Cancer cells release anaphylatoxin C5a from C5 by serine protease to enhance invasiveness

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Received April 17, 2014; Accepted May 29, 2014

DOI: 10.3892/or.2014.3341

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.

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Key words: C5a, cancer, protease, invasion, receptor, CD88

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-mercaptomethyl-3-guanidinoethylthiopropanoic 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 phospholamidon 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, Johns Hopkins University. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (40 U/ml), and streptomycin (40 μg/ml) and were maintained at 37˚C in 5% CO_2. HuCCT1 cells do not express C5aR but we prepared HuCCT1 cells stably expressing C5aR (HuCCT1/C5aR) by transfecting the plasmid carrying human C5aR cDNA (18).

Immunoblotting. To detect C5a released from human C5, cancer cells ($1x10^4$ 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 ($5x10^4$ cells/100 μl) were cultured in the medium at 37˚C for various periods. HuCCT1 ($5x10^4$ cells/100 μl) or MEC ($5x10^4$ 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 to immobilize the activation reaction of the complement system and 100 μl of the plasma supplemented with carboxypeptidase N inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (3 μM) was incubated with cancer cells ($1x10^4$) at 37˚C. At various incubation periods, 2 μl of the plasma was withdrawn. To characterize the C5a-releasing protease activity, C5 was incubated with HuCCT1 cells ($5x10^4$ cells/100 μl) in the presence of nontoxic doses of inhibitors specific for cysteine- (E64, 10 μM), serine- (aprotinin 10 μg/ml), acid- (pepstatin 1 μM) or metallo-proteases (GM, GM6001 5 μM; phospholamidon 10 μM). Twenty microliters of the supernatant were withdrawn. To obtain glycosylated C5a as a positive control, 5 ml of heparinized human plasma supplemented with the carboxypeptidase N inhibitor (3 μM) was incubated with 1 unit of cobra venom factor (Quidel Corporation, San Diego, CA, USA) at 37˚C for 30 min; 2 μl of the plasma was used (20). These samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with anti-human C5a goat IgG (x1,000 dilution), followed by HRP-conjugated anti-goat IgG rabbit antibody. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Blauvelt, NY, USA).

Invasion assay. To assess invasion of HuCCT1/C5aR cells *in vitro*, BioCoat Matrigel invasion chambers (22) (24-well plate, 8-μm pore) (BD Biosciences, San Jose, CA, USA) were used. Five hundred microliters of parental HuCCT1 cells ($5.0x10^4$) in the lower chamber were incubated at 37˚C for 24 h in serum-free RPMI-1640 medium supplemented with or without C5 (350 nM). Then, HuCCT1/C5aR ($3.75x10^4$) cell suspension in 0.75 ml of serum-free RPMI-1640 medium supplemented with 2.6 μg/ml of anti-C5a IgG or non-specific IgG was placed in the upper chamber and incubated at 37˚C for 24 h. 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 stained with 1% toluidine blue. Migrated cells were counted in five low power fields (x20). The invasion-enhancing effect was shown as the ratio of cell invasion by samples vs. serum-free RPMI-1640 medium in the lower chamber.

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

Results

Release of C5a from C5 by cancer cells. To explore cancer cell C5a release from C5, we cultured cancer cells 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-free cancer cell culture medium did not affect HuCCT1/C5aR cell invasion but the C5a-supplemented culture medium

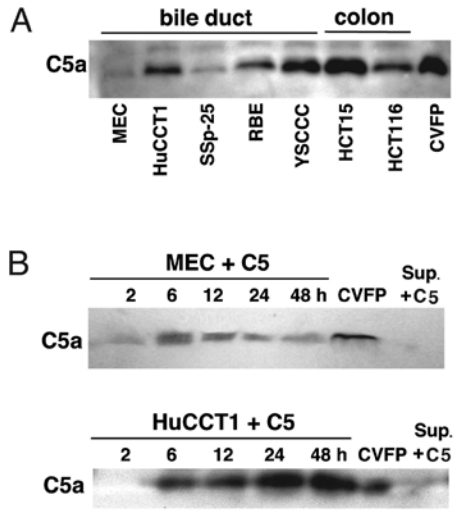

Figure 1. Release of C5a from C5 by cancer cells. (A) Cancer cells were cultured for 24 h in the presence of C5. (B) MEC or HuCCT1 cells were cultured for various periods in the presence of C5. Then, C5a in culture supernatants or reaction solutions was detected by immunoblotting. CVFP, cobra venom factor-treated plasma; Sup. + C5, the C5-supplemented medium incubated for 24 h with the C5-free medium in which cancer cells were cultured for 48 h.

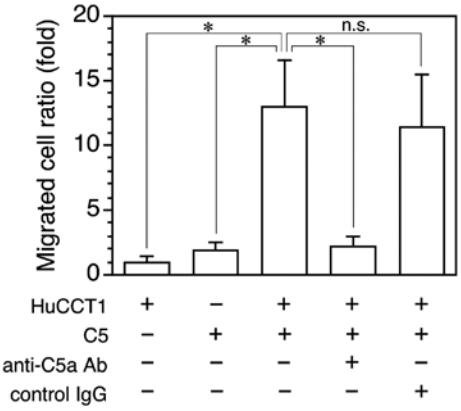

Figure 2. Invasion enhancement of C5aR-expressing cancer cells by cancer cell culture media. Matrigel invasion chamber assay was performed. HuCCT1 cells were incubated in the lower chamber for 24 h in the presence or absence of C5. Then, HuCCT1/C5aR cells were placed in the upper chamber and incubated for 24 h in the presence of anti-C5a antibody or non-specific IgG. *P<0.01; n.s., not significant.

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

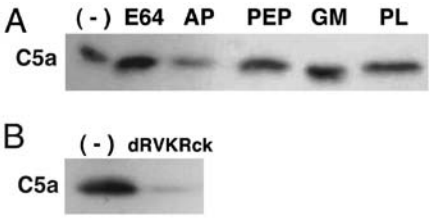

Figure 3. Inhibition of cancer cell C5a release by protease inhibitors. (A) HuCCT1 cells were cultured in C5-supplemented media for 24 h in the presence of either nontoxic doses of inhibitors specific for cysteine- (E64, 10 μM), serine- (AP, 10 μg/ml), acid- (PEP, 1 μM) or metallo-proteases (GM, GM6001 5 μM; PL, 10 μM). (B) HuCCT1 cells were cultured for 24 h in the C5 supplemented media in the presence or absence (-) of dRVKRck (2 μM). C5a in culture supernatants was detected by immunoblotting. AP, aprotinin; PEP, pepstatin; PL, phospholamidon.

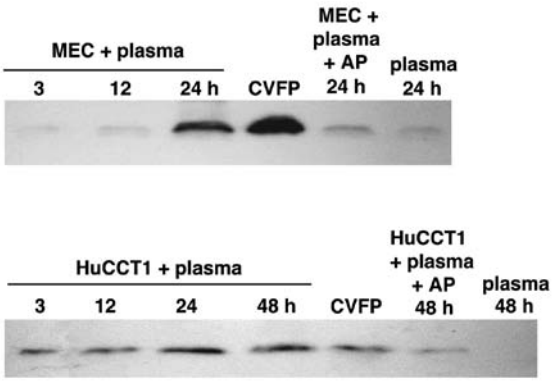

Figure 4. Release of C5a from complement-immobilized human plasma by cancer cells. MEC or HuCCT1 cells were cultured in the human plasma for various periods in the presence or absence of aprotinin (AP; 10 μg/ml). Then, C5a in the plasma was detected by immunoblotting. CVFP, cobra venom factor-treated plasma.

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. The two clones of cancer cells released C5a from the plasma in an incubation time-dependent manner and aprotinin inhibited the C5a release mostly (Fig. 4), showing complement activation-independent C5a release from C5 probably by the cancer cell serine protease. 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 dRVKRck (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 dRVKRck (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

1. Ricklin D, Hajishengallis G, Yang K and Lambris JD: Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11: 785-797, 2010.
2. Niculescu F, Rus HG, Retegan M and Vlaicu R: Persistent complement activation on tumor cells in breast cancer. Am J Pathol 140: 1039-1043, 1992.
3. Bjørge L, Hakulinen J, Vintermyr OK, Jarva H, Jensen TS, Iversen OE and Meri S: Ascitic complement system in ovarian cancer. Br J Cancer 92: 895-905, 2005.
4. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, Coukos G and Lambris JD: Modulation of the antitumor immune response by complement. Nat Immunol 9: 1225-1235, 2008.
5. Corrales L, Ajona D, Rafail S, *et al*: Anaphylatoxin C5a creates a favorable microenvironment for lung cancer progression. J Immunol 189: 4674-4683, 2012.
6. Müller-Eberhard HJ: Molecular organization and function of the complement system. Annu Rev Biochem 57: 321-347, 1988.
7. Yamakawa M, Yamada K, Tsuge T, Ohrui H, Ogata T, Dobashi M and Imai Y: Protection of thyroid cancer cells by complement-regulatory factors. Cancer 73: 2808-2817, 1994.
8. Niehans GA, Cherwitz DL, Staley NA, Knapp DJ and Dalmasso AP: Human carcinomas variably express the complement inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin). Am J Pathol 149: 129-142, 1996.
9. Jurians K, Ziegler S, Garcia-Schüller H, Kraus S, Bohana-Kashtan O, Fishelson Z and Kirschfink M: Complement resistance of tumor cells: basal and induced mechanisms. Mol Immunol 36: 929-939, 1999.
10. Morgan J, Spendlove I and Durrant L: The role of CD55 in protecting the tumour environment from complement attack. Tissue Antigens 60: 213-223, 2002.
11. Gancz D and Fishelson Z: Cancer resistance to complement-dependent cytotoxicity (CDC): problem-oriented research and development. Mol Immunol 46: 2794-2800, 2009.
12. Liu J, Miwa T, Hilliard B, Chen Y, Lambris JD, Wells AD and Song WC: The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. J Exp Med 201: 567-577, 2005.
13. Mikesch JH, Buerger H, Simon R and Brandt B: Decay-accelerating factor (CD55): a versatile acting molecule in human malignancies. Biochim Biophys Acta 1766: 42-52, 2006.
14. Guo RF and Ward PA: Role of C5a in inflammatory responses. Annu Rev Immunol 23: 821-852, 2005.
15. Markiewski MM and Lambris JD: The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am J Pathol 171: 715-727, 2007.
16. Nozaki M, Raisler BJ, Sakurai E, *et al*: Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci USA 103: 2328-2333, 2006.
17. Gerard NP and Gerard C: The chemotactic receptor for human C5a anaphylatoxin. Nature 349: 614-617, 1991.
18. Nitta H, Wada Y, Kawano Y, *et al*: Enhancement of human cancer cell motility and invasiveness by anaphylatoxin C5a via aberrantly expressed C5a receptor (CD88). Clin Cancer Res 19: 2004-2013, 2013.
19. Huber-Lang M, Sarma JV, Zetoune FS, *et al*: Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 12: 682-687, 2006.
20. Nitta H, Imamura T, Wada Y, Irie A, Kobayashi H, Okamoto K and Baba H: Production of C5a by ASP, a serine protease released from *Aeromonas sobria*. J Immunol 181: 3602-3608, 2008.
21. Huber-Lang M, Younkin EM, Sarma JV, *et al*: Generation of C5a by phagocytic cells. Am J Pathol 161: 1849-1859, 2002.
22. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM and McEwan RN: A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res 47: 3239-3245, 1987.
23. Kobayashi H, Utsunomiya H, Yamanaka H, Sei Y, Katunuma N, Okamoto K and Tsuge H: Structural basis for the kexin-like serine protease from *Aeromonas sobria* as sepsis-causing factor. J Biol Chem 284: 27655-27663, 2009.
24. Nitta H, Kobayashi H, Irie A, Baba H, Okamoto K and Imamura T: Activation of prothrombin by ASP, a serine protease released from *Aeromonas sobria*. FEBS Lett 581: 5935-5939, 2007.
25. Tian S and Jianhua W: Comparative study of the binding pockets of mammalian proprotein convertases and its implications for the design of specific small molecule inhibitors. Int J Biol Sci 6: 89-95, 2010.
26. Pintigny D and Dachary-Prigent J: Aprotinin can inhibit the proteolytic activity of thrombin. A fluorescence and enzymatic study. Eur J Biochem 207: 89-95, 1992.
27. Maeda H, Fang J, Inutsuka T and Kitamoto Y: Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications. Int Immunopharmacol 3: 319-328, 2003.
28. Angliker H, Wilkstrom P, Shaw E, Brenner C and Fuller RS: The synthesis of inhibitors for processing proteinases and their action on the Kex2 proteinase of yeast. Biochem J 293: 75-81, 1993.
29. DiScipio RG, Smith CA, Müller-Eberhard HJ and Hugli TE: The activation of human complement C5 by a fluid phase C5 convertase. J Biol Chem 258: 10629-10636, 1983.
30. Antonini E, Ascenzi P, Bolognesi M, Gatti G, Guameri M and Menegatti E: Interaction between serine (pro)enzymes, and Kazal and Kunitz inhibitors. J Mol Biol 165: 543-558, 1983.
31. Angliker H: Synthesis of tight binding inhibitors and their action on the proprotein-processing enzyme furin. J Med Chem 38: 4014-4018, 1995.
32. Szabo R and Bugge TH: Membrane-anchored serine proteases in vertebrate cell and developmental biology. Annu Rev Cell Dev Biol 27: 213-235, 2011.
33. Condeelis J and Pollard JW: Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 124: 263-266, 2006.
34. van der Bij GJ, Oosterling SJ, Meijer S, Beelen RH and van Egmond M: The role of macrophages in tumor development. Cell Oncol 27: 203-213, 2005.
35. Crowther M, Brown NJ, Bishop ET and Lewis CE: Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. J Leukoc Biol 70: 478-490, 2001.
36. Nozawa H, Chiu C and Hanahan D: Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci USA 103: 12493-12498, 2006.