Susceptibility of KSHV-Infected PEL Cell Lines to the Human Complement System

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Introduction

Primary effusion lymphoma (PEL) is a rare high-grade B-cell non-Hodgkin lymphoma with a poor prognosis and median survival time of around 6 months. PEL is caused by Kaposi’s sarcoma-associated herpesvirus, and is often co-infected with the Epstein Barr virus. The complement system is fundamental in the innate immune system against pathogen invasion and tumor development. In the present study, we investigated the activation of the complement system in PEL cells using human serum complements. Interestingly, two widely used PEL cell lines, BCP-1 and BCBL-1, showed different susceptibility to the complement system, which may be due to CD46 expression on their cell membranes. Complement activation did not induce apoptosis but supported cell survival considerably. Our results demonstrated the susceptibility of PEL to the complement system and its underlying mechanisms, which would provide insight into understanding the pathogenesis of PEL.

Keywords: Kaposi’s sarcoma-associated herpesvirus, human herpesvirus-8, pleural effusion lymphoma, complement system
expressed in lytic replication of KSHV, and only around 2% of PEL cells in normal culture conditions showed lytic replication [5]. Therefore, this viral protein does not seem to have a major effect on latently KSHV-infected cells. Endothelial cells (which are generally considered a precursor of spindle cells in the Kaposi’s sarcoma region) that were latently infected with KSHV showed activation of the complement system through the down-regulation of cellular complement regulatory proteins. MAC from complement activation supported the cell survival of KSHV-infected cells [10]. However, to the best of our knowledge, the activation of the complement system or its related control mechanism in latently KSHV-infected PEL cells has not been elucidated. In the present study, we investigated if the complement system can be activated in latently infected PEL cells (Fig. 1A). As previous reports have shown that B-cell lymphoma cells (BJAB) can activate the complement system in endothelial cells activate the complement system through alternative pathways [10]. To determine whether the activation of the complement system is also induced in PEL cell lines, the deposition of C5b-9 was analyzed by IFA after exposure to normal human serum complements (Fig. 1A). As previous reports have shown that B-cell lymphoma cells (BJAB) can activate the complement system [16], we used BJAB as a positive control. KSHV-infected BCP-1 cells had stronger signals of MAC than that of BJAB in IFA. However, no MAC deposition was detected in the KSHV-infected PEL cell line BCBL-1. Consistent results were observed from flow cytometry with anti-C3b antibodies (Fig. 1B). Although C3b depositions were detected in BJAB and BCP-1, we could not detect any in BCBL-1 cells. Together, these results indicated that the susceptibility against the

**Materials and Methods**

**Cell Culture and Reagents**

Burkit’s lymphoma cell line (BJAB cell) and PEL cell lines (BCBL-1 and BCP-1) were cultured in RPMI 1640 (Lonza, Allendale, NJ, USA) supplemented with 10% fetal bovine serum (FBS; Welgene, Seoul, South Korea). Human umbilical vein endothelial cells (HUVEC) were cultured in EGM2 with supplements (Lonza). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. Rat monoclonal anti-HHV8 [LN35] (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-CD46 (Santa-Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-CD55 (Santa Cruz Biotechnology), rabbit polyclonal anti-CD59 (Abcam, Cambridge, MA, USA), and anti-β-tubulin (Sigma) antibodies were used for immunofluorescence, western blot, and flow cytometry assays.

**Immunofluorescence Assay (IFA)**

Each cell line was seeded onto microscope cover glasses in 24-well tissue culture plates at a density of 5 × 10⁴ cells/well. After centrifugation at 2,500 rpm for 10 min, the culture medium was removed and cells were washed with PBS. The cells were fixed with 4% paraformaldehyde for 30 min and blocked with 3% bovine serum albumin in PBS for at least 30 min at 4°C. Cells were incubated with a primary antibody overnight at 4°C, and then incubated with Alexa Fluor 568 conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 15 min at 4°C. Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI). Cells were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and examined using an Eclipse E400 microscope (Nikon Instruments Inc., Melville, NY, USA). Images were captured using a Nikon Digital site DS-U2, and analyzed using NIS element F.

**Flow Cytometry**

The cells were washed three times with PBS. Cells suspended in 1% FBS/PBS were incubated with primary antibody at 4°C for 30 min. After washing, cells were treated with APC-conjugated secondary antibody at 4°C for 30 min. They were analyzed using a Guava easyCyte flow cytometer (Millipore, Billerica, MA, USA) and Incyte software (Millipore). Apoptosis was analyzed with the Annexin V apoptosis detection kit APC (eBioscience, San Diego, CA, USA) following the manufacturer’s instructions.

**Western Blot Assay**

Cell proteins were isolated using 1× SDS buffer containing 62.5 mM Tris-HCl at pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromophenol blue. The cell suspension was boiled for 10 min and centrifuged at 13,000 rpm for 8 min. The proteins were resolved by electrophoresis in a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare). The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20. Rabbit polyclonal anti-CD46, rabbit polyclonal anti-CD55, rabbit polyclonal anti-CD59, and anti-β-tubulin antibodies were used as primary antibodies. HRP-conjugated anti-rabbit or anti-mouse antibodies (Santa Cruz Biotechnology) were used as secondary antibodies. The results were visualized using an ECL detection reagent (Santa Cruz Biotechnology).

**Statistical Analysis**

All data are presented as the mean ± SD from at least three independent experiments and analyzed by a two-tailed unpaired Student’s t-test (Microsoft Excel). P-values < 0.05 were considered statistically significant.

**Results**

**Activation of the Complement System in BCP1 Cells**

We have recently shown that latently KSHV-infected endothelial cells activate the complement system through alternative pathways [10]. To determine whether the activation of the complement system is also induced in PEL cell lines, the deposition of C5b-9 was analyzed by IFA after exposure to normal human serum complements (Fig. 1A). As previous reports have shown that B-cell lymphoma cells (BJAB) can activate the complement system [16], we used BJAB as a positive control. KSHV-infected BCP-1 cells had stronger signals of MAC than that of BJAB in IFA. However, no MAC deposition was detected in the KSHV-infected PEL cell line BCBL-1. Consistent results were observed from flow cytometry with anti-C3b antibodies (Fig. 1B). Although C3b depositions were detected in BJAB and BCP-1, we could not detect any in BCBL-1 cells. Together, these results indicated that the susceptibility against the
Cell Death Activates the Complement System in BCBL-1 Cells

To confirm the results in Fig. 1, we repeated the same experiments many times because two KSHV-infected PEL cell lines showed different complement activation. Activation of the complement system was always observed in BCP-1 cells. Most times, BCBL-1 cells did not show complement activation. However, we observed some MAC-deposited BCBL-1 cells in some experiments (Fig. 2A). To investigate why MAC was deposited in BCBL-1 cells, dual staining with KSHV-LANA and MAC was performed. Interestingly, all MAC-positive cells did not express KSHV LANA protein. In addition, these cells showed shrinkage or were not stained with DAPI, as they were dead cells. These dead cells did not result from MAC deposition because LANA-negative and shrunk nuclei-containing cells were also observed in heat-inactivated serum-treated conditions. Therefore, these dead cells were possibly the result of unfavorable cell culture conditions. Apoptosis can induce complement activation, which is related to the clearance mechanism of the human body [15, 28]. Therefore, BCBL-1 seems to suppress complement system activation, but dead cells can induce MAC deposition through complement system activation.

Activation of the Complement System in BCP-1 Cells Is Mediated by an Alternative Pathway

To determine which pathway of the complement system
is activated in BCP-1 cells, we exposed the cells to normal human serum in the presence of 10 mM EGTA and 20 mM MgCl\(_2\), which spared the alternative pathway but inactivated the other pathways, or 20 mM EDTA, which inactivated the alternative pathway [3, 8]. Whereas the addition of EGTA and MgCl\(_2\) to normal human serum did not affect the detection of C5b-9 on BCP-1 cells, the addition of EDTA eliminated the detection of C5b-9. Thus, this result indicated that an alternative complement pathway was activated by BCP-1 cells (Fig. 3).

Inverse Correlation Between Cd46 Expression and Complement Activation

In the alternative pathway, complement regulatory proteins have an important role in complement system activation. In a previous study, latently KSHV-infected endothelial cells showed decreased CD55 and CD59 expression, which is an important mechanism of complement activation [10]. To investigate if complement activation of BCBL-1 and BCP-1 is also mediated by the expression of the complement regulatory proteins, the expression levels of total complement regulatory proteins in BCBL-1 and BCP-1 were analyzed by western blotting (Fig. 4A). CD55 and CD59 expression was not detected in both cells, which suggests that these proteins are not related to the suppression of complement activation in BCBL-1 cells. Interestingly, large amounts of CD46 were observed in BCBL-1 cells, but we could not detect CD46 expression in BCP-1. CD46 is a negative regulator for the activation of the complement system by working as a cofactor for factor I in the degradation of C3b/C4b [11]. Consistent results were observed in flow cytometry (Fig. 4B). Whereas BCBL-1 cells expressed CD46 on the cell surface, no CD46 expression in BCP-1 cells was detected. CD46 expression showed a negative relationship with the activation of the complement system in BCP-1 and BCBL-1 cells. Therefore, CD46 may play an important role in regulating the activation of the complement system in BCBL-1 and BCP-1 cells.

**Fig. 2.** Dead cells in BCBL-1 activated the complement system.

In unfavorable culture condition, BCBL-1 showing MAC deposition was analyzed by IFA. (A) After exposure to normal human serum, BCBL-1 cells were dual stained with KSHV LANA (red) and MAC (green). Nuclei were stained with DAPI (blue). Magnification, 400×. (B) Heat-inactivated human serum or normal human serum-exposed BCBL-1 cells were analyzed for KSHV LANA and the MAC deposition. Cells were visualized using a fluorescence microscope at 1,000× magnification.
The Activated Complement System Confers a Survival Advantage to BCP-1 Cells

To investigate whether the complement system activation induced cell death on PEL, BCP-1 and BCBL-1 treated with normal human serum complement or heat-inactivated serum for 1 h were analyzed using an apoptosis detection kit. As like endothelial cells in a previous study [10], we did not find definitive evidence for cell death in complement-activated PEL cells in the current study. Both BCP-1 and BCBL-1 showed similar amounts of apoptosis and the difference between heated and normal human serum complement-treated cells was not significant (Fig. 5A). Sublytic levels of activation of the complement system have been associated with enhanced cell survival [12, 27]. To examine the role of the activated complement system in BCP-1 cells, we cultured the cells with heat-inactivated or normal human serum for a longer time (Fig. 5B). The live numbers of BCBL-1 cells cultured in heat-inactivated and normal human serum were very similar. That of BCP-1 cells showed no significant difference up to 24 h of culture between heat-inactivated and normal human serum. However, the total number of live cells in heat-inactivated serum decreased by 25% and 35% compared with that in normal human serum at 48 and 72 h of culture, respectively. Consistently, a significantly higher percentage of apoptotic cells was observed in heated serum- than normal human serum-treated BCP-1 cells at 48 h of culture (Figs. 5C and 5D). These results suggested that the activated complement system would support survival in BCP-1 cells.

Discussion

To the best of our knowledge, this is the first study to demonstrate that the activation of the complement system is regulated in KSHV-infected PEL cells by the expression of complement regulatory proteins. An alternative pathway of the complement system was activated in BCP-1 cells by the suppression of a complement regulatory protein, CD46. Our results suggest that each PEL cell line showed different susceptibility to complement activation, which might be associated with different responses of each PEL cell to the host immune system or pathogenesis of cancer development. Activation of the complement system has been considered as a part of the body’s immunosurveillance against cancer. However, recent studies suggest that complement elements can promote tumor growth in various ways [21, 22]. The exact meaning of the complement activation in PEL cells should be further examined.

Most blood contacting cells, including red blood, white blood, and endothelial cells, express complement regulatory proteins on their cell surface to protect them from the complement system [7, 18]. CD46, CD55, and CD59 are expressed on the cell membrane and inhibit C4b deposition and MAC formation on the cell surface [18]. Secreted proteins, Factor H and Factor I, also participate in the inhibitory effects on the complement system [21]. In the present study, we focused on complement regulatory proteins on the cell surface because MAC deposition was clearly observed only on the IFA cell surface. Although the classical and lectin pathways have definite initiation.
factors for complement activation, the alternative pathway is dependent on the spontaneous hydrolysis of minute amounts of C3. Therefore, the complement regulatory mechanism seems to be more important in this pathway. Previously, latently KSHV-infected endothelial cells showed the induction of complement activation through an alternative pathway. CD55 and CD59 down-regulation is an important factor for complement activation in KSHV-infected endothelial cells [10]. Consistently, we found CD55 and CD59 expression was not observed in both latently KSHV-infected PEL cell lines, BCBL-1 and BCP-1. Since KSHV-negative B-cell lymphoma (the BJAB cell) expresses CD55 and CD59 [6], KSHV might regulate their expression in B-cell lymphomas as in human endothelial cells. However, CD46 was expressed in BCBL-1 but not in BCP-1 cells. Interestingly, the deposition of C3b and C5b-9 was inversely correlated with the CD46 expression, which suggest that CD46 expression might be an important factor.

Fig. 4. Expression of complement regulatory proteins in PEL cells.
Expression of the complement regulatory proteins CD46, CD55, and CD59 was analyzed. (A) Total protein levels of complement regulatory proteins were analyzed by western blotting. Human umbilical vein endothelial cells were used as the positive control. (B) Surface protein levels of CD46, CD55, and CD59 were analyzed by flow cytometry. Gray: isotype control; white: anti-complement regulatory protein antibody.
suppressing complement activation in PEL cells. As no association was found between LANA and CD46 expression in the current study, CD46 expression seems to be regulated by tumor cell characteristics and not by KSHV.

MAC accumulation on the cell surface can induce cell death through transmembrane channels. The complement system in KSHV-PEL.
system is also important in macrophage-mediated clearance of dead cells, which is an important physiological mechanism of dead cell clearance and preventing the release of inflammatory cellular content [27]. Some viruses have induced complement activation and the complement system is an important mechanism for protection from pathogens [4, 19, 23]. However, limited MAC formation sometimes does not cause cell death, especially in some cancer cells [21, 27]. This sublytic C5b-9 activates the cell cycle and enhances survival through the signal pathways activated by increased Ca\(^{2+}\) influx [22]. In our previous study, complement activation in latently KSHV-infected endothelial cell did not induce cell death, but enhanced cell survival via the STAT3 pathway [10]. This study also showed that the activated complement system conferred a survival advantage to BCP-1 cells. The mechanism might be similar to that of the survival advantage in KSHV-infected endothelial cells through the STAT3 pathway by MAC. However, as the complement system is related to enhanced cell survival in various ways [9, 22, 27], further study is required to determine the exact mechanisms of enhanced cell survival in BCP-1 cells.

In summary, KSHV-infected PEL cell lines showed different susceptibility to the complement system. CD46 expression had an inverse correlation with the activation of the complement system but CD46 could not prevent complement activation in apoptotic or dead cells. Additionally, the activated complement system significantly promoted the survival of PEL cells. This work provides further insights into the roles of the complement system in the pathogenesis of KSHV-infected PEL cells.

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**References**


17. Mettler TN, Cioc AM, Singleton TP, McKenna RW,


