by SDS-PAGE under reducing conditions using 10% polyacrylamide gels and were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon Transfer Membranes; Millipore). After blocking with 5% fat-free milk, the membranes were incubated with anti-human C5aR rabbit IgG (1000-fold dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal anti-actin rabbit IgG (500-fold dilution; Santa Cruz Biotechnology). This was followed by incubation with HRP-conjugated anti-rabbit IgG goat antibody (1000-fold dilution; Amersham Biosciences, Blauvelt, NY) and bands were visualized via enhanced chemiluminescence (ECL), according to the manufacturer's instructions.

Establishment of C5aR stably expressing HuCCT1 cells.

Full-length human C5aR cDNA of 1053 bp was amplified by PCR using human macrophage cDNA library and subsequently subcloned into the pENTR/D-TOPO-vector (Invitrogen, Carlsbad, CA). After confirming the sequence, the cDNA was inserted into pCAG-IRES-puro vector using the Gateway system (Invitrogen). The purified plasmid was transfected into HuCCT1 cells using

Lipofectamine 2000 (Invitrogen). After 48 h, medium was replaced with selection medium supplemented with puromycin (1 μg/mL) to be cultured for 2 weeks. Puromycin resistant cells were collected and were subjected to cell sorting by FACS Vantage to isolate those cells highly expressing C5aR (designated HuCCT1/C5aR). HuCCT1 cells transfected with empty-pCAG-IRES-puro vectors were used as the control (HuCCT1/mock).

Flow cytometric analysis.

MEC, HuCCT1/mock or HuCCT1/C5aR cells were treated for 30 min with a murine monoclonal FITC-conjugated anti-C5aR antibody (Serotec Ltd., Oxford, UK), or a FITC-conjugated isotype matched control antibody (Serotec Ltd.), followed by washing with PBS twice. C5aR antigen was quantified by FACScan (BD Biosciences).

Immunofluorescence analysis.

Filamentous actin (F-actin) formation was visualized as previously described (24). Cells were seeded at a low density on glass coverslips and were incubated for 24 h. After 2 h

serum starvation, cells were stimulated with 100 nM human C5a (Sigma, St Louis, MO) for the stated time periods. 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 (Molecular Probes, Eugene, OR) for 40 min, followed by washing with PBS. Images were obtained and processed by FluoView 300 Laser Scanning Confocal Microscope (Olympus, Melville, NY).

Time-lapse video analysis.

Cells (1 x 10^4/well in RPMI1640) were cultured in a 24 well-glass bottom plate (Iwaki, Funabashi, Japan) for 24 h. After addition of C5a (final concentration: 100 nM), cells were maintained at 37˚C in 5% CO_2 within the chamber set under the camera during the observation. Images were obtained using 20X UPlan SApo objective (Olympus IX81, Tokyo, Japan). The camera, shutters, and filter wheel were controlled by MetaMorph imaging software (Molecular Devices, Sunnyvale, CA), and images were collected every 10 min with an exposure time of 50 ms. Cell migration distance was measured by tracing individual cells using MetaMorph imaging software according to the

manufacturer's instructions.

Invasion assay *in vitro*.

To assess invasion of cancer cell lines *in vitro,* BioCoat Matrigel invasion chambers were utilized (24-well plate, 8-μm pore; BD Biosciences) (25). HuCCT1-derived (3.75×10^4 cells), or MEC (7.5×10^4 cells) cells were suspended in serum-free RPMI 1640 then seeded into the upper chamber. RPMI 1640 supplemented with either C5a or carrier solution (PBS) was placed in the lower chamber. To block C5aR-mediated signaling, anti-human C5aR rabbit IgG (10 μg/mL) or nonspecific control IgG (10 μg/mL) was added to the cell suspension before seeding. For analyzing the effect of discontinuous stimulation with C5a, cells were cultured at 37°C in serum-free RPMI 1640 supplemented with C5a for 12 h (HuCCT1-derived cells) or 24 h (MEC) at indicated concentrations. Cells were then washed with serum-free RPMI 1640 and were seeded into the upper chamber. RPMI 1640 containing 10% FBS was set in the lower chamber. Chambers were incubated for 24 h (HuCCT1-derived cells) or 36 h (MEC) at 37°C. Cells on the upper surface of the filter were removed with a cotton wool swab,

and cells that migrated to the lower surface were fixed in 100% methanol and were stained with 1% toluidine blue. Invaded cells were counted in five power fields (×20). The invasion-enhancing effect was shown as the ratio of cell invasion by C5a stimulation versus PBS controls. To determine whether MMPs were involved in C5a-elicited cancer cell invasion, GM6001 (5 μM; Merck, Darmstadt, Germany) was added to the cell suspension when cell invasion activity of 100 nM C5a was measured. For checkerboard analysis for C5a cancer cell invasion activity, various concentrations of C5a were added to the HuCCT1/C5aR cell suspension in the upper chamber together with the lower chamber, and cell invasion was assessed as described above.

Invasion assay *in vivo*.

HuCCT1/mock and HuCCT1/C5aR were incubated in serum-free medium in the presence or absence of C5a (10^{-7} M) at 37°C for 12 h. This was followed by labeling with CellTracker™ Orange CMTMR (20 μM) or CellTracker™ Green BODIFY (25 μM) (Molecular Probes) for HuCCT1/C5aR and HuCCT1/mock, respectively, at 37°C for 45 min. After washing with serum-free medium, HuCCT1/mock cells and

HuCCT1/C5aR cells were equally mixed to create a concentration of 3×10^7 cells/mL each. The cell mixture (50 μL) was injected intradermally into 7-week-old BALB/cA Jcl-nu/nu mice (CLEA Japan, Tokyo). After 1, 2, or 3 days, the nude mice were sacrificed by cervical dislocation, and the cell injection sites including surrounding tissues were excised to prepare frozen sections in liquid nitrogen. Labeled cells in 4-μm-thick sections were observed with a fluorescence microscope (BIOREVO; KEYENCE, Osaka, Japan). To quantify the distribution of HuCCT1-derived cells, regions of fluorescent dots of labeled cells were encircled (Fig. 6*A*) then the area of each region was measured using an imaging processor (VH-Analyzer; KEYENCE). The ratio of the distribution area of HuCCT1/C5aR versus HuCCT1/mock was calculated. Some endogenous green fluorescence background was observed in mice skin, therefore these spots were avoided and cancer cells were specifically encircled, which was confirmed by observation of the adjacent section HE-stained. This experiment was performed according to the criteria of animal experiments of the Kumamoto University Animal Experiment Committee.

Measurement of MMP concentration in culture supernatant of cells.

MMP concentration in supernatant of cancer cells stimulated with or without C5a was measured using the Quantibody Human MMP Array 1 kit (RayBiotech, Norcross, GA). Culture supernatant was taken from MEC (1×10^6 cells) or HuCCT1/C5aR (5×10^5 cells) grown in a 6-well plate for 24 h in the presence or absence of C5a (100 nM). The supernatant was diluted at 1:3 with PBS then MMP concentrations were determined according to the manufacture's instructions.

Statistics.

Statistical analyses were performed using the unpaired Student's *t*-test. Values are expressed as means ± SD and experiments were performed in triplicate, unless otherwise stated.

RESULTS

Aberrant expression of C5aR in human cancer cells.

We first investigated C5aR expression in human cancer specimens from 225

patients by immunohistochemistry. C5aR expression was observed in cancer cells from all the organs examined and in all the three cancer cell types, squamous cell carcinoma, adenocarcinoma and transitional cell carcinoma (Fig. 1*A* and supplementary Fig. 1*A*). Generally, C5aR was robustly expressed in a significant proportion of cancer samples. On the other hand, null or only faint reaction of C5aR immunohistochemistry was observed in their normal counterparts (Fig. 1, *A* and supplementary Fig. 1*B*) except kidney tubular epithelial cells (Supplementary Fig. 1*B*), which is in line with the previous report (16). Percentage of C5aR-positivity in cancer cases varied among organs. In colon, bile duct, kidney and prostate carcinomas, more than 50% of cases examined were C5aR-positive (Fig. 1*B*). In bile duct-derived cancer in the liver, C5aR was positive in 26 patients. Among them, vascular invasion was found in 18 patients, whereas vascular invasion was seen in only 4 cases out of 16 C5aR-negative patients. This result indicates a significant relationship between cancer cell C5aR expression and vascular invasiveness ($p = 0.010$ by Fisher's exact test). Since vascular invasion of bile duct cancer is closely linked to metastasis and prognosis (26), C5aR expression may correlate with those clinical endpoints. Next, we examined a panel of cancer cell lines

for C5aR expression. RT-PCR revealed that several human cancer cell lines originated from bile ducts (MEC and RBE) and colon (HCT15, COLO205, and HCT116) expressed C5aR mRNA (Fig. 2*A*). Out of these cell lines, those except RBE also expressed C5aR protein (Fig. 2*B*). The localization of C5aR on the cell-surface was shown by flow cytometry (Fig. 2*C*). These results suggest that aberrant C5aR expression observed in human cancer specimens is actually conserved in some human cancer cell lines. It is intriguing that only MEC cells express C5aR at the protein level among the bile duct-derived cancer cell lines (Fig. 2*B*), whereas bile duct carcinomas showed the highest positive ratio of C5aR expression (Fig. 1*B*). This fact seems paradoxical to the result of immunohistochemistry at a glance (Figs. 1, *A* and *B*), implying that C5aR expression was lost during the process of cell line establishment from primary culture of human cancer cells, as those cells can prioritize expression of other essential proteins for clonal development in the context of two-dimensional culture *in vitro*, which is usually in the absence of C5a.

Cytoskeletal rearrangement and enhanced motility of C5aR-expressing cancer

cells by C5a stimulus.

In order to analyze the biological effects of C5aR expression in cancer cells under C5a stimuli, we chose C5aR-negative HuCCT1 cells derived from bile duct carcinomas of the highest C5aR expression ratio (Fig. 1*B*). HuCCT1/C5aR cells but not HuCCT1/mock cells expressed C5aR (Figs. 2, *A* and *B*). The cell surface expression of C5aR in HuCCT1/C5aR cells was confirmed by flow cytometric analysis (Fig. 2*C*), which was comparable with that in MEC (Fig. 2*C*). Since the chemoattractant C5a causes cytoskeletal rearrangement and stimulates migration of leukocytes (27, 28), we hypothesized that cancer cells may exploit this mechanism to gain the ability of migration and invasion by activation of aberrantly expressed C5aR on their cell surface. To test this, the effect of C5aR activation on actin rearrangement was analyzed by F-actin immunofluorescence labeling. The majority of cells at the outer edges of HuCCT1/C5aR cell clusters clearly showed strong filopodia formation 30 min after C5a treatment (Fig. 3*A*), which was followed by development of membrane ruffling and dissolution of stress fibers (Fig. 3*A*). Three hours after the treatment, such ruffles disappeared and formation of stress fibers became evident again (Fig. 3*A*). Some cells at

the periphery of the cluster even changed their morphology to spindle-like shape and protruded from the cluster to the vacant area (Fig. 3*A*). On the other hand, HuCCT1/mock cells did not show any remarkable changes in both cell morphology and actin cytoskeleton at any time points after C5a stimulation (Fig. 3*A*). The time-lapse video analysis of HuCCT1/C5aR cell movement demonstrated that C5a activated motility of the cells (Fig. 3*B* and supplementary Fig. 2 video). Tracing of cell movement revealed that C5a enhanced motility of HuCCT1/C5aR cells in a dose-dependent manner, increasing motility 3-fold at 100 nM (Figure 3*C*), whereas motility of HuCCT1/mock cells was not significantly affected by C5a (Figs. 3*B* and 3*C*). This experiment confirmed that C5a enhances motility of cancer cells in a C5aR-dependent fashion.

Enhanced invasiveness of C5aR-expressing cancer cells by C5a stimulus *in vitro*.

Experiments using Matrigel chambers revealed that C5a stimulated invasion of HuCCT1/C5aR cells through the matrix layer in a C5a concentration-dependent manner and enhanced approximately 13-fold over carrier control (PBS) at 10 nM (Fig. 4*A*). The

enhancing effect of C5a on invasion of HuCCT1/mock cells was not seen (Fig. 4*A*). Intriguingly, cancer cells pretreated with C5a also demonstrated enhanced invasiveness even in the absence of a C5a concentration gradient (Figs. 4*C*). This result indicates that stimulation with C5a during pretreatment is sufficient for enhancing invasion of C5aR-expressing cancer cells *in vitro*, and suggests that neither concentration gradient nor continuous exposure to C5a is required for activating invasion of C5aR-expressing cancer cells. Similarly, C5a enhanced invasion of MEC cells that endogenously express C5aR (Fig. 2*C*), in a C5aR-dependent manner, since this enhancement was abrogated by a C5aR antagonist (Supplementary Fig. 3) and by the neutralizing antibody against C5aR, but not by nonspecific IgG (Figs. 4, *B* and *D*). Dose-dependent effect of C5a on invasion was also the case observed in MEC cells (Figs. 4, *B* and *D*). To determine whether C5a-elicited cancer cell migration is dependent on the C5a concentration gradient, we performed the checkerboard analysis. In addition to C5a concentration-dependent invasion in the absence of C5a in the upper chamber, HuCCT1/C5aR cell invasion by 100 nM C5a in the lower chamber was inhibited by C5a in the upper chamber in a dose-dependent manner (Fig. 4*E*). Moreover, invasion by

10 nM C5a in the lower chamber was completely inhibited by 100 nM C5a in the upper chamber (Fig. 4*E*). These results suggest that C5a-induced cancer cell invasion is explained partly by chemotaxis, particularly in the presence of 100 nM C5a. However, when C5a concentration in the upper chamber was equal to that in the lower chamber, C5a was still able to induce HuCCT1/C5aR invasion to the significant extent. The invasion by 10 nM C5a in the lower chamber was not affected by addition of 10 nM C5a in the upper chamber (Fig. 4*E*). Furthermore, when 100 nM C5a was added to the cell suspension in the upper chamber, it triggered significant cancer cell migration even in the absence of C5a in the lower chamber (Fig. 4*E*). These results suggest that enhanced random cell locomotion plays a vital role in the C5a-elicited cancer cell invasion.

C5a elicits MMP secretion from C5aR-expressing cancer cells.

Degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is an essential process for cancer cell invasion (29). The interaction of the chemokine CXCL12 and its receptor CXCR4 has been shown to increase MMP expression and

invasion of prostate cancer cells (30). While C5a induces MMP-9 release from human neutrophils (31), such C5a-elicited MMP release from cancer cells has not been reported. Interestingly, C5a-enhanced invasion of C5aR expressing cancer cells in the transwell chambers was significantly hindered by an MMP inhibitor GM6001 (32) (Figs. 4*A* and 4*B*), indicating that enzymatic activity of MMPs plays a crucial role in the C5a-enhanced invasion of C5aR-expressing cancer cells. Hence we explored the possibility of C5a provoking MMP secretion from C5aR-expressing cancer cells. MMP expression array analysis showed that C5a significantly increased release of MMP-1, 3, 9, 10, and 13 from MEC cells, and MMP-8 and 10 from HuCCT1/C5aR cells (Table 1). These MMPs are known to be associated with both cancer invasion and patient prognosis (33, 34). Together with inhibition of C5a-enhanced invasion by GM6001 (Figs. 4*A* and 4*B*), increased secretion of MMPs by C5a (Table 1) indicates that MMPs contribute to the C5a-enhanced invasion of C5aR expressing cancer cells. Among specific MMP inhibitors, an MMP-8 inhibitor exhibited the most significant effect to impede the MEC cell invasion enhanced by C5 (Supplementary Fig. 3). Intriguingly, about a 3.2-fold increase in MEC cell invasion induced by C5a at 10 nM (Fig. 4B)

appeared to correlate with a 3.7-fold increase in MMP-8 (Table 1). These results suggest that MMP-8 is the most responsible MMP for C5a-elicited MEC cell invasion.

Enhanced invasiveness of C5aR-expressing cancer cells by C5a *in vivo*.

To evaluate the effect of C5a stimulation on the invasiveness of C5aR expressing cancer cells *in vivo*, HuCCT1/mock and HuCCT1/C5aR cells were pretreated with C5a, labeled with green or orange fluorescent dyes respectively, then mixed to be injected into nude mice skin. This assay system enables direct comparison of spreading *in situ* between two different sublines. This assay revealed that C5a-treated HuCCT1/C5aR cells spread more broadly than C5a-treated HuCCT1/mock cells in nude mice skin tissue (Fig. 5*A*). The most evident induction of the spreading of HuCCT1/C5aR by C5a was observed at day 2, which was a 1.8-fold increase over HuCCT1/mock (Fig. 5*B*). In contrast, there was no difference in spreading between those two sublines of HuCCT1 when they were not treated with C5a (Figs. 5*A* and 5*B*). This result suggests that C5a enhances invasion of C5aR expressing cancer cells *in vivo* as well as *in vitro*, and again stimulation with C5a before injection is sufficient for C5aR expressing cancer cells to

demonstrate such enhanced invasiveness.

DISCUSSION

C5-derived fragments were reported to enhance cancer cell locomotion, however, these were not identified as C5a when such reports were made. Rather, they appeared not to be C5a because of its molecular weight and lack of chemotactic activity for leukocytes (35, 36). When these studies were performed, the C5aR molecule had not been identified, and neither recombinant C5a nor C5aR-specific antibodies were available to prove the activity of C5a to enhance cancer cell migration. In the present study, we have demonstrated several lines of evidence indicating a crucial role of C5a-C5aR interaction in cancer cell invasion: [1] C5aR expression was observed in cancer cells from patients' tissues and in various human cancer cell lines (Figs. 1 and 2), [2] recombinant C5a enhanced cancer cell motility (Fig. 3) and invasion both *in vitro* (Fig. 4) and *in vivo* (Fig. 5), and [3] enhanced cancer cell invasiveness elicited by the C5a-C5aR axis was dependent on the increased release of MMPs (Fig. 4 and Table 1), proteases that are indispensable for cancer cell invasion towards surrounding tissues

(29). C5aR expression is essential for cancer cells of epithelial origin to enhance motility and invasiveness by C5a, given that C5aR expression is required for any remarkable changes in cell morphology (Fig. 3*A*) and enhanced invasiveness (Figs. 4*A* and 4*C*) in HuCCT1 cells after C5a stimulation (Figs. 5*A* and 5*B*). In addition, a C5aR antagonist (Supplementary Fig. 3) and by a neutralizing antibody against C5aR (Figs. 4, *B* and *D*) abrogated C5a enhanced invasion of MEC cells. These data are consistent with the phenomenon that C5a enhances cancer cell invasion via C5aR, and to our knowledge, this is the first report that shows the biological role of the C5a-C5aR axis in human cancer cell invasion.

C5aR being expressed in cancerous cells but not normal epithelial cells except kidney proximal tubular epithelial cells in human tissue specimens (Fig. 1*A*, and supplementary Fig. 1*A*) may indicate C5aR expression to be a consequence of malignant transformation. A similar example is CXCR4: the receptor of CXCL12 that is a potent chemoattractant like C5a and is produced in the cancer microenvironment (20). This receptor is commonly found in cancer cells and its expression is induced by factors such as hypoxia, vascular endothelial growth factor and estrogen in the cancer

microenvironment. CXCR4 expression is also activated by mutations in genes that alter levels of hypoxia-inducible factors, and gene fusion events. IL-6-induced C5aR expression in rat hepatocytes (37) suggests that C5aR can be expressed in response to specific cytokines that are rich in the cancer microenvironment (20, 38). However, IL-6 and IFN-γ did not induce C5aR expression in HuCCT1 cells (unpublished data). This may suggest that C5aR expression is dependent on genetic events characteristic in individual cancers. Such differences might reflect variation in C5aR-positivity in different primary organs (Fig. 1*B*).

Leaky cancer vasculature facilitates the supply of the complement system components from the bloodstream to cancer tissues (39), where as shown in an animal cancer model (2), C5a is generated through activation of the complement system in response to cancer cells (3), although they are protected from complement attack by complement regulators (6). Indeed, C5a is detectable in human plasma incubated with MEC or HuCCT1 cells *in vitro* (Supplementary Fig. 4). Besides this pathway, C5a is possibly generated directly from C5 through thrombin-dependent cleavage (12), following the coagulation reaction initiated by tissue factor that can be expressed on

cells in cancer tissues including cancer cells, fibroblasts and activated leukocytes (13). C5a can also be generated from C5 by a serine protease from activated phagocytes (14) recruited to the cancer tissue. In addition, compared with tightly adhering non-cancerous epithelial cells, the loose cell-to-cell contact of cancer cells allows the generated C5a to access the cancer cell membrane, enabling it to bind to C5aR. Thus, C5a produced in the cancer microenvironment can be predicted to activate C5aR-positive cancer cells to promote migration from the primary site.

C5a induced dynamic sequential reorganization of actin cytoskeleton in C5aR-expressing cancer cells, namely, filopodia formation, membrane ruffling then formation of stress fibers (Fig. 4*A*). These processes have been reported to be provoked by activation of Cdc42, Rac1 and RhoA, respectively (40). Such sequential activation of these small G proteins, which is a robust driving force for cell movement, has been documented previously (41). In fact, C5a induces activation of Cdc42 and Rac1 in neutrophils, leading to actin reorganization of the cell (42). We are currently studying if the C5a-C5aR axis can activate upstream signaling pathways of those small G proteins in cancer cells.

Together with motility stimulation in the C5a-containing culture medium (Fig. 3), increased invasiveness of C5a-treated C5aR-positive cancer cells in the matrix gel (Fig. 4) and in nude mouse skin (Fig. 5) in the absence of a C5a concentration gradient suggest that C5a enhances cancer cell random locomotion instead of inducing chemotaxis, which is supported by the checkerboard analysis (Fig. 4*E*). If C5a were only chemotactic for cancer cells, stimulated cells would be expected to assemble in the primary cancer site where C5a is released and enriched; thus C5a would hinder cancer cell spread. In contrast, enhanced random cell locomotion would be more relevant for promoting cancer cell invasion and spreading, namely, the phenomenon that cells leave away from the source of the stimulant. Accordingly, such C5a activity is presumed to favor cancer cell dissemination from the primary site (Fig. 5).

This study implies that the C5a-C5aR axis could be a target for anti-cancer therapy. For instance, depleting C5 with anti-C5 antibodies would suppress C5a generation in cancer tissues. Therefore this suggests that C5aR may also become a possible and feasible target for molecular-based medicine by generating specific antagonists. Such agents may provide useful therapeutic options for cancer treatment in the future.