 h at room temperature with secondary HRP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:250 in PBS Tween 0.1%. After several PBS rinses, the bound antibody was detected using Nova RED (Vector Labs, Burlingame, CA) and the slides were subsequently counterstained with Harris hematoxylin (Sigma, St. Louis, MO) and mounted using VectaMount AQ (Vector Labs).

The staining intensity of the RGC-32 deposits was evaluated independently by two investigators in a blinded fashion. The intensity of staining was graded as follows: negative (−), slightly positive (+), positive (++), or highly positive (+++).

2.2. Double-staining immunohistochemistry

Paraffin sections of brains from adult patients with MS were double-stained for RGC-32 and CD4, CD68, alpha smooth muscle actin (α-SMA), or C5b-9 as previously described (Tegla et al., 2015). Sections were initially processed for RGC-32 immunostaining as described above and the reactions were developed with NovaRed. The sections were treated with BLOXALL blocking solution and then incubated for 2 h at RT with mouse monoclonal anti-CD4 (NovaCastra), diluted 1/50, anti-CD68 (KP1) (Covance), anti- alpha smooth muscle cell actin (Biotechne), or anti C5b-9 (Calbiochem). The slides were washed several times in PBS and reacted with alkaline phosphatase-conjugated goat anti-mouse IgG (Vector). The reaction was developed using a Vector Alkaline Phosphatase Substrate Kit III (Vector Labs). Control sections were prepared by immunostaining without the primary antibody or by using control isotype IgG instead of the primary antibody. The immunostained slides were independently evaluated by two investigators.

Table 1
Quantification for RGC-32 and C5b-9 in human aortic atherosclerotic wall.

No.	Type	Age	Sex	Area	RGC-32	C5b-9
1	IT	60	F	Intima	++	++
				Media	++	+
2	IT	51	F	Intima	++	++
				Media	+++	+++
3	IT	84	M	Intima	++	++
				Media	+++	+++
4	IT	54	M	Intima	++	++
				Media	+++	+++
5	IT	69	M	Intima	++	+
				Media	+++	++
6	FP	73	F	Intima	+++	+
				Media	+++	+++
7	FP	59	M	Intima	+++	++
				Media	+++	+++
8	FP	57	M	Intima	++ ++	+++
				Media	+++	+++
9	FP	74	F	Intima	++	++
				Media	++	+++

IT = Intimal thickening.
FP = Fibrous plaque.