Abstract. Anaphylatoxin C5a indirectly fosters cancer cells through recruitment of myeloid-derived suppressor cells (MDS) for inhibiting antitumor CD8+ T cells and induction of neovascularization. We recently found activation of cancer cells by C5a directly via the C5a-receptor (C5aR; CD88) to enhance invasiveness. Thus, C5a possibly contributes to cancer progression rather than elimination. C5a generation in cancer tissues has been reported; however, the mechanism is not fully elucidated. Cancer cell expression of complement regulatory molecules suggests inefficient C5a generation through activation of the complement system in response to cancer cells. To explore another C5a generation mechanism in cancer tissues, we examined cancer cells for C5a-releasing activity from C5. C5a was present in C5-supplemented culture media of cancer cells including C5aR-expressing cells, and the media enhanced C5aR-expressing cancer cell invasion, which was abolished by anti-C5a antibody. The C5a-releasing activity was absent in the supernatants of the media and was inhibited by aprotinin, a serine protease inhibitor, and decanoyl-Arg-Val-Lys-Arg-chloromethylketone but not by inhibitors specific for cysteine, acid, or metal proteases. These results indicated C5a release from C5 by a cancer cell membrane-bound serine protease that can cleave peptide bonds at the carboxy-terminal site of paired basic amino acid residues. Cancer cell C5a release from the complement-immobilized plasma supported feasibility of this cancer cell protease-dependent C5a generation in cancer tissues. The new mechanism of C5a generation suggests self-activation of C5aR-expressing cancer cells to enhance invasiveness and induction of MDS recruitment and neovascularization to create a microenvironment favorable for cancer progression.

Introduction

The complement system is one of the defense mechanisms against microorganisms and is also involved in various immune and inflammatory diseases (1). In response to microbes, the complement system is activated through any of the three pathways, eventually causing cytolysis of pathogens. Emerging evidence suggests that the complement system is activated in cancer tissues in human specimens (2,3) and animal models (4,5). Activation of the complement system may be involved in cancer immune surveillance by its direct cytolytic effect (2) and the sensitization of cancer cells to the immune effector cells via release of chemoattractants (6). However, cancer cells can evade damaging complement attack by expressing either soluble or membrane-associated complement regulators (7-9), such as CD55, which protects cancer cells from complement-dependent cytolysis (10,11) and anticancer immune responses (12,13). It is unlikely that the complement system works for cancer cell elimination.

Anaphylatoxin C5a is an N-terminal 74 amino acid fragment of the α-chain of the complement fifth component (C5) and acts as a leukocyte chemoattractant and inflammatory mediator (14,15). A previous report that C5a recruited myeloid-derived suppressor cells for inhibiting the antitumor CD8+ T cell response (4) suggests its indirect role in fostering cancer cells by protecting them from cytotoxic T cells. C5a induces endothelial cell chemotaxis and blood vessel formation (5), promoting neovascularization (16). Thus, C5a creates a favorable microenvironment for cancer progression.

C5a activities are triggered by its binding to C5a receptor (C5aR; CD88) which was originally identified in leukocyte cell lines (17). We recently demonstrated aberrant C5aR expression in cancer cells originated from various organs and revealed enhancement of cancer cell invasiveness via the C5a-C5aR system (18), indicating a supportive role of the anaphylatoxin in cancer progression. However, C5a generation in cancer tissues has not been fully elucidated. C5a generation through activation of the complement system has been reported (4), however cancer cell expression of complement regulatory molecules (7-9) suggests inefficient C5a generation through activation of the complement system in response to cancer cells. In addition to complement activation, thrombin (19) and proteases from bacteria (20) and phagocytes (21) can release C5a directly.
from C5. Cancer cell-derived protease(s) may be capable of releasing C5a. Therefore, we investigated C5a release from C5 by cancer cells as a new C5a generation mechanism in the cancer tissues.

Materials and methods

Materials. Human C5, aprotinin, GM6001, and decanoyl-Arg-Val-Lys-Arg-chloromethylketone were purchased from Calbiochem (San Diego, CA, USA) and the carboxypeptidase N inhibitor (DL-2-mercaptopentyl-3-guanidothiopropionic acid) from Merck (Darmstadt, Germany). Recombinant human C5a was purchased from EMD Millipore (Billerica, MA, USA). Anti-human C5a goat antibody was obtained from R&D Systems (Minneapolis, MN, USA). E-64, pepstatin and phosphoramidon were products of the Peptide Institute (Minoh, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cells. Human bile duct cancer cell lines HuCCT1 and MEC, and the human colon cancer cell line HCT15 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). Human cholangiocarcinoma cell lines, SSp-25, RBE and YSCCC were obtained from the Riken Cell Bank (Tsukuba, Japan). The human colon cancer cell line HCT116 was a gift from Dr B. Vogelstein, John Hopkins University. Other cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan). Other cell lines were obtained from the Riken Cell Bank (Tsukuba, Japan).

Immunoblotting. To detect C5a released from human C5, cancer cells (1x10⁴ cells/100 µl) were cultured in serum-free RPMI-1640 medium supplemented with C5 at the normal plasma concentration (350 nM) at 37˚C for 24 h. MEC and HuCCT1 cells (5x10⁴ cells/100 µl) were cultured in the medium at 37˚C for various periods. HuCCT1 (5x10⁴ cells/100 µl) or MEC (5x10⁴ cells/100 µl) cells were cultured for 24 h at 37˚C in serum-free RPMI-1640 medium and supernatants were incubated at 37˚C for 24 h in the presence of C5 (350 nM). To detect C5a generated from human plasma, citrated human plasma was treated at 56˚C for 30 min; 2 µl of the plasma was used (50).

Release of C5a from C5 by cancer cells. To explore cancer cell C5a release, C5a-negative cancer cells were cultured in RPMI-1640 medium supplemented with C5 and examined the culture medium for C5a. All tested cancer cell lines from bile ducts or colon released C5a from human C5 at its plasma concentration (20) and the C5a-releasing activity varied in cell lines (Fig. 1A). MEC, HCT15 and HCT116 cells, but not the other cell lines, express C5aR (18), suggesting that cancer cells do not always express C5aR together with the protease responsible for the C5a release. By culturing MEC or HuCCT1 cells, C5a concentrations in the C5-supplemented medium reached a maximum in 6-12 h and 24-48 h, respectively (Fig. 1B). Since C5a was not detected in the C5-supplemented medium treated with the culture supernatant of MEC or HuCCT1 cells (Fig. 1B), the C5a-releasing protease is associated with cancer cells but is not secreted into the culture medium. It is likely that a cancer cell membrane-bound protease releases C5a from C5.

Invasion enhancement by cancer cell-released C5a. C5a enhances invasion of C5aR-expressing cancer cells (18). To determine whether cancer cell-released C5a is active for C5aR-expressing cancer cells, the C5-supplemented medium in which HuCCT1 cells were cultured was examined for invasion enhancing activity using HuCCT1/C5aR cells. The C5-negative cancer cell culture medium did not affect HuCCT1/C5aR cell invasion but the C5-supplemented culture medium
enhanced cancer cell invasion >10-fold, which was equivalent to the activity of ~10 nM C5a (18) and was almost completely inhibited by anti-C5a IgG, but not control IgG (Fig. 2). The result indicates that the cancer cell-released C5a is active and sufficient to enhance C5aR-expressing cancer cell invasion.

**Inhibition of cancer cell C5a release by protease inhibitors.** To characterize the C5a-releasing protease, C5a release from C5 by HuCCT1 cells was investigated in the presence of various types of protease inhibitors. Aprotinin, a serine protease inhibitor, inhibited C5a release by HuCCT1 cells but inhibitors specific for cysteine, acid or metal proteases did not affect C5a release by the cells (Fig. 3A), indicating that the protease is serine-type. We previously reported C5a release from human C5 by ASP (Aeromonas sobria serine protease) (20) belonging to the kexin subfamily (23). Similar to other members of the subfamily, ASP cleaves peptide bonds at the C-terminal side of paired basic amino acid residues (24) and proteases of such substrate specificity are inhibited by decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dRVKRCk) (25). Therefore, we examined effects of dRVKRCk on cancer cell C5a-releasing activity. This inhibitor almost completely suppressed C5a release from C5 by HuCCT1 cells (Fig. 3B), suggesting that the cancer cell C5a-releasing protease has substrate specificity similar to that of the kexin subfamily proteases.

**C5a release from immobilized plasma by cancer cells.** C5 is a plasma protein. Under physiological conditions, plasma leaks from the blood stream and fills the interstitial tissue space as lymph fluid. To address cancer cell C5a release from C5 in the lymph fluid that is present in contact with cancer cells, human plasma treated to immobilize the cascade reaction of complement activation was incubated with MEC or HuCCT1 cells and then the plasma was examined for C5a. No or negligible C5a was released from the plasma.
incubated in the absence of cancer cells. Aprotinin at the concentration used does not inhibit thrombin (26), excluding C5a release by thrombin. The result suggests that the cancer cell protease can release C5a from C5 in the lymph fluid in cancer tissues.

**Discussion**

In the present study, we demonstrated C5a release directly from human C5 by cancer cells (Fig. 1A and B). This is a new mechanism of C5a generation in the cancer microenvironment. Since activation of the complement system is not involved in this mechanism, cancer cell-associated complement regulators (7-11) do not hinder the C5a release. Recently, C5 binding to cancer cells cultured in serum-supplemented media and C5a production by washed cancer cells cultured in serum-free conditions were shown by flow cytometry and ELISA, respectively (5). However, the concentrations of the C5a were <1 nM, the minimal concentration at which C5a enhances cancer cell invasion (18). As the molecular weight and activity of the C5a were not shown in the present study, a possibility that the C5a antigens are derived from non-functional fragments of cancer cell-bound C5, cannot be excluded. The result that cancer cell culture medium not supplemented with C5 did not enhance invasiveness of C5aR-expressing cancer cells (Fig. 2) suggests the necessity of C5 in the release of significant amounts of active C5a. In accordance with our result (Fig. 3), partial inhibition by serine protease inhibitors suggested involvement of serine protease(s) in the C5a production by mouse lung cancer cells, but localization of the protease(s) was not determined (5).

We demonstrated that the C5a antigen released in the cancer cell culture medium supplemented with C5 had a molecular weight identical to that of the C5a antigen released in the cobra venom-treated human plasma (Fig. 1). Furthermore, the released C5a exhibited cancer cell invasion enhancing activity that corresponds to the activity of 10 nM C5a (18) and its release requires both cancer cells and C5 (Fig. 2). As the C5a-releasing activity was absent in the cancer cell culture medium (Fig. 1B), the protease that contributes to the C5a release is possibly present on the cell membrane but is not secreted into the medium. Collectively, the present study has clearly shown that active C5a is released from C5 by a cancer cell membrane-anchored protease and the C5a concentration is sufficient to enhance invasion of C5aR-expressing cancer cells.

C5 is present in the interstitial fluid that is plasma leaked from capillaries into the interstitial space under physiological conditions. Plasma leakage is enhanced by leaky vasculature in cancer tissues (27). Loose cell-to-cell contact of cancer cells and change from the mass to free cells by epithelial-mesenchymal transition facilitate interstitial fluid C5 access to the cell surface, which enables C5 cleavage by the cell-membrane protease. Binding of the released C5a to C5aR on the same cell and/or neighboring cells lessens C5a dilution by diffusion and allows C5a to efficiently activate cancer cells. In contrast, non-cancerous epithelial cells do not release C5a from C5 (5). Tight adhesion among epithelial cells prevents interstitial fluid C5 from accessing to the cell surface, which makes C5a release unlikely, even if the cells possess the C5a-releasing protease. C5a release by cancer cells from the complement-immobilized plasma (Fig. 4) indicates that C5 cleavage by the protease can occur in cancer tissues. C5a release from human C5 at its plasma concentration by all of the cancer cell lines examined, including C5aR-expressing cells (Fig. 1A), suggests that various types of cancer cells may have this activity and continuously release C5a. Thus, there may be a self-activation circuit via the C5a-C5aR system in cancer cells that express C5aR and the protease on the cell surface.

Almost complete inhibition of HuCCT1 cell C5a release by dRVK/Rck (Fig. 3B) may indicate that the cancer cell C5a-releasing protease belongs to the kexin subfamily proteases (28). A kexin subfamily serine protease ASP cleaves peptide bonds at the carboxy-terminal side of -Ile-Glu-Gly-Arg- and -Leu-Ser-Thr-Arg- more efficiently than those of paired basic residues -Arg-Thr-Lys-Arg- and -Glu-Lys-Lys- (24). In fact, this bacterial protease activates prothrombin (24) and releases C5a from C5 (18), which requires cleavage at the carboxy-terminal side of Arg residues of the two substrates (24,29).

Inhibition of the C5a-releasing protease by aprotinin (Figs. 3 and 4) that inhibits trypsin (30) appears to be consistent with the cancer cell protease activity to cleave proteins at the carboxy-terminal side of Arg residues to release C5a. These results support that the cancer cell C5a-releasing protease is possibly a membrane-anchored kexin-like protease. Furin, a membrane-anchored protease, is, thus far, an only kexin subfamily member of human cell origin and is inhibited by dRVK/Rck (31). Although recombinant human furin released C5a from C5, C5a-releasing activity of HuCCT1 cells did not change by furin knockdown using siRNA. Other candidates may be cell membrane-anchored trypsin-like serine proteases (32), but the antibody against a representative protease matriptase did not affect the HuCCT1 cell activity to release C5a. Further study is required to identify the cancer cell C5a-releasing protease.

As macrophages, neutrophils and myeloid derived suppressor cells express C5aR (4,14,15,17), cancer cell-released C5a probably recruits these leukocytes to cancer tissues in addition to activation of C5aR-expressing cancer cells. The macrophages, known as tumor-associated macrophages (TAMs), promote cancer development and metastasis by secreting several factors associated with angiogenesis, tumor cell growth, migration and invasion, such as MMP-9, VEGF and EGF (33-35). Infiltrating neutrophils can play an important role in activating angiogenesis in the tissue vasculature in carcinogenesis (36). C5a also recruit myeloid-derived suppressor cells that suppress the antitumor CD8+ T cell-mediated response, thereby augmenting tumor growth (4). Thus, the cancer cell protease-released C5a could contribute to create a microenvironment favorable for cancer progression by gathering such cancer cell-supporting leukocytes to cancer tissues in addition to inducing neovascularization (5,16,36).

C5a activities that help cancer progression (4,5,18) suggest that agents targeting the C5a-C5aR system, such as anti-C5a antibody, anti-C5aR antibody and C5aR antagonists, can interrupt cell activation via the system, inhibiting C5aR-expressing cancer cell invasion, cancer cell-supporting leukocyte recruitment and neovascularization. Thus, these agents are potentially available for anticancer therapy. In the present study, we revealed cancer cell membrane-bound protease-mediated C5a release as a new C5a generation mechanism in cancer tissues.
Inhibitors specific for the cancer cell C5a-releasing protease suppress C5a release, thus may also provide a useful therapeutic option for cancer treatment in the future.

Acknowledgements

The authors thank Dr A. Irie for the technical assistance. This study was supported by JSPS KAKENHI grant nos. 22590363 and 25460498 to T.I.

References