C5aR is frequently expressed in metastatic renal cell carcinoma and plays a crucial role in cell invasion via the ERK and PI3 kinase pathways

YOSHIHIRO MAEDA[1*], YOSHIAKI KAWANO[1*], YOSHIHIRO WADA[1], JUNJI YATSUDA[1], TAKANOBU MOTOSHIMA[1], YOJI MURAKAMI[1], KEN KIKUCHI[2], TAKAHISA IMAMURA[3] and MASATOSHI ETO[1]

[1]Department of Urology, Faculty of Life Sciences, Kumamoto University, Kumamoto 860-8556; [2]Medical Quality Management Center, Kumamoto University Hospital, Kumamoto 860-0811; [3]Department of Molecular Pathology, Faculty of Life Sciences, Kumamoto University, Kumamoto 860-8556, Japan

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Abstract. The anaphylatoxin C5a is a chemoattractant for leukocyte migration via the C5a receptor (C5aR). We recently reported that C5aR was aberrantly expressed in a wide variety of human related cancers, while it also promotes cancer cell invasion by C5a stimulation. However, the biological significance of C5aR expression in renal cell carcinoma (RCC) has not yet been clarified. In the present study, we aimed to elucidate the biological role of C5aR in RCC progression. Clinical RCC specimens were analyzed for C5aR expression and its relationship with baseline demographic data and clinicopathological parameters was analyzed. Moreover, renal carcinoma Renca cells stably expressing C5aR were generated and used to assess the effects of C5a-C5aR axis activation on various cellular phenomena in culture. Immunohistochemistry revealed that 96.7% of the metastatic RCCs (mRCCs) showed C5aR expression, whereas only 50.5% of the non-metastatic RCCs expressed C5aR (P<0.001). Although C5a stimulation did not significantly alter anoikis of C5aR-expressing Renca cells, C5a elicited cell morphological change and scattering of those cells accompanied with dynamic actin rearrangement, which was not observed in the Renca cells harboring the empty vector only. Moreover, C5a triggered ERK and PI3K-dependent invasion of the C5aR-expressing renal carcinoma cells. These results are consistent with the idea that the C5a-C5aR axis plays a crucial role in renal carcinoma cell invasion, which may be one of the key steps for RCC metastasis. The present study provides proof-of-concept that the C5a-C5aR axis may be a useful therapeutic target for preventing RCC progression.

Correspondence to: Dr Yoshiaki Kawano, Department of Urology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto, Kumamoto 860-8556, Japan
E-mail: ykawano@kumamoto-u.ac.jp

[*] Contributed equally

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Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults. Surgical removal of the cancer, either by radical or partial nephrectomy, is a standard therapeutic option for organ-confined RCC. However, ~30% of patients eventually manifest metastatic disease after surgery (1). Recent studies have clarified the key molecular events of oncogenesis for renal cell carcinoma such as aberrant hypoxia-inducible factor (HIF)-α activation by Von Hippel-Lindau (VHL) gene mutation/deletion and the resultant overexpression of vascular endothelial growth factor (VEGF) in clear cell carcinoma, which provided the rationale for the development of VEGF receptor (VEGFR) inhibitors such as sunitinib and sorafenib. Although contemporary emergence of such molecular targeting agents has contributed to the prolonged survival of metastatic RCC (mRCC) patients, attainment of complete response is extremely rare (2) and improvement in overall survival is still limited. Therefore, detailed mechanisms of how RCC cells spread and eventually metastasize must be clarified in order to develop better therapeutic options.

Cancer cell invasion and metastasis share many similarities with leukocyte trafficking, which is crucially regulated by soluble factors and their receptors (3). Anaphylatoxin C5a is an N-terminal 74 amino acid fragment derived from the α-chain of the complement fifth component (C5), which serves as a leukocyte chemoattractant and inflammatory mediator (4). C5a promotes leukocyte migration by interacting with the membrane-associated C5a receptor (C5aR; CD88). C5aR is one of the G protein-coupled receptors (GPCRs), and its association with the C5a ligand provokes activation of intracellular signaling pathways such as the Raf/MEK/ERK and

PI3K/Akt pathways (5,6), which play pivotal roles in leukocyte migration (7,8). Recently, we reported that C5aR is aberrantly expressed in a wide variety of human cancers presumably due to the consequence of malignant transformation while the C5a-C5aR axis promoted cancer cell invasion by eliciting matrix metalloprotease (MMP) secretion and cytoskeletal reorganization (9). In that study, we briefly mentioned that C5aR was also expressed in renal cell carcinoma specimens. However, the number of specimens in that study was limited, and the clinical significance and biological role of the C5aR expression in renal cell carcinoma remains unclear.

Here, we analyzed the relationship between the C5aR expression and the clinical parameters in renal cell carcinoma specimens. We observed that C5aR was expressed in a vast majority of the mRCC cases, whereas only half of the organ-confined RCCs expressed C5aR. Furthermore, we provided *in vitro* evidence that the C5a-C5aR axis provoked renal cancer cell invasion. Our results suggest that the C5a-C5aR axis-elicited renal cancer cell invasion may be one of the critical steps for establishing renal cancer cell metastasis.

Materials and methods

Cell line. Renca cells (ATCC: CRL-2947) were obtained in 2013 from the American Type Culture Collection. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), penicillin (40 U/ml) and streptomycin (40 μg/ml) and were maintained at 37˚C in 5% CO_2.

Reagents. Recombinant C5a was purchased from Hycult Biotech (Plymouth, PA, USA). G418 was purchased from InvivoGen (San Diego, CA, USA). U0126 was purchased from Promega (Madison, WI, USA). PI-103 was purchased from Merck (Darmstadt, Germany). Anti-C5aR antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies for phospho-ERK and total ERK, phospho-Akt and total Akt and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Tissue samples, immunohistochemistry and retrospective analysis. Renal cell carcinoma tissue samples were obtained by surgical resection or core needle biopsy from 127 patients in Kumamoto University Hospital, and the usage of those samples for the present study was approved by The Internal Review Board of Kumamoto University Hospital. Immunohistochemistry for C5aR in the RCC samples was performed according to a previously described protocol (9). The relationship between the C5aR expression and baseline demographic data/clinicopathological parameters was analyzed by Fisher's exact test.

Immunoblotting. Immunoblotting was performed as previously described (10). Briefly, cell lysates were analyzed by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Protran; GE Healthcare Life Sciences, Pittsburgh, PA, USA). After blocking with blocking buffer (1% TBS-T buffer containing 5% bovine serum albumin), the membranes were incubated with the primary antibodies indicated according to the manufacturer's instructions, followed by incubation with appropriate HRP-conjugated secondary antibodies. The bands were visualized by ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Establishment of C5aR stably expressing Renca cells and in vivo study. pCMV-C5aR that encodes full-length murine C5aR was purchased from OriGene Technologies (Rockville, MD, USA). Renca cells were transfected with pCMV-C5aR using the ProFection Mammalian Transfection System (Promega). After 48 h, the medium was replaced with a selection medium supplemented with G418 (400 μg/ml) then cultured for 2 weeks. G418-resistant cells were isolated by limiting dilution and propagated. The cells were subjected to flow cytometric analysis by FACSCan (BD Biosciences, San Jose, CA, USA) as previously described (9) to select cells expressing C5aR (designated as Renca/C5aR cells). Renca cells transfected with the empty plasmid pCMV and resistant to 400 μg/ml G418 were isolated and then designated as Renca/empty cells. For the *in vivo* study, the Renca-derived cells were injected into the renal subcapsular space in mice according to Shvarts *et al* (11). This experiment was approved by the Kumamoto University Animal Experiment Committee.

Anoikis assay. Anoikis assay was performed based on the protocol reported by Berezovskaya *et al* (12) with minor modifications. Renca-derived cells were dissociated by Accutase (Millipore, Billerica, MA, USA), and then washed with serum-free medium and suspended with medium containing 0.5% FCS. The viable cells were counted using the trypan blue dye exclusion method to confirm that the initial viability of the cells after dissociation by Accutase was >95%. A suspension of $1x10^6$ viable cells was treated with or without 10 nM recombinant C5a (rC5a) then plated in 2.0 μl of serum-free medium in 6-well ultra-low-attachment polystyrene plates (Corning, Tewksbury, MA, USA) and incubated at 37˚C in 5% CO_2 overnight. The numbers of the total and viable cells after incubation were counted as described above, and then the percentage of the viable cells was calculated.

Immunofluorescence analysis. Cells ($5x10^4$) were seeded on glass coverslips and incubated for 24 h. After serum starvation overnight, the cells were stimulated with 10 nM rC5a for the stated time periods. The cells were then fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 for 5 min, and were incubated with 5 U/ml Alexa 488-phalloidin (Invitrogen Life Technologies, Carlsbad, CA, USA) for 40 min, followed by washing with PBS. Nuclei were counterstained with 1.0 mM TO-PRO®-3 (Invitrogen Life Technologies) for 15 min. Images were obtained and processed by FluoView 300 laser scanning confocal microscope (Olympus, Melville, NY, USA).

Invasion assay in vitro. BioCoat Matrigel invasion chambers were utilized (24-well plates, 8-μm pores; BD Biosciences) as previously described (9). Renca-derived cells ($5x10^4$) were suspended in serum-free RPMI-1640, which were then seeded into the upper chamber. RPMI-1640 supplemented with either 10 nM rC5a or carrier solution (PBS) was placed in the lower chamber. PI-103, U0126 or carrier control (DMSO) was added to the medium in both the upper and lower well at the indi-

Table I. Patients and tumor characteristics.

	C5aR(-) (n=49)	C5aR(+) (n=78)	P-value
Age (years)	61.8	61.490	0.8962
Gender, n (%)			
Male	36 (73.5)	50 (64.1)	0.3313
Female	13 (26.5)	28 (35.9)	
Histological subtypes, n (%)			
Clear cell	48 (100)	72 (92.3)	0.0812
Chromophobe	0 (0)	3 (3.8)	0.2835
Papillary	0 (0)	3 (3.8)	0.2835
Fuhrman grade, n (%)			
G1	23 (46.9)	17 (21.8)	0.0056
G2	24 (49.0)	50 (69.2)	0.0259
G3	1 (2.0)	5 (6.4)	0.4044
Unknown	1 (2.0)	2 (2.6)	NA
Stage[a], n (%)			
T1	46 (93.9)	55 (70.5)	**0.0056**
T2	2 (4.1)	10 (12.8)	0.1270
T3	1 (2.0)	11 (14.1)	**0.0283**
T4	0 (0)	2 (2.6)	NA
N0	48 (98.0)	68 (87.2)	**0.0497**
N1	0 (0)	5 (6.4)	0.1555
N2	1 (2)	5 (6.4)	0.4044
M0	48 (98.0)	49 (62.8)	**<0.0001**
M1	1 (2)	29 (37.2)	
Microscopic invasion[b], n (%)			
Vascular	3 (6.3)	20 (29.0)	**0.0038**
Lymphatic	0 (0)	3 (4.7)	0.2587

Significant P-values are indicated in bold style. NA, not available. [a]T staging was based upon the pathological assessment of surgical specimen except for a single case, for which histology (clear cell subtype) was diagnosed by needle biopsy and staging (T4) were radiographically assessed. N and M staging are based upon radiographical assessment. [b]Due to diagnostic limitation, not all cases were available for assessment (a: 48 cases, b: 69 cases and c: 64 cases). C5aR, C5a receptor.

cated concentrations when appropriate. The numbers of cells that migrated through the membrane were counted in 4 microscopic fields (x20 magnification) per membrane. The average was calculated from triplicate samples, and statistical analyses were performed by two-tailed t-tests.

Results

Frequent expression of the C5a receptor in metastatic renal cell carcinoma. First, we analyzed C5aR expression in 127 primary RCC specimens using surgically removed or needle biopsy samples. Overall, the C5aR expression was observed in 78 out of 127 samples (61.4%). This sample cohort consisted of 97 RCCs without metastasis and 30 mRCC (Fig. 1). As shown in Table I, there was no significant difference between the C5aR-positive and -negative group in regards to age, gender and histological subtype. Regarding Fuhrman grade, although the grade 3 population was quite low (n=6) in this sample cohort, C5aR-positive tumors tended to exhibit a higher grade than the C5aR-negative tumors (G1: 21.8 vs. 46.9%, G2: 69.2 vs. 49.0%). Regarding T staging, the C5aR-positive group contained significantly less T1 tumors (P=0.0056) and more T3 tumors (P=0.0283) than the C5aR-negative group. As for N staging, more C5aR-negative tumors were without lymph node metastasis (N0) than the C5aR-positive tumors (98.0 vs. 87.2%) with marginal significance (P=0.0497). Distant metastasis was more frequently observed in the C5aR-positive group than in the C5aR-negative group (37.2 vs. 2%). Of note, 96.7% (29/30) of the patients with metastatic disease showed C5aR expression at their primary sites. With regards to microscopic invasion, more C5aR-positive tumors manifested microvascular invasion than C5aR-negative tumors (29.0 vs. 6.3%). These results suggest that C5aR expression contributes to local invasion and distant metastasis of renal cell carcinoma.

C5a-C5aR axis is not involved in Renca cell proliferation and anoixis. To study the biological role of the C5a-C5aR axis in renal cell carcinoma, we employed Renca cells since these cells are the established model for studying renal cell carcinoma metastasis (11,13). Interestingly, both western blotting and flow cytometric analysis showed that C5aR was not expressed in Renca cells (data not shown). Such an observation in cancer cell lines, which does not seemingly reflect the characteristics in clinical specimen, was described and discussed in our previous study, suggesting that C5aR expression was lost during the process of cell line establishment from primary culture of cancer cells in order to prioritize the expression of other essential proteins for clonal development in the context of 2-dimensional culture (9). To establish renal cell carcinoma cells expressing C5aR, C5aR cDNA was stably introduced into Renca cells, and clones expressing C5aR were isolated and propagated. Cells expressing C5aR on their cell surface, confirmed by flow cytometric analysis (Fig. 2A), were selected and designated as Renca/C5aR cells. Cells stably transfected with the empty plasmid were designated as Renca/empty cells. The original purpose of establishing Renca/C5aR cells was to study if C5aR can enhance the metastatic behavior of renal carcinoma cells in a syngeneic orthotopic murine model using BALB/c mice since Renca cells are known to establish multiple lung metastasis in this model (11). However, the Renca/C5aR cells, which were stimulated by recombinant C5a and then injected into the renal subcapsular space, did not exhibit any increase in either the number or the size of lung metastatic nests compared to the Renca/empty cells (data not shown). This may be due to the difficulty of attaining sustained C5aR activation by recombinant C5a until establishing lung metastasis, considering the stability of C5a and the period required for tumor cell implantation into the lungs after subcapsular injection. Because of such technical difficulty of the *in vivo* study to analyze the biological role of C5aR expression in renal cell carcinoma metastasis, we analyzed the effect of C5aR on crucial steps of cancer metastasis *in vitro* instead. Anoikis is a subtype of apoptosis induced upon cell detachment from the extracellular matrix, which is an indispensable step for

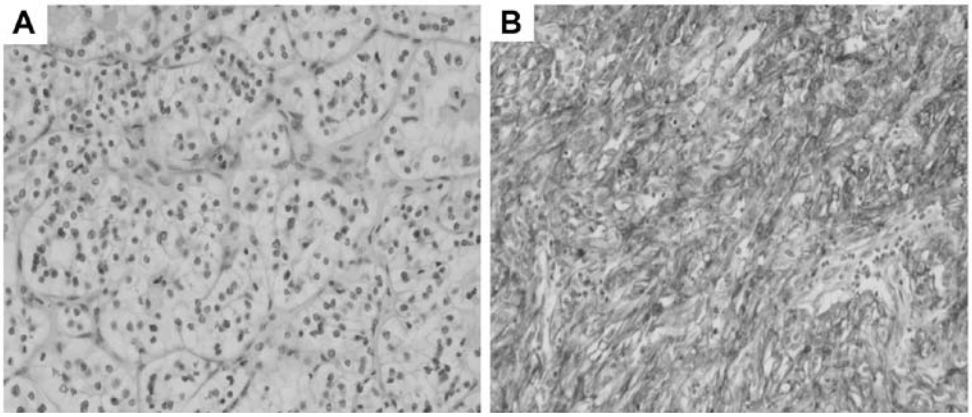

Figure 1. Immunohistochemistry of C5aR in the RCC specimens. FFPE samples of RCC were stained with the anti-C5aR antibody. Representative examples of C5aR-negative RCC without metastasis (A) and C5aR-expressing mRCC (B) are shown. Original, x200 magnification. C5aR, C5a receptor; RCC, renal cell carcinoma; mRCC, metastatic RCC; FFPE, formalin-fixed paraffin-embedded.

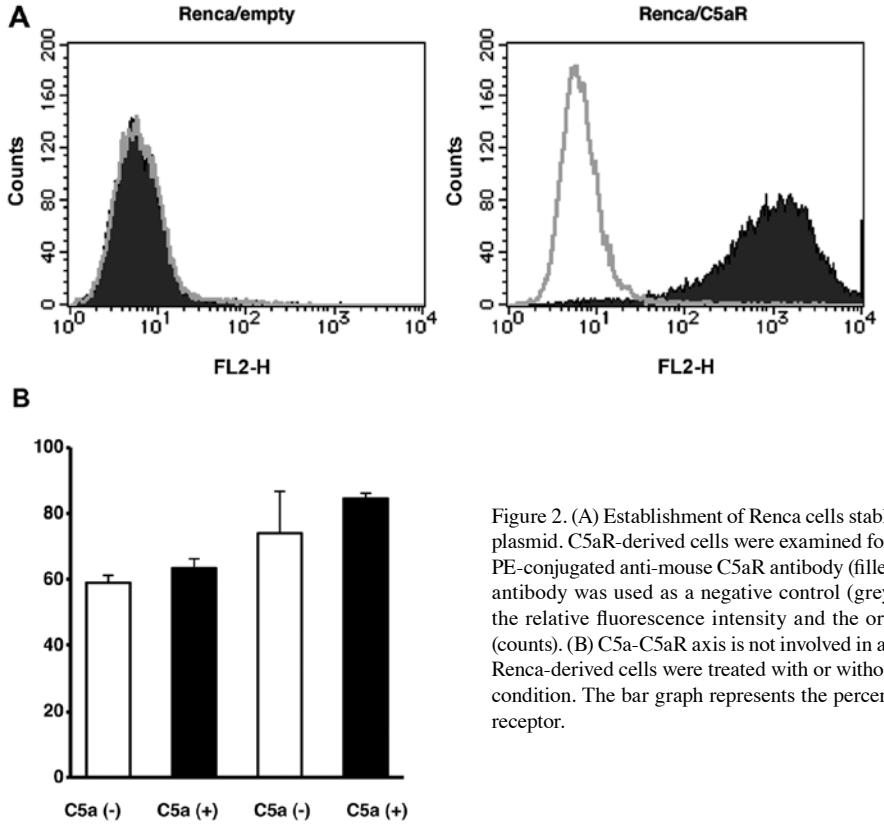

Figure 2. (A) Establishment of Renca cells stably expressing C5aR or the empty plasmid. C5aR-derived cells were examined for C5aR surface expression using PE-conjugated anti-mouse C5aR antibody (filled area). Isotype matched control antibody was used as a negative control (grey line). The abscissa represents the relative fluorescence intensity and the ordinate the relative cell number (counts). (B) C5a-C5aR axis is not involved in anoixis resistance by Renca cells. Renca-derived cells were treated with or without C5a and cultured in a floating condition. The bar graph represents the percentage of viable cells. C5aR, C5a receptor.

metastasis (14). To analyze whether renal carcinoma cells are able to acquire resistance to anoikis by C5a-C5aR axis activation, Renca-derived cells were treated with or without C5a and then cultured in suspension on ultra low-attachment plates and a number of viable cells was assessed. Fig. 2B shows that the percentage of Renca/C5aR cell survival in suspension culture was slightly higher than that of the Renca/empty cells, which was not significantly enhanced by C5a treatment. This result suggests that, although C5aR expression itself may have a marginal effect on cell survival in an adhesion-independent condition, it is unlikely that anoikis plays an important role in the metastasis of C5aR-expressing renal carcinoma cells.

C5a elicits cytoskeletal rearrangement and changes in cellular morphology in C5aR-expressing Renca cells. It is known that the chemoattractant C5a causes actin rearrangement and stimulates the migration of leukocytes (15,16). We previously showed that cancer cells can exploit this mechanism to acquire the ability of migration and invasion by activation of aberrantly expressed C5aR using bile duct carcinoma cells (9). To test the hypothesis that C5aR expressed in renal cell carcinoma facilitates actin reorganization by C5a stimulation as well, the effect of C5aR activation on actin rearrangement in Renca cells was analyzed by F-actin immunofluorescent staining. Without C5a stimulation, both Renca/empty and Renca/C5aR cells showed

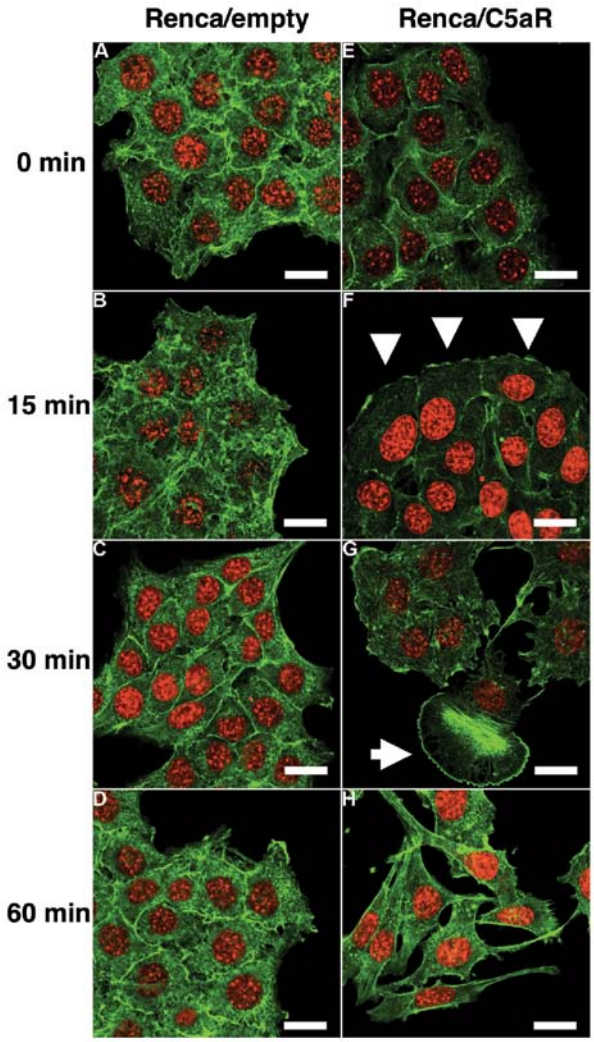

Figure 3. C5a elicits cytoskeletal rearrangement and changes in cellular morphology in the C5aR-expressing Renca cells. Renca/empty cells (A-D) and Renca/C5aR cells (E-G) were incubated with C5a (10 nM) and fixed at the indicated time-points. F-actin was visualized by immunofluorescent staining with Alexa 488-conjugated phalloidin (green), and nuclei with TO-PRO-3 (red). Scale bars, 20 μm. Arrowheads and the arrow indicate membrane ruffing and lamellipodia, respectively. C5aR, C5a receptor.

staining of cortical F-actin bundles at the borders of the cells (Fig. 3A and E). As early as 15 min after C5a stimulation, Renca/C5aR cells revealed membrane ruffling formation at the periphery of the cell clusters with reduced F-actin bundles at the cell-cell border (Fig. 3F). Thirty minutes after stimulation, the cell-cell contact became more loosened and some cells started to manifest lamellipodia formation (Fig. 3G). This was followed by marked change in cell shape such as stretched morphology and dissociation of cell clusters accompanied by stress fiber formation (Fig. 3H). In contrast, Renca/empty cells did not show any significant changes in both cytoskeleton and cellular morphology during observation despite C5a stimulation (Fig. 3A-D). These results suggest that C5a elicits cytoskeletal rearrangement and cellular morphological change in renal carcinoma cells via C5aR, leading to their dissociation and scattering.

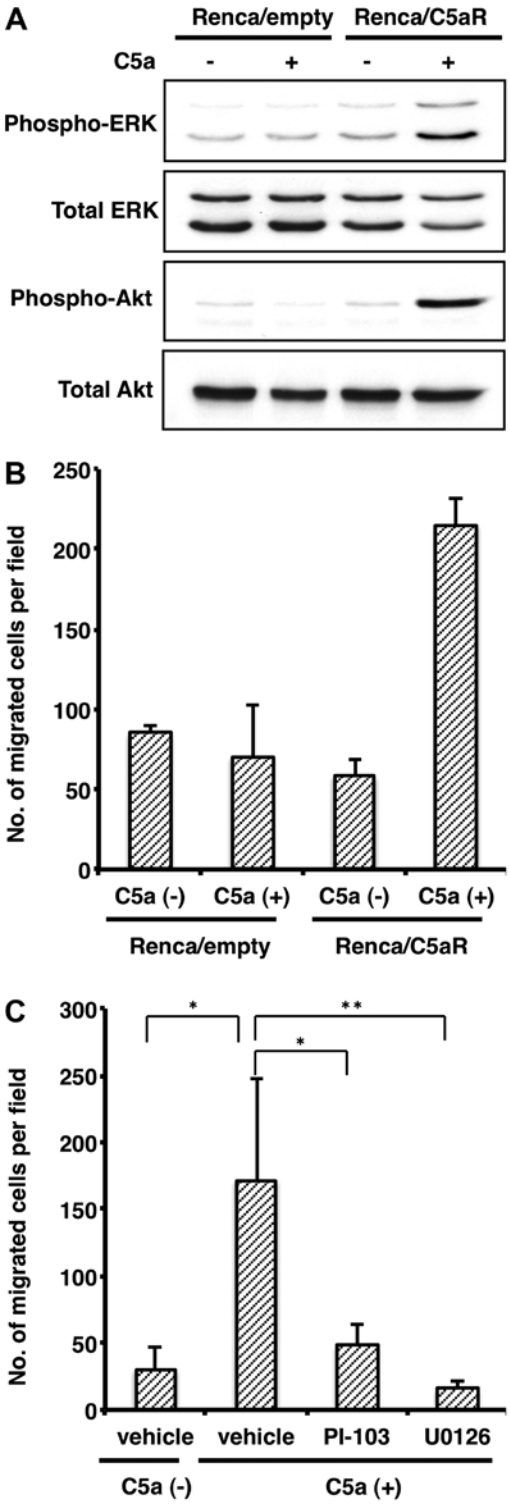

Figure 4. C5a-C5aR axis triggers ERK and PI3K-dependent cellular invasion. (A) Renca-derived cells were stimulated with or without 10 nM C5a for 15 min after serum starvation for 24 h. Samples were harvested and subjected to immunoblotting using antibodies indicated. (B and C) Invasion assays were carried out using (B) Renca/empty and Renca/C5aR cells or (C) Renca/C5aR cells only. (C) Medium in both the upper and lower wells were mixed with the indicated kinase inhibitors or vehicle (DMSO). U0126 was used at 20 μM and PI-103 was used at 0.5 μM. Data are presented as the mean ± SD. *P<0.02 and **P<0.01. C5aR, C5a receptor.

C5a-C5aR axis enhances Renca cell invasion via the ERK and PI3K pathways. C5a is known to induce ERK (5,17) and PI3K activation (6,18) in inflammatory/immune cells in a C5aR-dependent manner, leading to manifestation of a variety

of biological phenomena including leukocyte migration (19). Since ERK and PI3K are crucial mediators of extracellular stimuli-induced actin reorganization (20,21) which was observed in Fig. 3, we investigated if C5a provokes ERK and PI3K activation in C5aR-expressing renal carcinoma cells. As shown in Fig. 4A, Renca/C5aR cells showed a robust increase in ERK and Akt, substrate of PI3K, phosphorylation by C5a stimuli, whereas such phosphorylation did not occur in the Renca-empty cells. This result indicates that the C5a-C5aR axis can activate ERK and PI3K kinase pathways in renal carcinoma cells. We previously reported that the C5a-C5aR axis enhances cancer cell invasion both *in vitro* and *in vivo* (9). To test if this is also the case in renal carcinoma cells, we performed an invasion assay using a Matrigel-coated Boyden chamber. Fig. 4B shows that C5a stimuli elicited invasion of the Renca/C5aR cells, but not the Renca-empty cells, in the Matrigel-coated boyden chamber, indicating that the C5a-C5aR axis can enhance invasion of renal cell carcinoma as well. In addition, treatment with either an MEK inhibitor U0126 or a PI3K inhibitor PI-103 significantly diminished Renca cell invasion promoted by the C5a-C5aR axis (Fig. 4C). This result indicates that the ERK and PI3K pathways are indispensable for C5a-triggered C5aR-expressing renal carcinoma cell invasion. All things considered, these results suggest that the C5a-C5aR axis elicits renal carcinoma cell metastasis by triggering actin reorganization and invasion induced by ERK and PI3K pathway activation.

Discussion

C5aR was originally identified in cells with a myeloid origin, and it has been shown by numerous studies that it mediates a wide variety of biological phenomena in myeloid cells induced by C5a such as leukocyte migration upon inflammation. However, Cao *et al* (5) reported an intriguing finding that the C5aR is also expressed in epithelial cells, which suggested the involvement of C5aR in other biological processes in non-myeloid cells. Recently, we showed that C5aR is aberrantly expressed in various types of human cancers (9), which was the first study regarding the biological significance of C5aR expression in cancer cells. In that study, we also showed that ~60% of RCC specimens expressed C5aR. However, the sample number of RCC specimens in that study was limited (n=11) and not sufficient to analyze the relationship between C5aR expression status and clinical parameters. In this study, we investigated 127 RCC samples using immunohistochemistry and found a similar frequency of C5aR expression (61.4%; 78/127) as in the previous study. It is of note that around half of adjacent normal kidney tubular epithelial cells already express C5aR (9), which was confirmed in this expanded analysis (data not shown). At the moment, the biological significance of C5aR expression in normal renal tubular epithelial cells is unknown. The fact that around 40% of RCC samples (49/127) did not express C5aR, implies that C5aR could be a sublineage marker of renal tubular epithelial cells and the expression status of C5aR in renal cell carcinoma may be stochastic and reflect its sublineage rather than a consequence of malignant transformation. Angelotti *et al* (22) reported that there are 2 subpopulations of renal progenitors with the potential to regenerate tubular epithelial cells. It would be intriguing if C5a-positive renal tubular epithelial cells represent either of those populations.

In the study cohort we examined by immunohistochemistry, C5aR-positive RCCs manifested both a locally invasive and metastatic phenotype with a higher incidence of microvascular invasion. In addition, we demonstrated that C5a-C5aR axis activation elicited invasion using an *in vitro* invasion assay with a renal carcinoma cell line. Hence C5aR appears to facilitate local invasion to adjacent tissues and microvascular infiltration thereby promoting distant metastasis of renal carcinoma cells. Generally, metastasis requires a number of steps including dissociation of cancer cells from primary sites, invasion through basement membrane and into blood/lymphatic vessels, survival when floating in blood/lymphatic stream, and implantation and proliferation in distant target organs (23). From both clinical and experimental results shown in the present study, it is plausible that C5aR expression contributes at least to the invasion steps in this metastasis model.

We performed renal subcapsular injection of Renca-derived cells in BALB/c mice to examine if C5aR expression promotes spontaneous renal carcinoma cell metastasis in a syngeneic orthotopic murine model. Although we were able to observe lung metastasis using this model as reported in the literature (11), we could not find any difference in either the size or the number of metastatic foci in the lung between Renca/empty and Renca/C5aR cells regardless of recombinant C5a treatment. Our previous study showed an increased invasion of subcutaneously injected C5aR-expressing HuCCT1 cells in nude mice compared to HuCCT1 cells harboring empty plasmid (9). In the latter assay, the 2-day period was sufficient to observe a significant increase in local invasion. However, in the case of the syngeneic orthotopic murine model used in the present study, it required up to 3 weeks to observe spontaneous lung metastasis (11), which may be sufficiently long to offset the effect of the recombinant C5a stimulation to Renca/C5aR cells before injection. In order to appropriately assess the effect of C5a-C5aR axis activation on spontaneous metastasis in this murine model, improved experimental methods may be required for sustained activation of C5aR.

Acquisition of anoikis resistance by cancer cells is also an important step for establishing cancer metastasis because cancer cells have to survive in the floating condition during their travel through the circulatory and lymphatic systems until implantation to target organs (14). These facts led us to analyze further whether C5a-C5aR axis activation contributes to this cellular phenomenon. However, C5a stimulation did not have a significant impact on anoikis resistance in the renal carcinoma cells regardless of C5aR expression. Therefore we concluded that the C5a-C5aR axis does not play a significant role in acquiring anoikis-resistance in mRCC.

We showed that C5a induced dynamic reorganization of actin cytoskeleton in the C5aR-expressing renal carcinoma cells, namely, lamellipodia and stress fiber formation, resulting in dissociation of cell clusters and scattering. It is well known that these processes are mediated by activation of Rho family small G proteins such as Rac1 and RhoA (24). Li *et al* (25) previously reported that C5a induced activation of Rho family small G proteins in neutrophils, leading to actin reorganization of the cell, suggesting that the C5a-C5aR axis is one of the upstream switches of Rho family protein activation. It would

be of interest to analyze if Rho family proteins are activated when actin rearrangement is induced in renal carcinoma cells expressing C5aR by C5a stimulation.

In addition to inducing actin rearrangement, C5a elicited invasion of C5aR-expressing renal carcinoma cells in a Matrigel-coated Boyden chamber. Therefore, it is plausible that C5a-C5aR may be involved in the metastasis of renal cell carcinoma by prompting dissociation of cancer cells from primary sites and invasion through the basement membrane. Furthermore, C5a stimulation activated the ERK and PI3K pathways and inhibition of these kinase pathways by specific inhibitors negated the C5aR-expressing renal carcinoma cell invasion. These pathways are known to be activated by C5a stimulation in cells with myeloid origin to regulate numerous biological phenomena (19). In the present study, we showed that such activation does occur in renal carcinoma cells. This is the first study to show that the C5a-C5aR axis does trigger activation of these kinase pathways and invasion in cancer cells. Campbell *et al* (26) reported that phosphorylated ERK is an independent prognostic biomarker that significantly predicts the onset of metastasis in clinically confined RCC, and Horiguchi *et al* (27) showed that phosphorylated Akt is significantly associated with RCC metastasis. These studies are consistent with our finding that C5aR, which can trigger ERK and PI3K activation by C5a stimulation, is expressed in a vast majority of clinical mRCC specimens.

The present study provides the proof-of-concept that the C5a-C5aR axis can be a novel target for preventing renal cell carcinoma progression as well as further support of the current therapeutic concept to target the ERK and PI3K/mTOR pathways in mRCC (28). Clinical application of this concept may contribute to develop novel therapeutic strategies for advanced RCC in the future.

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