

C5a stimulates the proliferation of breast cancer cells via Akt-dependent RGC-32 gene activation

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Abstract. Complement system activation contributes to various immune and inflammatory diseases, as well as cancers. However, the role of complement activation in the proliferation of cancer cells is not clear. In the present study, we investigated the consequences of complement activation on the proliferation of breast cancer cells and its possible mechanisms. We focused our study on the potential roles of the anaphylatoxins C3a and C5a in the proliferation of human breast cancer, as two important immune mediators generated after complement activation. Our study revealed that C5a stimulation, but not C3a, enhanced the proliferation of human breast cancer cells *in vitro*. Moreover, the expression of response gene to complement 32 (RGC-32) was pronounced in breast cancer cells in response to C5a stimulation. Notably, blockade of the C5a receptor markedly reduced the expression of RGC-32 and the proliferation of breast cancer cells stimulated by C5a. Meanwhile, silencing of RGC-32 expression reduced the proliferation of breast cancer cells induced by C5a treatment. Further investigation revealed that Akt activation was involved in C5a-induced RGC-32 expression and breast cancer cell proliferation. In conclusion, the present study indicates that C5a may promote the proliferation of breast cancer cells through Akt1 activation of the RGC-32 gene.

Introduction

It is well established that the human complement system is a central part of the innate immune response. Complement is known to play a major role as a first defense against microbes (1,2), and also participates in diverse physiological

processes and contributes to various immune and inflammatory diseases (3-6). There are three conventional mechanisms of complement activation, known as the classical, lectin and alternative pathways (7,8). Activation of complement causes recruitment of immune cells; opsonization of coated cells; and direct killing of affected cells through a membrane attack complex (MAC). During complement activation, soluble proinflammatory peptide fragments C3a and C5a are released from C3 and C5 molecules, respectively. C3a and C5a are referred to as anaphylatoxins and exhibit a variety of biological activities in the immune response (9-11).

There is increasing evidence for the contribution of complement activation to cancer progression (12,13). During carcinogenesis, tumor cells acquire genetic and epigenetic alterations that promote their malignant growth. As a result, the complement system can recognize these tumor cells. For decades, complement has been recognized as an effector arm of the immune system that contributes to the destruction of tumor cells (14,15); however, cancer cells can resist the harmful effects of complement by different extracellular and intracellular mechanisms. In fact, new findings on the contribution of complement to tumor growth have challenged the paradigm that complement always protects against tumors. For example, some studies have revealed that the generation of anaphylatoxins C3a and C5a in the tumor microenvironment leads to significant tumor progression (12,13,16,17). Nevertheless, the precise roles of C3a and C5a in human breast cancer are still controversial.

Response gene to complement 32 (RGC-32) is a novel gene whose expression is induced by complement (18-20). The RGC-32 gene is abundantly expressed in the placenta, skeletal muscle, kidney and liver. Upregulation of RGC-32 gene expression has been demonstrated in a wide range of cell lines in response to a variety of stimuli, such as sublytic C5b-9, steroid hormones and growth factors (18,19,21). It has been reported that RGC-32 plays an important role in cellular proliferation and differentiation (18,22), particularly in human pancreatic cancer and colon cancer (21,23). Based on these findings, the role of the RGC-32 gene in C5a-induced proliferation of breast cancer cells and its regulation by signaling pathways warrant further investigation.

In the present study, we evaluated the role of C3a and C5a in human breast cancer cells. We found that breast cancer cells

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are able to enhance proliferation in response to C5a stimulation compared with non-malignant breast epithelial cells. Blockade of the C5a receptor (C5aR) strongly slowed the proliferation of breast cancer cells exposed to C5a. Further investigation revealed that the upregulation of the RGC-32 gene could contribute to C5a-induced breast cancer cell proliferation. Meanwhile, Akt activation was found to be necessary for C5a-induced RGC-32 expression and breast cancer cell proliferation. These results provide novel information concerning the relationship between complement activation and breast cancer, which may influence the development of future therapeutic strategies.

Materials and methods

Reagents. Polyclonal antibodies against RGC-32 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against β -actin were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG antibodies, 20X LumiGLO[®] reagent and 20X peroxide were purchased from Cell Signaling Technology. RIPA lysis buffer was purchased from Thermo Scientific (Fremont, CA, USA). The Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). The incision enzymes *Hind*III and *Eco*RV as well as T4 DNA ligase were purchased from Fermentas (Burlington, Ontario, Canada). The QIAprep[®] Spin Miniprep kit was obtained from Qiagen (Hilden, Germany). The mammalian expression vector of pcDNA3.1 and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA). The shRNA expression plasmids of pGPU6/GFP were purchased from GenePharma (Shanghai, China). pGL3-basic vector and the dual luciferase assay system were from Promega (Madison, WI, USA). High Capacity cDNA reverse transcription kits and TaqMan[®] Fast Advanced Master Mix were obtained from ABI (Foster, CA, USA). Human C3a and C5a were from R&D Systems (Minneapolis, MN, USA).

Cell culture. Established human malignant breast cancer cell lines (MCF-7 and MDA-MB-231) as well as a human non-malignant breast epithelial cell line (HBL-100) were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μ g/ml). Cells were incubated at 37°C in a 5% CO₂ incubator.

Construction of the plasmids. To silence the human RGC-32 gene, different shRNA sequences against RGC-32 mRNA (NM_014059.2) were designed. The expression plasmids of RGC-32 shRNA were then constructed by using the plasmids of pGPU6/GFP. The most effective shRNA expression plasmids against the RGC-32 gene and the corresponding scrambled shRNA expression plasmids as a negative control were chosen for further functional experiments. In addition, pcDNA3.1/RGC-32 was constructed by inserting the ORF of human RGC-32 cDNA (NM_014059.2) into the plasmids of pcDNA3.1. The PCAF gene containing an HA tag was amplified by polymerase chain reaction (PCR) from cDNA

of normal human breast epithelial cells. The PCR products and the vector of pcDNA3.1 were digested with *Hind*III and *Eco*RV, and then ligated by using T4 DNA ligase. Meanwhile, the human RGC-32 promoter (-1600 nt ~ -1 nt before ATG) was constructed into the pGL3-basic vector.

Cellular transfection. MCF-7 and MDA-MB-231 cell lines were transfected using Lipofectamine 2000 according to the manufacturer's instructions (24).

Luciferase. MCF-7 and MDA-MB-231 cells at 80% confluency in 96-well plates were transfected with the pGL3-basic vector containing a human RGC-32 promoter driving luciferase, using Lipofectamine 2000. After 36 h of transfection, the cells were stimulated with C5a for 9 h, and then lysed for luciferase assays using the dual luciferase assay system.

Quantitative reverse transcriptase-PCR. After isolation of total RNA from cells using TRIzol reagent, an equal amount (2 μ g) of total RNA from each sample was synthesized as a first-strand cDNA using the High Capacity cDNA reverse transcription kits. The cDNA was then amplified using TaqMan[®] Fast Advanced Master Mix to detect the level of RGC-32 mRNA. The 7300 Real-Time PCR system (ABI) was used for the experiment. The reaction program consisted of an initial denaturation step at 95°C for 10 min, denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec for 40 cycles. The β -actin gene was used as an internal control. The relative level of gene expression was obtained by calculating the ratio of the cycle numbers of the initial exponential amplification phase as determined by the sequence detection system for RGC-32 and β -actin using the $2^{-\Delta\Delta C_t}$ formula. Each sample was assayed in triplicate.

Western blot analysis. The proteins (40 μ g) were subjected to 12% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) by a semi-dry electrophoretic transfer cell (Bio-Rad). The membranes were incubated for 1 h at room temperature in blocking buffer (5% skimmed milk in TBS-T), and were then incubated with the antibodies against RGC-32 and β -actin, respectively, overnight at 4°C. After washing with TBST-T three times, the membranes were incubated with HRP-conjugated anti-rabbit and anti-mouse antibodies for 1 h at 37°C. The bands were then visualized by the ECL detection system (Bio-Rad) with 3 to 10 min exposure after washing the membranes. Finally, the radiographic band density was measured using Quantity One software (Bio-Rad). Control for protein loading was verified by using β -actin as the internal standard. Similar results were obtained in three independent experiments.

CCK-8 assay. MCF-7, MDA-MB-231 and HBL-100 cells were incubated with CCK-8 for the final 3 h before detection. The formazan product was then visualized at the absorbance of 450 nm, and the absorbance was directly proportional to the number of living cells (25,26). Each sample was assayed in triplicate.

Statistical analysis. All data are expressed as mean \pm SD. All statistical analysis was carried out using SPSS 11.5 software.

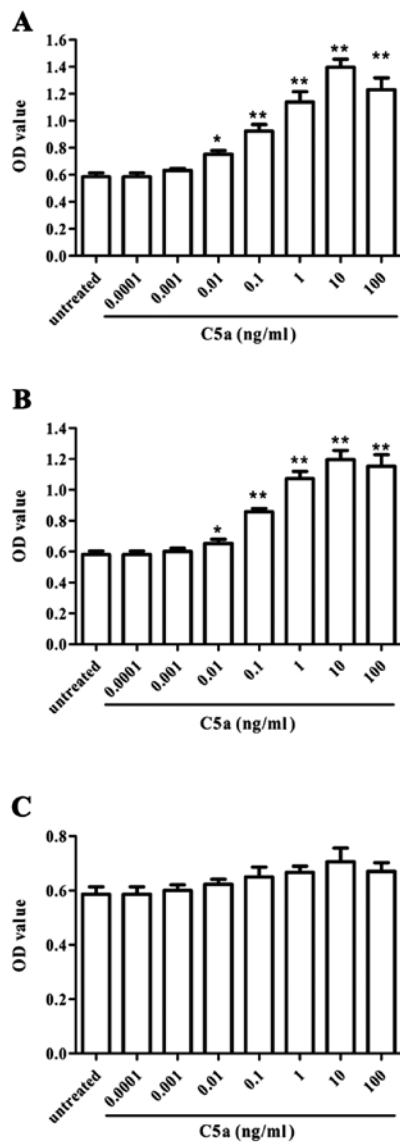


Figure 1. The role of C5a in the proliferation of human breast cancer cell lines. The two human breast cancer cell lines (MCF-7 and MDA-MB-231) and the non-malignant breast epithelial cell line (HBL-100) as a control were cultured *in vitro*. Purified human C5a was then added to the MCF-7, MDA-MB-231 and HBL-100 cells, respectively, during this time period. CCK-8 assay revealed that C5a stimulation for 48 h enhanced the proliferative response of both (A) MCF-7 and (B) MDA-MB-231 cell lines in a dose-dependent manner with a prominent effect at the dose of 10 ng/ml. (C) In contrast, C5a stimulation had no significant effect on the proliferation of the HBL-100 cell line. Results are representative of 3 independent experiments. Data are presented as means \pm SD. *P<0.05; **P<0.01 vs. untreated group.

One-way analysis of variance (ANOVA), followed by post hoc Bonferroni test with adjustment for multiple comparisons, was used to test the significance of the differences between groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

C5a promotes the proliferation of human breast cancer cells. In order to determine the role of C3a and C5a in the proliferation of human breast cancer cells, two human breast cancer cell lines (MCF-7 and MDA-MB-231) and a non-malignant

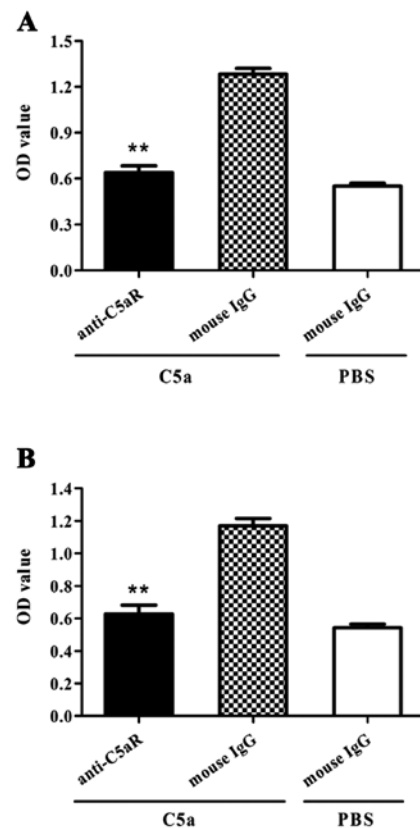


Figure 2. Effects of C5aR blockade on C5a-induced proliferation of breast cancer cell lines. The anti-C5aR neutralizing antibody was added into the medium and cultured for 30 min. C5a was then added into the medium and cultured for 48 h, and the proliferation of breast cancer cells was subsequently detected by the CCK-8 assay. The results revealed that blockade of C5aR with the anti-C5aR neutralizing antibody markedly reduced the proliferation of (A) MCF-7 and (B) MDA-MB-231 cells exposed to C5a. Results are representative of 3 independent experiments. Data are presented as means \pm SD. **P<0.01 vs. the mouse IgG + C5a group.

breast epithelial cell line (HBL-100) as a control were cultured *in vitro*. Purified human C3a and C5a were then added to the human breast cancer cell lines and the non-malignant breast epithelial cell line, respectively, to evaluate their ability to induce the proliferation of breast cancer cells. The results showed that C5a stimulation enhanced the proliferative response of both human breast cancer cell lines *in vitro*, but had no marked effect on the proliferation of the non-malignant breast epithelial cell line (Fig. 1A-C). No significant increase in cellular proliferation in response to C3a was observed in the breast cancer cell lines MCF-7 and MDA-MB-231 (data not shown).

Blockade of C5aR slows the proliferation of breast cancer cells. Since the breast cancer cell lines showed a significant increase in proliferation in response to C5a stimulation, the influence of C5a on the proliferation of breast cancer cells was further studied using the anti-C5aR neutralizing antibody as a C5aR antagonist. The result revealed that blockade of C5aR with the anti-C5aR neutralizing antibody markedly reduced the proliferation of the two breast cancer cell lines cultured with human C5a (Fig. 2A and B). Taken together, these findings (Figs. 1 and 2) indicate that anaphylatoxin C5a promotes the proliferation of the human breast cancer cell lines.

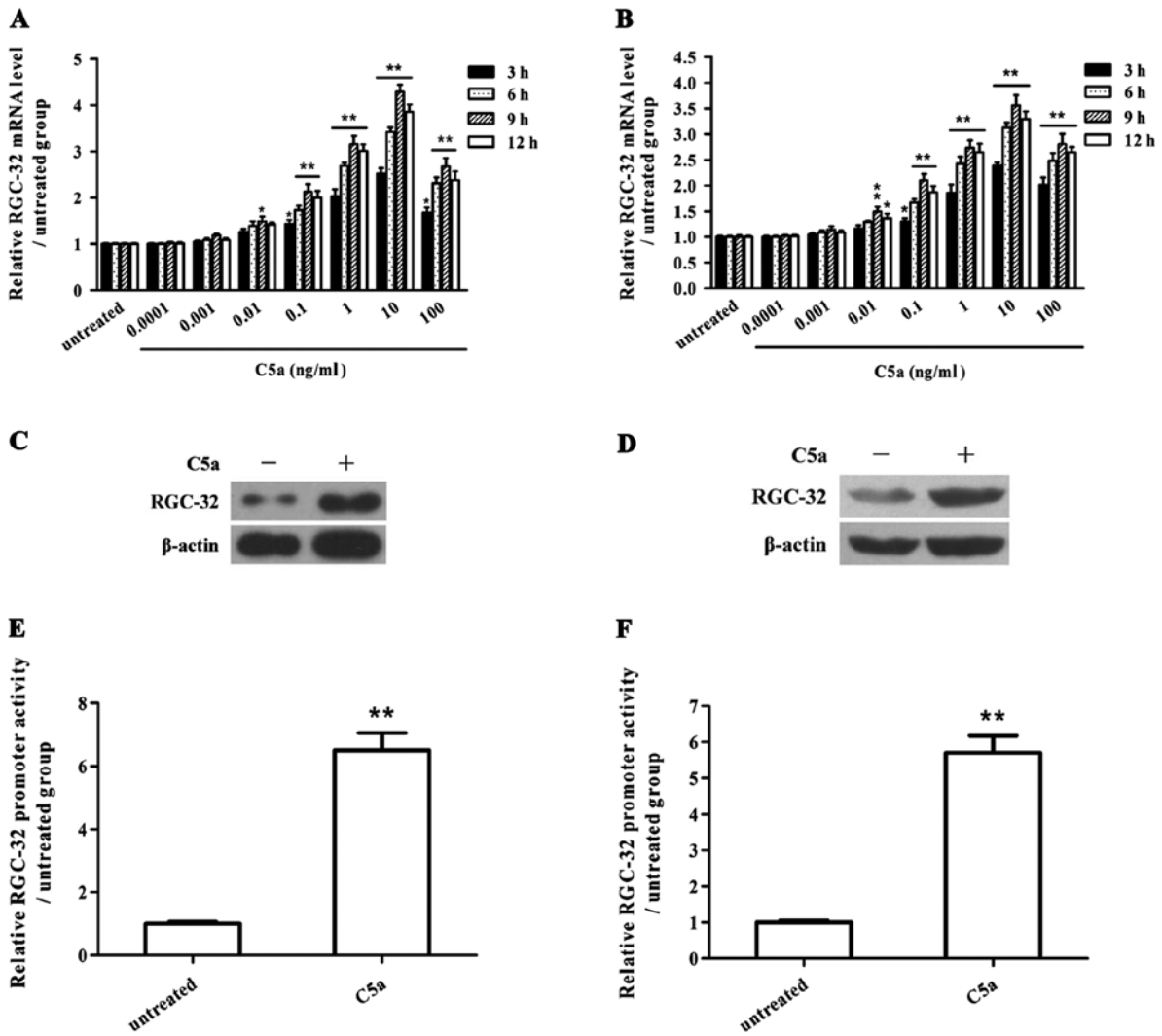


Figure 3. RGC-32 expression in C5a-induced breast cancer cell lines. Expression of the RGC-32 gene was detected in the breast cancer cell lines exposed to C5a. (A and B) Real-time PCR assay showed that the level of RGC-32 mRNA was markedly elevated not only in the (A) MCF-7 cell line but also in the (B) MDA-MB-231 cell line in response to C5a stimulation. (C and D) Western blot analysis showed that the expression of RGC-32 was significantly increased at the protein level in both (C) MCF-7 and (D) MDA-MB-231 cell lines in response to C5a stimulation. (E and F) Luciferase experiments revealed that transcription of the RGC-32 gene was markedly increased in both cell lines including (E) MCF-7 and (F) MDA-MB-231 after stimulation by C5a. Results are representative of 3 independent experiments. Data are presented as means ± SD. *P<0.05; **P<0.01 vs. the untreated group.

RGC-32 expression is induced in breast cancer cells exposed to C5a. It is well accepted that RGC-32 is a novel gene whose expression is induced by complement and other stimuli (18-20). Given that C5a may contribute to the proliferation of breast cancer cells, we further detected the expression of the RGC-32 gene in breast cancer cells exposed to C5a. The results showed that the expression of RGC-32 was markedly elevated in the breast cancer cells in response to C5a stimulation at both the mRNA and protein levels (Fig. 3A-D). The luciferase experiments further demonstrated that transcription of the RGC-32 gene was significantly increased in the breast cancer cells after stimulation by C5a (Fig. 3E and F). These findings indicate that C5a *in vitro* has the ability to promote RGC-32 gene expression in human breast cancer cell lines.

Expression of RGC-32 is required for C5a-induced proliferation in breast cancer cells. It remains to be determined

whether RGC-32 expression is necessary for the proliferation of breast cancer in response to C5a stimulation. Thus, breast cancer cells were transfected with RGC-32 shRNA-expressing plasmids, and then the cellular proliferation in response to C5a stimulation was determined by the CCK-8 assay. The results revealed that treatment with RGC-32 shRNA obviously suppressed the proliferation of breast cancer cells in response to C5a stimulation (Fig. 4). Remarkably, overexpression of the RGC-32 gene by plasmids of pcDNA3.1/RGC-32 promoted the proliferation of the breast cancer cells (Fig. 5), indicating that RGC-32 expression is involved in C5a-induced proliferation of breast cancer cells. These findings indicate that C5a-induced RGC-32 expression is required for the proliferation of breast cancer cells.

Akt activation is necessary for C5a-induced RGC-32 expression and breast cancer cell proliferation. Akt is a widely recognized contributory factor in the proliferation of cancer

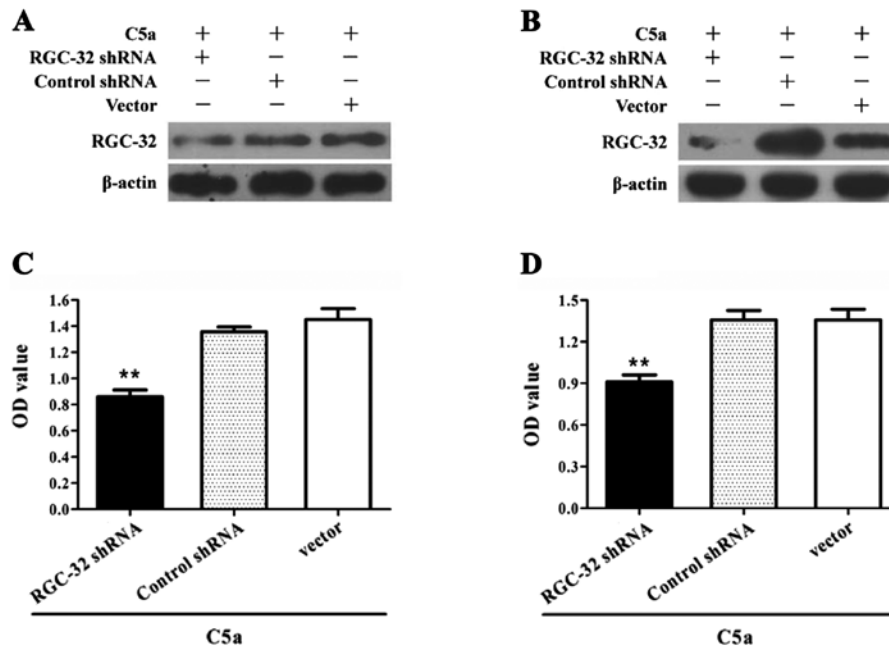


Figure 4. The role of RGC-32 in the proliferation of breast cancer cell lines exposed to C5a. Breast cancer cells were transfected with RGC-32 shRNA and cultured continually for 48 h, and then C5a was used to stimulate breast cancer cell proliferation. (A and B) Western blot analysis revealed that, in comparison with control shRNA, treatment with RGC-32 shRNA obviously suppressed the expression of RGC-32 in both the (A) MCF-7 and (B) MDA-MB-231 cell lines in response to C5a stimulation. (C and D) In comparison with control shRNA, treatment with RGC-32 shRNA obviously suppressed the proliferation of the (C) MCF-7 and (D) MDA-MB-231 cell lines in response to C5a stimulation as determined by the CCK-8 assay. Results are representative of 3 independent experiments. Data are presented as means \pm SD. ** $P < 0.01$ vs. control shRNA + C5a group and vector + C5a group.

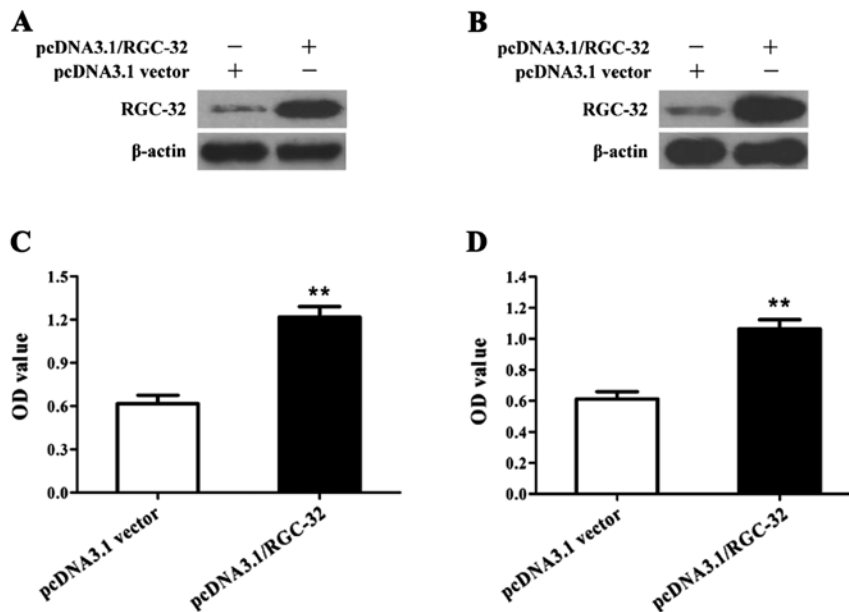


Figure 5. The effects of RGC-32 overexpression on the proliferation of breast cancer cell lines. The plasmids of the pcDNA3.1/RGC-32 and pcDNA3.1 vectors were transfected into the two breast cancer cell lines respectively, and cultured continually for 48 h. (A and B) Western blot analysis revealed that transfection of pcDNA3.1/RGC-32 increased RGC-32 expression in both the (A) MCF-7 and (B) MDA-MB-231 cell lines. (C and D) CCK-8 assay revealed that overexpression of the RGC-32 gene promoted the proliferation of the (C) MCF-7 and (D) MDA-MB-231 cell lines compared to the pcDNA3.1 vector control group. Results are representative of 3 independent experiments. Data are presented as means \pm SD. ** $P < 0.01$ vs. the pcDNA3.1 vector group.

cells. Since Akt activation has been reported to promote cell proliferation in response to complement stimulation (27-29), and both C5a stimulation and RGC-32 gene expression were involved in the proliferation of breast cancer cells (Fig. 5), the influence of Akt activation on C5a-induced RGC-32 expression and breast cancer cell proliferation was further explored

in the present study. The results revealed that inhibition of Akt activation with Ly294002 strongly reduced C5a-induced RGC-32 expression and breast cancer cell proliferation (Fig. 6). It was therefore confirmed that Akt activation contributes to C5a-induced RGC-32 expression and breast cancer cell proliferation.

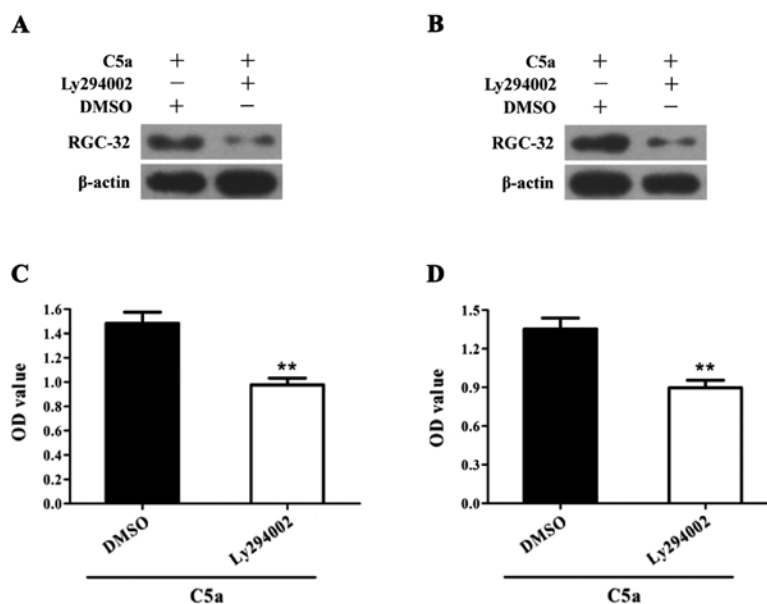


Figure 6. The roles of Akt activation in C5a-induced RGC-32 expression and breast cancer cell proliferation. Ly294002 (25 μ M) was used to inhibit Akt activation, and then C5a was added to induce RGC-32 expression and breast cancer cell proliferation. (A and B) Western blot analysis revealed that inhibition of Akt activation with Ly294002 significantly reduced C5a-induced RGC-32 expression in both the (A) MCF-7 and (B) MDA-MB-231 cell lines. (C and D) CCK-8 assay revealed that Ly294002 significantly reduced C5a-induced proliferation of the (C) MCF-7 and (D) MDA-MB-231 cell lines. Results are representative of 3 independent experiments. Data are presented as means \pm SD. ** $P < 0.01$ vs. the DMSO + C5a group.

Discussion

Breast carcinogenesis is a multi-step process in which normal breast cells acquire genetic and epigenetic alterations that result in the expression of many cancer-associated molecules (30-32). However, the mechanism by which breast cancer cells undergo proliferation still remains controversial.

Complement is known to play important roles as a first defense against microbes, and also participates in diverse physiological processes and contributes to various immune and inflammatory diseases (4,33-35). In addition, there is increasing evidence for the contribution of complement activation to cancer progression (12,16). Cancer cells appear to be able to establish a convenient balance between complement activation and inhibition, taking advantage of complement initiation without suffering its deleterious effects. Various studies have demonstrated that generation of anaphylatoxins C3a and C5a in the tumor microenvironment leads to significant tumor progression (12,16,17). During the development of tumors, complement C3 and C5 activation may support chronic inflammation, promote an immunosuppressive microenvironment, induce angiogenesis, and activate cancer-related signaling pathways. Little is known, however, concerning the roles of C3a and C5a in breast cancer.

In the present study, we provide evidence for the contribution of C5a to the proliferation of breast cancer cells. In particular, we found that breast cancer cells were able to proliferate in response to C5a stimulation much more efficiently than non-malignant breast cells, indicating that breast cancer cells are more sensitive to C5a stimulation than non-malignant breast cells. In contrast, C3a did not significantly affect the proliferation of breast cancer cells. Other effects of C3a on breast cancer cells need to be determined to explore the potential roles of C3a in breast cancer.

RGC-32 is a novel gene induced by complement and other stimuli (18-20). It is well known that upregulation of RGC-32 gene expression contributes to the proliferation of target cells (18,19,21). However, the precise role of the RGC-32 gene in C5a-induced proliferation of breast cancer cells needs to be explored. Our present study demonstrated that the expression of RGC-32 was markedly elevated in breast cancer cells in response to C5a stimulation. Further investigation revealed that gene transfer of RGC-32 shRNA obviously diminished the proliferation of breast cancer cells in response to C5a stimulation. Conversely, overexpression of the RGC-32 gene by transfection of pcDNA3.1/RGC-32 promoted the proliferation of breast cancer cells without C5a stimulation. These findings indicate that C5a-induced RGC-32 expression is required for the proliferation of breast cancer cells.

Further experiments were carried out to discover the possible mechanism by which C5a stimulates the expression of the RGC-32 gene in breast cancer cells. The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is one of the most frequently dysregulated pathways in human cancers (36,37). On the other hand, Akt activation has been reported to promote cell proliferation in response to complement stimulation (27-29). Thus, in a subsequent experiment, we sought to explore the role of Akt activation in regulating RGC-32 expression and cellular proliferation of breast cancer cells in response to C5a. The results revealed that inhibition of Akt activation suppressed RGC-32 expression and cellular proliferation of breast cancer cells induced by C5a. Further studies need to be performed to discover the possible regulatory mechanism by which Akt increases the expression of the RGC-32 gene in breast cancer cells.

In conclusion, we evaluated the roles of C3a and C5a in promoting the proliferation of breast cancer cells *in vitro*. We found that breast cancer cells are able to undergo proliferation

in response to C5a stimulation rather than C3a. We also found a significant increase in the expression of the RGC-32 gene in breast cancer cells induced by C5a. Further investigation revealed that upregulation of the RGC-32 gene could contribute to C5a-induced proliferation of breast cancer cells. Finally, Akt activation was shown to be necessary for C5a-induced RGC-32 expression and breast cancer cell proliferation. On the basis of these observations, we conclude that C5a promotes the proliferation of breast cancer cells through Akt-activated RGC-32 gene expression. These findings provide novel information concerning the relationship between complement activation and breast cancer, which may influence the development of future therapeutic strategies.

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