A Tumor-expressed Inhibitor of the Early but not Late Complement Lytic Pathway Enhances Tumor Growth in a Rat Model of Human Breast Cancer

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ABSTRACT

Membrane-bound complement inhibitors protect host cells from inadvertent complement attack, and complement inhibitors are often up-regulated on tumors, possibly representing a selective adaptation by tumors to escape elimination by a host antitumor immune response. Relevant in vivo studies using rodent models of human cancer have been hampered by the fact that human complement inhibitors are not effective against rodent complement. Using nude rats and MCF7 cells expressing different rat complement inhibitors, a model of human breast cancer was established to investigate the role of complement and complement inhibitors in tumor progression. Expression of rat CD59, an inhibitor of the terminal cytolytic membrane attack complex of complement, had no effect on the incidence or growth rate of MCF7 tumors. In contrast, expression of rat Crry, an inhibitor of complement activation, dramatically enhanced established to investigate the role of complement and complement inhibitors in tumor progression. Expression of rat CD59, an inhibitor of the terminal cytolytic membrane attack complex of complement, had no effect on the incidence or growth rate of MCF7 tumors. In contrast, expression of rat Crry, an inhibitor of complement activation, dramatically enhanced established to investigate the role of complement and complement inhibitors in tumor progression. Expression of rat CD59, an inhibitor of the terminal cytolytic membrane attack complex of complement, had no effect on the incidence or growth rate of MCF7 tumors. In contrast, expression of rat Crry, an inhibitor of complement activation, dramatically enhanced.

INTRODUCTION

Complement activation on a cell surface results in the formation of cell-bound C3/C5 convertases, enzyme complexes that cleave serum complement proteins C3 and C5. Cleaved C3 fragments can become covalently bound to the activating cell surface, where they can amplify the complement cascade and serve as opsonins for immune effector cells. C3 opsonization can be important for promoting and enhancing ADCC and NK cell-mediated lysis, effector systems that are believed to be important in immune resistance to tumors. It was shown recently that inhibitory Fc receptors can modulate in vivo cytotoxicity, confirming the physiological significance of ADCC in an antitumor immune response (1). Cleavage of C5 yields C5a and C5b fragments. C5a is a chemoattractant and powerful mediator of inflammation that may potentiate antitumor responses. C5b initiates the sequential recruitment of the terminal complement proteins (C6, C7, C8, and C9) to form the MAC on the activating cell surface. If deposited on a tumor cell in sufficient quantity, the MAC is directly cytolytic, but when deposited in sublytic concentrations, the MAC can also stimulate cells to release proinflammatory molecules. Complement activation on a tumor cell surface can result from the binding of exogenously administered tumor reactive antibodies (immunotherapy). In addition, a humoral immune response to tumors has been described, and there are reports of persistent complement activation on breast (2) and other tumor cells (3, 4).

Both normal and tumor cells are protected from complement attack by different membrane-bound complement inhibitory proteins. In humans, complement activation is controlled primarily by the membrane proteins DAF and MCP. A complement activation inhibitor termed Crry is expressed in rodents but not humans. Although rodent cells also express DAF and MCP, the expression of Crry is broader, and Crry is considered the rodent functional and structural analogue of human DAF and MCP (5–7). These regulators of complement activation act early in the pathway, inhibit the generation of C3 opsonins and the soluble inflammatory activation fragments C3a and C5a, and may thus modulate a cell-mediated antitumor effector response. Control of cytolytic MAC formation on cell membranes is provided by CD59, a cell surface glycoprotein that binds to the terminal proteins in the complement cascade (C8 and C9) and prevents their assembly into a lytic complex (8, 9). Therefore, CD59 has no direct effect on the generation of C3 opsonins and C3/C5 peptide activation products but has the potential to modulate direct complement-mediated lysis of tumor cells.

Complement inhibitors have been identified as tumor-associated antigens (10, 11), and their expression is up-regulated on some tumor cells. There is good in vitro evidence to support the hypothesis that complement inhibitors expressed on tumor cells can provide protection from immune surveillance and that they present a barrier to successful antibody-mediated immunotherapy (for reviews on the subject, see Refs. 12–14). However, there is a paucity of in vivo data to support these hypotheses, one reason being the species selectivity of complement inhibitors and the lack of a suitable rodent model. Here we report the development of a clinically relevant nude rat model of human breast cancer and its use to study the role of different types of tumor-expressed complement inhibitors on tumor progression.

MATERIALS AND METHODS

Cell Lines. The human breast cancer cell line MCF7 (American Type Culture Collection, Rockville, MD) and MCF7 transfectants were grown at 37°C in 5% CO₂ in Eagle’s modified essential medium, supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 0.1% nonessential amino acids, human insulin (10 μg/ml), and 2 mM glutamine. For preparation of transfected MCF7 cell lines, cDNA encoding rat CD59 (15) and rat Crry (6) was subcloned into the mammalian expression vector pCDNA3 and pCDNA3.1 (Invitrogen, Carlsbad, CA), respectively, and transfected into MCF7 cells using Lipofectamine plus (Life Technologies Inc., Grand Island, NY), according to the manufacturer’s instructions. Stably transfected MCF7

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3 The abbreviations used are: ADCC, antibody-dependent cell cytotoxicity; MCP, membrane cofactor protein; NK, natural killer; DAF, decay accelerating factor; mAb, monoclonal antibody; MAC, membrane attack complex; CR, complement receptor.
cells were selected after the cultivation of cells in the presence of G418 (PCDNA3) or zeocin (PCDNA3.1), and populations expressing uniform levels of each rat complement inhibitor were isolated by three rounds of cell sorting (16). Double rat Cb59/crry transfectants were prepared by transfecting stable Crry transfectants with rat CDS9.

**Antibodies and Serum.** Rabbit antisera to MCF7 cell membranes that was used to sensitize MCF7 cells to complement for *in vitro* assays was prepared by standard techniques (17). Antirat Crry mAb 5 I2 was described previously (18). Antirat CDS9 mAb 6D1 and anti-MUC1 mAb C959 were provided by Drs. B. P. Morgan (University of Wales, Cardiff, United Kingdom) and M. R. Price (University of Nottingham, Nottingham, United Kingdom), respectively. FITC-labeled goat antihuman C3 (cross-reactive with rat C3) was purchased from ICN Pharmaceuticals (Aurora, OH). Antibodies to rat immunoglobulin and serum and FITC-labeled antibodies for flow cytometry were purchased from Sigma Chemical Co. (St. Louis, MO). Rat serum was prepared from blood collected by heart puncture, and serum was processed after clotting for 3 h on ice. C6-deficient rat serum was the kind gift of Dr. W. M. Baldwin (Johns Hopkins University School of Medicine, Baltimore, MD).

**Complement Lysis Assay.** Rat complement-mediated lysis was determined using cells either in the presence or absence of anti-MCF7 complement activating antibodies. For antibody sensitization, cells were incubated in 10% anti-MCF7 antisera for 30 min on ice, washed, and resuspended in assay medium. Cell lysis was determined by 51Cr release as described previously (19).

**Flow Cytometry.** Analysis of cell surface expression of rat complement inhibitors on transfected MCF7 cells was performed using appropriate antibodies as described (16). For analysis of rat C3 deposition on MCF7 cells *in vitro*, versene-detached cells were first incubated in different concentrations of anti-MCF7 antisera as described above for lysis assays. Washed cells were then incubated in 40% C6-deficient rat serum (to prevent MAC-mediated lysis) for 30 min at 37°C, washed, fixed with 2% paraformaldehyde, and analyzed for deposited C3 by means of FITC-conjugated antirat C3. A similar procedure was also followed for measuring C3 deposition on MCF7-transfected cells that were exposed to 5% rat serum in the absence of exogenously added antibody. For analysis of rat IgG and IgM binding to tissue culture-derived MCF7, cells were incubated in 40% heat-inactivated serum (56°C at 30 min) isolated from either naïve or tumor-bearing rats. After 60 min incubation on ice, cells were washed, fixed, and stained with appropriate FITC-labeled secondary antibody. For analysis of tumor-derived cells, cell suspensions were prepared by gentle teasing of isolated tumor tissue with scalpels (in RPMI/10% FCS), followed by centrifugation through Ficoll to remove tumor pieces and aggregates. Isolated cells were then washed by centrifugation in RPMI/10% FCS before use. Viability was checked by trypan blue exclusion. Procedure for staining for flow cytometry was described previously (16). In all assays, control experiments were performed using isotype-matched antibodies of irrelevant specificity.

**Rat Tumor Model.** Four-week-old female athymic nu/nu (nude) rats were obtained from the National Cancer Institute (Frederick, MD). Rats were housed in a clean room, and food and water were sterilized. Five mg of 90-day release 17β-estriadiol pellets (Innovative Research, Saratoga Springs, FL) were implanted with a trocar between scapulae of anesthetized rats. Five days later, rats were injected in left ventral mammary pad with 5 × 10⁶ cells suspended in 0.2 ml of PBS. Groups of rats received MCF7 cells transfected with either rat CDS9, rat Crry, or both, and stably expressing populations were isolated (Fig. 1). MCF7 cells expressing rat CDS9 or Crry were highly resistant to rat complement-mediated lysis (Fig. 2). Rat Crry-transfected cells, but not rat CDS9-transfected cells, were also resistant to rat complement C3 deposition (Fig. 3). When measuring C3 deposition, C6-deficient rat serum was used to prevent cytolytic MAC formation. The resistance of the rat CDS9 and Crry-transfected MCF7 cells to rat complement parallels the resistance of MCF7 cells to human complement.

Crry, but not CDS9, Promotes Tumor Growth in Nude Rats. To investigate the effect of rat complement inhibitors on the growth of MCF7 tumors in rats, nude rats were inoculated s.c. with 5 × 10⁶ transfected or mock-transfected MCF7 cells, and tumor growth was monitored. This inoculum was below the previously determined minimum tumorigenic dose (50% tumor take) of 1 × 10⁷ cells. As expected, only 15% of rats inoculated with mock-transfected MCF7 grew tumors (Fig. 4). Similarly, <15% of rats inoculated with rat CDS9-transfected MCF7 developed tumors (Fig. 4). In sharp contrast, however, 100% of rats inoculated with MCF7 cells expressing rat Crry, either alone or together with CDS9, developed tumors (Fig. 4). There was also a dramatic difference in the rate of tumor growth in rats inoculated with Crry expressing MCF7 cells compared with rats inoculated with either mock- or rat CDS9-transfected MCF7 cells (Fig. 5), although the incidence of tumors was low with mock- and rat CDS9-transfected cells. The expression of rat CDS9 on MCF7 cells did not enhance tumor growth, but its coexpression with rat Crry on MCF7 further enhanced the effect of Crry on tumor growth; analysis of the mean difference in tumor size on each day of tumor measure-
accomplished with control and transfected MCF7 cells, incubated in serum from naïve or MCF7 tumor-bearing nude rats, and antibody deposition was analyzed. There was evidence of an IgM but not an IgG response (Fig. 8). There was no difference between antibody binding to either mock-transfected or transfected MCF7 cells (data not shown). IgM is an effective activator of complement, and in correlation with increased anti-MCF7 IgM concentration in serum from tumor-bearing rats, we found that immune serum was more effective at lysing unsensitized MCF7 cells; serum from naïve and tumor-bearing rats at a concentration of 40% resulted in 12 ± 2.5% and 18 ± 3.2% lysis, respectively (mean ± SD, n = 4, P < 0.05).

Together, these data indicate that natural antibodies in rat serum and antibodies to MCF7 antigens activate complement on MCF7 cells in vivo and that opsonization with C3 and subsequent C3 receptor-dependent, cell-mediated cytotoxicity is more important at limiting tumor growth than direct complement-mediated lysis. To further test this conclusion, we determined whether Crry-transfected MCF7 cells have increased resistance to cell-mediated cytotoxicity in vitro. MCF7 cells transfected with either rat CD59 (does not effect C3 deposition) or rat Crry were assayed in the presence of rat serum for their susceptibility to lysis mediated by nude rat splenocyte preparations enriched for NK cells and depleted of B cells. Fig. 9a shows that CD59-transfected MCF7 cells are susceptible to cell-mediated cytotoxicity but that Crry expression results in an inhibition of cell-mediated cytotoxicity. Expression of either of these complement inhibitors will prevent direct complement-mediated cytolysis in these assays (and is the reason mock-transfected MCF7 cells were not used in these assays). The data shown in Fig. 9b confirms that the incubation of MCF7/rCD59 cells in vitro in serum derived from tumor-bearing nude rats resulted in C3 deposition and that C3 deposition was inhibited on MCF7/Crry cells. These results correlate with C3 deposition found on MCF7 tumor-derived cells (Fig. 7) and with C3 deposition on cultured MCF7 cells incubated in C6-deficient rat serum (Fig. 3).

Analysis of Tumor-derived MCF7 Cells. Transfected tumor-derived MCF7 cells continued to express rat complement inhibitory proteins (Fig. 6). When compared with cells used for inoculation, there was no change in the expression levels of rat CD59 or rat Crry in either single or double transfectants after in vivo growth. Tumor-derived MCF7 cells, whether mock transfected (Fig. 6) or transfected with rat complement inhibitors (data not shown), also continued to express the tumor-associated antigen MUC1 on their surface. We had found previously that in vivo growth of a human neuroblastoma cell line in rats resulted in an up-regulation of endogenously expressed (human) DAF (22), but we found no up-regulation of any of the endogenously expressed complement inhibitors (DAF, MCP, or CD59) on MCF7 after in vivo growth (data not shown). To determine whether the expression of Crry on MCF7 has functional consequences with regard to complement deposition in vivo, tumor-derived MCF7 cells were examined for deposited C3. There was a negative correlation between Crry expression and C3 deposition, with deposited C3 being detected only on mock-transfected MCF7 cells. No C3 was detected on Crry-expressing MCF7 cells (Fig. 7). Similar data were obtained when control and transfected MCF7 cells were incubated in nude rat serum in vitro (see Fig. 3).

To investigate the mechanism of in vivo complement activation by MCF7 cells, tumor-derived MCF7 cells were analyzed for the deposition of rat antibody. By flow cytometry, moderate amounts of bound IgM and lower levels of bound IgG were detected (Fig. 6). To determine whether there was an antibody response to MCF7 (presumably T-cell independent), tissue culture-derived MCF7 cells were incubated with heat-inactivated serum from naïve or MCF7 tumor-bearing nude rats, and antibody deposition was analyzed. There was evidence of an IgM but not an IgG response (Fig. 8). There was no difference between antibody binding to either mock-transfected or transfected MCF7 cells (data not shown). IgM is an effective activator of complement, and in correlation with increased anti-MCF7 IgM concentration in serum from tumor-bearing rats, we found that immune serum was more effective at lysing unsensitized MCF7 cells; serum from naïve and tumor-bearing rats at a concentration of 40% resulted in 12 ± 2.5% and 18 ± 3.2% lysis, respectively (mean ± SD, n = 4, P < 0.05).

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Dependent, and it is also possible that this finding may be an artifact. Nevertheless, this property of mouse serum has been ascribed to deficiencies in different terminal complement proteins and the inability of the mouse classical pathway C5 convertase to cleave C5 and initiate the terminal lytic pathway (23-26). In addition, a comparison of in vitro antibody-targeted cytotoxicity using mouse, rat, and human effectors indicated that the rat is a more relevant rodent model than the mouse for testing in vivo antitumor activity of at least some antibodies (27).

Complement activation on a tumor cell will result in the deposition of activation products C3b and its longer-lived degradation product iC3b. Only C3b can participate further in amplification of the complement cascade, but both proteins are ligands for CRs found on immune effector cells in rats and humans. Cry (DAF and MCP in humans), but not CD59, will control C3 opsonization. CR1 is expressed on phagocytes and binds C3b with high affinity and iC3b (and C4b, a classical pathway fragment) with low affinity. CR3 (CD11b/CD18) binds iC3b with high affinity and is expressed on phagocytes and NK cells. These C3 receptors, in particular CR3, can promote the adhesion of C3-opsonized tumor cells with effector cells, promote and enhance ADCC/NK lysis, and play an important role in leukocyte adhesion and migration during an inflammatory process (for review of C3 receptors, see Ref. 28). In this study, MCF7 tumorigenicity in nude rats was enhanced significantly when C3 deposition was inhibited, but inhibition of cytolytic MAC formation alone had no effect on tumor growth. We infer from these results that only cell-mediated mechanisms, promoted or enhanced by the deposition of C3 fragments generated during the complement activation phase, are involved in controlling the growth of MCF7. This inference is further supported by the in vitro study demonstrating that in the presence of rat serum, CD59-transfected MCF7 cells are susceptible to rat effector cell-mediated cytotoxicity but that Cry-transfected MCF7 cells have increased resistance to lysis. In these in vitro cell-mediated cytotoxicity assays, we used B-cell-depleted rat splenocyte preparations that were enriched for NK cells. NK cells express inhibitory receptors that are

DISCUSSION

Endogenously expressed DAF and CD59 provide the human breast cancer cell line MCF7 with resistance to human complement deposition and lysis in vitro, even in the presence of complement-activating anti-MCF7 antibodies. However, MCF7 cells are susceptible to rat complement in vitro, illustrating the fact that human complement inhibitors are less effective against rat complement. The resistance of the rat CD59- and Crry-transfected MCF7 cells to rat complement parallels the resistance of MCF7 cells to human complement and establishes the clinical relevance of using these transfected cells to study complement inhibitors and complement effector mechanisms in rats. For studies on complement, the rat may be a better model than the mouse, because isolated mouse serum appears to contain only low levels of hemolytic complement. The reason for this may be strain dependent, and it is also possible that this finding may be an in vitro artifact. Nevertheless, this property of mouse serum has been ascribed variably to deficiencies in different terminal complement proteins

Fig. 6. Analysis of tumor-derived MCF7 cells by flow cytometry. Tumor cells obtained from rats inoculated with either rat CD59- or Crry-transfected MCF7 cells were analyzed for continued expression of rat complement inhibitor. Tumor-derived, mock-transfected MCF7 was also analyzed for the continued endogenous expression of MUC1 antigen and for deposition of rat IgG and IgM. Analyses were performed on cells derived from tumors 28–30 days after inoculation. Panels, data obtained with isotype control antibody (gray trace) and specific antibody (black trace).

Fig. 7. Relationship between Cry expression and C3 deposition on tumor-derived, transfected and mock-transfected MCF7 cells. Cell suspensions obtained from MCF7 tumors were analyzed by two color flow cytometry using FITC and PE-conjugated anti-C3 and anti-Cry antibody, respectively. Relative fluorescence shown on each axis.

Fig. 8. Deposition of rat IgG and IgM on tissue culture-derived MCF7 cells. MCF7 was incubated in heat-inactivated serum obtained from naive (gray trace) or tumor-bearing rats (black trace) and analyzed separately for either IgG or IgM deposition by flow cytometry. Control antibodies gave a mean fluorescence <10.

Fig. 9. Effect of rat complement inhibitor expression on rat cell-mediated cytotoxicity and C3 deposition in the absence of exogenously added antibody. a, cell-mediated lysis. MCF7 cells expressing either rat Cry or CD59 were exposed to a NK cell-enriched nude rat splenocyte preparation depleted of B cells (E:T, 100:1) in the presence of 10% rat serum, and lysis was determined (mean ± SD, n = 3). b, C3 deposition. MCF7 cells expressing either rat Cry or CD59 were incubated in rat serum, and C3 deposition was analyzed by flow cytometry. Serum was derived from tumor-bearing nude rats.
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specific for MHC class I alleles, and if rat NK cells are the important effector cells in the model used here, it is unlikely that rat NK inhibitory receptors will recognize MHC class I molecules expressed on human MCF7 cells. Nevertheless, because the majority of human metastatic mammary carcinomas do not express MHC class I, our model may be relevant to human breast cancer, and cytotoxicity may be mediated by human NK cells.

The complement activation product C5a, the generation of which will not be affected by CD59, may also contribute to cell-mediated effector functions. C5a is a chemoattractant and a powerful mediator of inflammation that may potentiate an antitumor response. It should nevertheless be noted that compared with human cell types, complement-mediated recruitment of rat leukocytes and associated rat CRs are less well defined and represent a topic for further study. As evidenced by the lack of any effect of rat C59 expression on MCF7 tumorigenesis, the terminal cytolytic phase of the complement pathway does not alone play any direct role in controlling tumor growth in this model, although there were some synergy when both Cry and CD59 were expressed together on MCF7 cells. Interestingly, a previous study indicated an important role for CD59 in the survival of a neuroblastoma cell line in rats (22), indicating that different complement-associated mechanisms operate in controlling growth of different tumors. The reason for this difference has not been investigated but could be attributable to several factors, including relative levels of endogenous complement inhibitors, differences in quantity or isotype of tumor-reactive antibodies present in nude rat serum, and differential susceptibility of tumor cells to ADCC and NK cell lysis.

Complement was deposited on MCF7 cells grown in rats, although complement activating anti-MCF7 antibodies were not exogenously administered. This observation is explained by the finding that nude rat serum contained naturally occurring IgG and IgM reactive to MCF7 cells. There was also an anti-MCF7 IgM immune response in tumor-bearing rats. IgM is an effective activator of complement, but only IgG will bind to Fc receptors on phagocytes and NK cells. MCF7 cells express the tumor-associated antigen MUC1.4, and, interestingly, antibodies to MUC1 occur naturally in healthy individuals (29–31). Further, as in our rat model, an IgM humoral immune response occurs in patients with MUC1 tumors (30–32), and deposited complement has also been detected in tissue samples from breast and other tumors (2,4). Thus, the data reported here raise the possibility that regulating the function of complement inhibitors of activation on a tumor cell surface may augment the effector phase of a normally ineffective humoral immune response to MUC1 antigen or indeed to other tumor antigens. Enhancing C3 deposition by suppressing complement inhibitor function may also enhance the induction phase of a humoral immune response, because C3 opsonization of antigen can provide a costimulatory signal via CR2 (CD21) expressed on B cells (33). In this context, enhancing C3 deposition by suppressing complement inhibitor function may also be a useful strategy to potentiate a tumor vaccine strategy. CR2 is also expressed on some T cells, although a role for CR2 in T-cell immunity has not been reported. Whether or not tumor-expressed complement inhibitors and their blockade can effect the outcome of an immune response, the data provide strong direct in vivo evidence to support a hypothesis that inhibiting DAF and/or MCP will enhance the outcome of immunotherapy of certain tumors using complement-activating antitumor antibodies. The species’ selective activity of complement inhibitors and the data presented here also provide an explanation for the disappointing results obtained in the clinic with antibodies that were successful at treating tumors in rodent models of human cancer.

At the present time, blocking the function of complement inhibitors specifically on tumor cells in vivo is a technically difficult proposition, although significant progress is being made in cell-targeting strategies. Nevertheless, the specificity required for targeting is less critical than it would be for targeting toxins, because blocking the function of complement inhibitors on normal tissue is unlikely to present a significant health risk in the absence of a specific complement-activating signal (such as antitumor antibody). Of potential significance, a mAb that has been used successfully for tumor imaging was recently found to be specific for DAF (34). The expression of DAF on normal tissue raises the question of how it was possible to successfully image with this antibody and why only background levels of the antibody were found on endothelium and blood cells. A possible explanation for this finding comes from a more recent study showing that DAF is up-regulated by as much as 100-fold on some tumors (11). Thus, for some tumors, these findings may bode well for strategies aimed at tumor-targeted regulation of this complement inhibitor of activation.

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