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CD13/aminopeptidase N and murine cytomegalovirus infection

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Abstract

CD13/aminopeptidase N is a membrane-bound metalloproteinase implicated in human cytomegalovirus (HCMV) infection and pathogenesis. Anti-CD13 antibodies can neutralize HCMV infectivity, and HCMV viremia after bone marrow transplantation induces anti-CD13 autoantibodies which correlate with development of chronic graft vs. host disease. We examined whether murine CD13/APN was similarly implicated in murine cytomegalovirus (MCMV) disease. MCMV infection did induce anti-CD13 antibodies in mice in a strain-specific manner. ICR and 129S mice developed high titers of anti-CD13 antibodies and anti-MCMV antibodies after MCMV infection, whereas CBA and CBAXC57BL/6 fl hybrid mice produced antibodies against MCMV only. Unlike HCMV, no evidence was found for a correlation between host cell CD13/APN expression and infection, or for the presence of CD13/APN on MCMV particles, although APN inhibitors decreased MCMV plaque formation. Reproduction of CD13/APN autoantibody production in the murine system should make it possible to determine if these antibodies contribute to CMV pathogenesis.

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Introduction

Cytomegaloviruses are beta-herpesviruses which establish life-long infections in their hosts. Several findings have implicated CD13, also known as aminopeptidase N (APN), in human cytomegalovirus (HCMV) infection and pathogenesis. In a series of bone marrow transplant patients, CD13/APN-specific autoantibodies were found only in patients with confirmed CMV disease after transplant and were highly associated with later development of chronic graft vs. host disease (cGVHD) (Soderberg et al., 1996a, 1996b). Patients seropositive for CMV but negative for the presence of virus did not develop anti-CD13/APN antibodies or cGVHD (Soderberg et al., 1996b). On the molecular level, several groups have shown that human CMV incorporates host cell proteins, one of which is CD13/APN, into its viral envelope (Homman-Loudiyi et al., 2003; Michelson et al., 1989; Naucler et al., 1996; Wright et al., 1995). Some monoclonal antibodies raised against CD13/APN can neutralize human CMV viral particles, and immunoblots of purified human CMV virions detect CD13/APN (Soderberg et al., 1993a). When viral particles are labeled with immunogold-anti-CD13 antibodies, the immunogold particles are later seen on the surface of infected cells (Giugni et al., 1996). Consistent with viral sequestration of CD13/APN, cell surface CD13/APN is down-regulated after human CMV infection in fibroblasts (Phillips et al., 1998). Association of CD13/APN with the viral particle has been suggested as a mechanism for the generation of anti-CD13/APN antibodies after CMV infection by autoimmunization (Naucler et al., 1996). That virus-associated CD13/APN is essential for HCMV infection is suggested by anti-CD13 antibody inhibition of infection (Soderberg et al., 1993a); however, human neuroblastoma cells lacking CD13 expression were susceptible to HCMV infection (Watanabe, 1998), and recently the epidermal growth factor receptor has been shown to be an important mediator of HCMV entry into cells (Wang et al., 2003).
CD13/APN is a membrane-bound metalloproteinase widely expressed on mammalian cells including monocytes, macrophages, endothelial cells, epithelial cells, and fibroblasts (Curnis et al., 2002; Phillips et al., 1998; Plakidou-Dymock et al., 1993; Riemann et al., 1999). It exists as a heavily glycosylated homodimer with a constitutively active proteinase activity external to the plasma membrane (Riemann et al., 1999). In addition to numerous tissue-specific functions, CD13/APN serves as a major receptor for type I coronaviruses (Breslin et al., 2003; Yeager et al., 1992), including possibly the SARS virus (Yu et al., 2003). More recently, CD13/APN has been shown to be an important mediator of angiogenesis when expressed on endothelial cells of nascent blood vessels (Bhagwat et al., 2001). Antibodies raised against CD13/APN and inhibitors of aminopeptidase activity both block angiogenesis in models of neovascularization (Pasqualini et al., 2000). This may have significance with regard to CMV, since CMV infection is associated with many chronic vascular diseases (Borchers et al., 1999; Hamamdzic et al., 2002; Nieto et al., 1996; Pandey and LeRoy, 1998). CMV-induced anti-CD13/APN antibodies may represent one mechanism by which the virus initiates vascular pathology.

Like most herpesviruses, cytomegaloviruses are species specific, but the link between CMV and vascular disease seen in humans has been reproduced repeatedly in mice infected with murine cytomegalovirus (Berencsi et al., 1998; Hamamdzic et al., 2001; Hummel et al., 2001; Presti et al., 1998). Therefore, in this study, we have examined whether CD13/APN is also implicated in MCMV infections in mice.

Results

MCMV infection induces anti-CD13/APN antibodies in a strain-specific manner

Four mouse strains were tested for the induction of MCMV and CD13/APN immunity after MCMV infection. These strains, CBA, CBAXc57BL/6 f1 hybrids, ICR, and 129S mice deficient in the interferon gamma receptor (IFNgR−/−), vary in their H-2 haplotype and in their reported resistance to MCMV infection. Groups of 4–6 animals of each of these strains were infected with 10^5 PFU MCMV by IP injection, which is a sublethal dose in adult animals of each of these strains were infected by intraperitoneal injection with 10^5 PFU MCMV. Sera collected 14 days post-infection were assayed in duplicate at a 1:10 dilution for anti-MCMV (gray bars) and anti-CD13 (black bars) antibodies by ELISA. Figure is representative of two similar experiments. Dashed line indicates mean antibody response in mock-infected mice.

levels anti-CD13 antibodies in mice, cyclosporine A was used to induce an immunocompromised state in ICR mice. Three groups of 6 ICR mice were treated with either cyclosporine A alone, cyclosporine A and 10^5 PFU MCMV, or 10^5 PFU MCMV + mock cyclosporine A injections. Mice treated with cyclosporine A and MCMV had increased signs of illness over those given MCMV alone (not shown). However, levels of antibodies to both MCMV and CD13 were not significantly different between the two groups (Fig. 2).

Cell surface APN activity and MCMV susceptibility

To investigate whether expression of murine CD13/APN correlates with MCMV susceptibility as reported for HCMV (Soderberg et al., 1993a, 1993b), five murine cell lines were characterized with respect to MCMV progeny production and cell surface aminopeptidase N/CD13 activity. NIH 3T3, 3T12, and primary mouse embryonic fibroblasts (MEF) are fibroblast in phenotype. Py-41 and SVEC-4 cells are mouse endothelial cell lines immortalized by the polyoma T antigen and the SV40 T-antigen, respectively (Dubois et al., 1991; O’Connell and Edidin, 1990). In an assay of MCMV progeny production after inoculation at low multiplicity of infection, marked differences were seen between cell lines in susceptibility to productive MCMV infection (Fig. 3A). Peak MCMV titers in supernatants from the three fibroblast lines were close to 10^6 PFU/ml, while SVEC-4 cells produced 10^5 PFU/ml, and Py-41 cells produced less than 1000 PFU/ml. Similar relative results were seen in a plaque assay on the five cell lines (Fig. 4).

In contrast to human cells, attempts to compare CD13/APN expression on murine cell lines by immunological methods were unsuccessful. Two commercially available antibodies specific for murine CD13/APN failed to recognize any proteins on Western blotted cell extracts (not shown). Immunofluorescent flow cytometry staining of cell surface proteins with antibody R3-242 conjugated to phycoerythrin showed that CD13/APN was present on both endothelial and fibroblast cells. However, relative expression
cannot be compared by this method in different cell types, so an alternative to immunological techniques was used.

To quantitate cell surface CD13/APN, APN enzymatic activity was measured by a standard procedure using the colorimetric substrate, ala-β-nitroalanine hydrochloride (Plakidou-Dymock et al., 1993). To determine the linearity of the assay with adherent cells, Py-41, SVEC-4, 3T3, 3T12, and MEF cells were plated at densities of 5000, 10,000, 20,000, and 40,000 cells/well and incubated 18 h later as adherent monolayers with APN substrate. Substrate conversion was directly proportional to the number of cells in all cell lines (Fig. 3B), with slight decreases at higher densities as expected. Cells were plated at 10,000 cells/well in 96-well plates for subsequent assays. To compare amounts of cell-surface CD13/APN exposed to MCMV in a standard inoculation with subsequent plaque formation, parallel sets of 6 wells of each cell type were assayed for APN enzyme activity, or inoculated with MCMV-lacZ and incubated a further 48 h later as adherent monolayers with APN substrate. Substrate conversion was directly proportional to the number of cells in all cell lines (Fig. 3B), with slight decreases at higher densities as expected. Cells were plated at 10,000 cells/well in 96-well plates for subsequent assays. To compare amounts of cell-surface CD13/APN exposed to MCMV in a standard inoculation with subsequent plaque formation, parallel sets of 6 wells of each cell type were assayed for APN enzyme activity, or inoculated with MCMV-lacZ and incubated a further 48 h, followed by staining and counting of plaques. Three dilutions of MCMV-lacZ virus were used in three sets of 6 wells to detect the range of susceptibilities observed. There was no correlation between CD13/APN expression and MCMV plaque formation (Fig. 4). CD13/APN enzyme activity was similar on all five cell lines whereas plaque formation and progeny virus production were markedly different, especially between endothelial and fibroblast cells. Whether or not CD13/APN is necessary for MCMV infection cannot be determined from this experiment since none of the murine cell lines tested were negative for CD13/APN, however, CD13 expression is clearly not sufficient for productive MCMV infection of Py-41 endothelial cells.

**APN inhibitors reduce MCMV infection**

EDTA and 1,10-phenanthroline monohydrate are both APN inhibitors which act by chelating divalent metals. At effective concentrations, both have the effect of causing adherent cells, such as 3T3 cells, to lift. Therefore, cells were purposely incubated with MCMV-lacZ virus in suspension in the presence or absence of APN inhibitor. After 1-h incubation, cells were washed clean of unbound virus and inhibitor and plated. Plaques visualized at 48 h post-infection with X-Gal were counted in 6 parallel wells/ treatment. Both EDTA and 1,10-phenanthroline significantly decreased the number of plaques formed on treated cells in a dose-dependent manner (Fig. 5A). Exposure to concentrations of 1,10-phenanthroline above 1.5 mM or EDTA above 5 mM for 1 h killed 3T3 cells. In contrast, when virus was exposed to the same inhibitor concentrations for 30 min prior to dilution and inoculation of cells,
little effect was seen on plaque formation, indicating that the MCMV particles themselves were not dependent on metalloproteinase activity for stability or activity (Fig. 5B).

Effect of anti-CD13/APN antibody on MCMV infection in vitro

CD13/APN has been detected with antibodies in HCMV viral envelopes and some anti-CD13/APN antibodies neutralize human CMV and prevent infection in vitro (Giugni et al., 1996). Both anti-murine CD13/APN antibodies were tested for their ability to block MCMV infection of NIH3T3 cells. Neither preincubation of antibody with virus nor antibody with host cells reduced plaque formation (Figs. 5A and B). Since not all CD13/APN epitopes give rise to neutralizing antibodies of HCMV (Giugni et al., 1996), this did not rule out a role for APN in MCMV infection. Strangely, the presence of antibody R3-242 during infection significantly enhanced the number and size of plaques formed on NIH3T3 cells (Fig. 5A), while antibody H-300 had a similar effect when preincubated with virus (Fig. 5B). Although antibody-dependent enhancement of viral infection is well documented, it generally requires antibodies from the same species as the host cells and depends on Fc or complement receptor expression of the host cell (Tirado and Yoon, 2003). R3-242 and H-300 are rat and rabbit antibodies, respectively, and NIH 3T3 cells are not known to express Fc receptors.

Two other methods were used to attempt to detect APN incorporation into MCMV viral particles. APN activity assays of salivary gland-derived virus vs. uninfected salivary gland supernatants were compared and found to be identical when normalized for protein content (Table 1). In addition, salivary gland-derived MCMV, tissue culture-derived MCMV, relevant uninfected control extracts, and murine brush border membrane vesicles (BBMV) were spotted onto nitrocellulose at several dilutions and probed with antibody R3-242. The antibody readily detected an 80-fold dilution of BBMV, while failing to detect any protein in the viral or control preparations at any dilution (data not shown). In summary, no evidence for incorporation of CD13/APN into MCMV viral envelopes was found.

Table 1

| APN activity in MCMV virus preparation | APN activity (substrate cleaved/mg protein) |
|----------------------------------------|--------------------------------------------|
| BBMV (μmol/min)                        | 53.7                                       |
| Uninfected salivary gland homogenate (μmol/min) | 3.0                                        |
| MCMV-infected salivary gland homogenate (μmol/min) | 2.8                                        |
Human CMV has been reported to reduce CD13/APN expression in infected human fibroblasts approximately 30% within 24 h of infection, as determined by immuno-fluorescence staining and APN activity assays. Expression is further reduced to up to 50% over the next 2–3 days of infection and appears to be due to ER retention and degradation of the CD13 protein (Phillips et al., 1998). To determine if MCMV has a similar effect on murine CD13/APN, cells from all five murine cell lines were assayed for APN activity 18 and 72 h after infection. Cells were infected with an MOI of approximately 20, or mock infected with an equivalent concentration of salivary gland homogenate from uninfected mice. In contrast to human fibroblasts, all three mouse fibroblast lines had slightly (10–30%) increased APN cell surface activity 18 h after infection, while mouse endothelial cell lines Py-41 and SVEC-4 had slightly (3–9%) decreased APN activity (Fig. 6). At 72 h, cell death was apparent in all infected wells, and APN activity was reduced compared to mock-infected wells, however, APN activity per well was increased compared to 18 h post-infection in all cases due to increases in cell number (not shown). Whole cell ELISA gave similar results, except that differences between infected and non-infected cells were even smaller (not shown). Therefore, unlike human CMV infection, MCMV infection seems to have only a small effect if any on CD13/APN expression in fibroblasts and endothelial cells, which varies according to cell type.

Discussion

In agreement with previous reports that human CMV infection induces anti-CD13/APN antibodies in immuno-compromised individuals, we found that murine CMV induced antibodies against murine CD13/APN in susceptible strains of mice. However, treatment of susceptible mice with the immunosuppressant Cyclosporine A did not significantly increase anti-CD13/APN antibody production, suggesting that immunosuppression is not necessary for induction of these autoantibodies. Whether immunocompetent humans can develop anti-CD13 antibodies after CMV infection has not been investigated to our knowledge.

The strain producing the highest ratio of anti-CD13 to anti-MCMV antibodies was 129S-IFNgR−/− which has been used as a model of persistent CMV infection because the mice actively shed virus for at least 6 months after infection (Presti et al., 1998). In contrast, CBA and CBA/B6 hybrids are known to be genetically resistant to MCMV-induced mortality. MCMV resistance in mice is determined largely by the gene cmv1, which encodes Ly49H, an NK cell receptor which is activated by the MCMV protein m157 (Arase et al., 2002; Lee et al., 2002; Swihart et al., 1995). MCMV-resistant mouse strains have peak spleen viral titers that are 2–3 logs lower and LD50 doses that are 1–2 logs higher than susceptible strains. CBA and C57BL/6 mice express Ly49H on their NK cells and are resistant whereas 129S mice do not express Ly49H and are susceptible. The ICR mice were not genotyped for cmv1 and were intermediate in their susceptibility to MCMV morbidity, as well as autoantibody production. This suggests that high spleen titers may be associated with development of anti-CD13 antibodies. MHC type does not seem to be correlated with anti-CD13 antibody production in our experiments, since the haplotype H-2b is shared by both productive 129S and non-productive C57BL/6 hybrid strains.

In the human system, it has been proposed that anti-CD13/APN antibodies occur as a result of autoimmunization by CD13/APN incorporated into HCMV viral envelopes. We were unable to find evidence of CD13/APN incorporation into MCMV viral particles, but this may be due to the limited variety of anti-CD13 antibodies available for the murine system. Neither antibody tested was able to detect CD13/APN on Western blots, and sc-15360 failed to detect CD13/APN even in native BBMV containing APN readily detected at a 1:80 dilution by antibody R3-242. It is possible that a more comprehensive array of anti-murine CD13/APN antibodies would have detected CD13/APN in MCMV particles or have been able to neutralize MCMV infectivity, however, APN enzymatic assays also failed to detect a difference between virus and control lysates. Cell-surface CD13/APN also did not correlate with MCMV susceptibility. And while inhibitors of APN enzyme activity did reduce MCMV plaque formation, the inhibitors used are not especially specific for APN. Taken together, a role for CD13/APN as an entry mediator for MCMV seems unlikely.

Virus-induced autoantibodies, including anti-endothelial cell and anti-phospholipid antibodies following cytomegalovirus infections, have been demonstrated before in both
mice and humans (Bartholomaeus et al., 1988; Cheng and Khairullah, 1997; Fairweather et al., 1998; Hebart et al., 1996; Hiemstra et al., 2001; Lipes et al., 2002; Lunardi et al., 2000). With regard to the mechanisms of their induction, it has been proposed that these antibodies are directed against viral antigens which by coincidence resemble host cellular proteins (molecular mimicry), or that virus-induced tissue damage results in immunological processing and recognition of self-antigens normally sequestered from immune surveillance (Lawson, 2000; Naucler et al., 1996). In this study, several factors argue against a simple case of molecular mimicry. Although human and mouse CD13/APN are 76% identical at the amino acid level (Chen et al., 1996), HCMV and MCMV share less homology, CD13/APN is glycosylated and genetically polymorphic (Vijgen et al., 2004; Wentworth et al., 1998). The fact that CMV induction of anti-CD13/APN antibodies is now reproduced in mice should make it possible to examine whether such antibodies play a part in CMV-induced vascular pathology.

Materials and methods

Cells, medium, antibodies, FACS, immunoblotting

SVEC-4, NIH 3T3, and NIH 3T12 cells were obtained from the ATCC (Rockville, MD) and grown as recommended. Mouse embryo fibroblasts were prepared from Balb/c mouse embryos as described (Brune et al., 1999). Py-41 cells were generously provided to our lab by V.L. Bauch (University of North Carolina, Chapel Hill, NC) and propagated on gelatin-coated plates (Biocoat, Becton Dickinson) in low glucose DMEM + 10% fetal calf serum (Dubois et al., 1991). All cells were grown in medium containing Gibco antibiotic–antimycotic solution (Invitrogen, Inc.). Two anti-murine CD13 antibodies were used: R3-242, a rat anti-mouse monoclonal (Research Diagnostics, Inc.), and sc-15360, a rabbit IgG (Santa Cruz Biotechnologies, Inc.).

Virus and plaque assays

A recombinant murine cytomegalovirus expressing the Escherichia coli lacZ gene (RM461), generously provided by Edward Mocarski (Stanford University), was used in all experiments. This virus expresses lacZ mid to late infection so that the enzyme accumulates throughout infection (Stoddart et al., 1994). Salivary gland passaged virus was prepared as described previously (Hamamdzic et al., 2001) and titered on 3T3 cells. Plaque assays were performed in 96-well plates. Briefly, NIH 3T3 cells were plated the day before the assay at 10^3 cells/well. Dilutions of virus were made in minimal essential medium containing 0.1% porcine gelatin, and 20 µl diluted virus/well was incubated with cells at 37 °C for 2 h. Inoculum was aspirated and cells covered with 100 µl/well 0.6% methylcellulose in complete medium. After incubation for 48–72 h, 100 µl 10% neutral-buffered formalin was added to each well for 10 min, followed by three washes with phosphate-buffered saline to remove methylcellulose. Plaques were visualized by overlay with X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and counted under a dissecting scope. Inoculations of mice were by intraperitoneal injection of salivary gland-passaged virus in a volume of 0.2 ml using minimal essential medium as a diluent.
Reagents

Cyclosporine A was purchased from LC laboratories (Woburn, MA) and 14 mg was solubilized in 0.5 ml 100% ethanol and 0.2 ml Tween 80, then diluted to 2 mg/ml in PBS for injection. Ethylenediaminetetraacetic acid (0.5 M solution, pH 8.0) was purchased from GibcoBRL (Cat. No. 15575-038). 1,10-Phenanthroline monohydrate (P 9375) and L-alanine-p-nitroanilide hydrochloride were purchased from Sigma.

Mice

CBA, CBA/C57BL6 f1 hybrids, and 129S IFNγR−/− mice were obtained from Jackson Laboratories (Bar Harbor, Maine). ICR mice were purchased from Harlan Laboratories. All experiments described in this study conform with “The guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the MUSC institutional committee for the care and use of laboratory animals. Food and water were provided ad libitum. All mice were housed in hepa-filtered cages in the approved animal facility and monitored daily.

Crude aminopeptidase N preparation

To prepare murine CD13, small intestines from nine ICR mice were dissected free of mesenteric fat and flush cleaned with cold saline. The drained tissue was homogenized in 9 volumes cold sucrose buffer (2 mM Tris, pH 6.8, 50 mM sucrose) with a Tissumizer SDT-1810 (Tekmar, Cincinnati, Ohio) for 1 min keeping the tube on ice. Brush border membrane vesicle formation was induced by adding CaCl2 to a final concentration of 10 mM and stirring continuously for 15 min at 4 °C. Debris was pelleted by centrifugation at 4000 × g for 10 min. BBMVs were pellet by centrifugation of the supernatant for 30 min at 27,000 × g at 4 °C. BBMVs were resuspended in a small volume of sucrose buffer and stored at −80 °C. Gut APN has been shown to be serologically identical to CD13/APN expressed in splenocytes and macrophage (Riemann et al., 1999).

Aminopeptidase N activity assays

Aminopeptidase N activity assays were performed by incubating living adherent cells at 37 °C in phosphate-buffered saline (pH 7.2) containing 6 mM alanine-p-nitroanilide hydrochloride. Absorbance was read at 1-h intervals at 405 nm.

ELISA

ELISAs were performed on Reacti-Bind maleic anhydride activated clear 96-well plates (Pierce, Rockford, IL). Viral antigen for ELISA was intact MCMV grown in tissue culture and prepared by ultracentrifugation through sucrose by standard protocol (Brune et al., 1999). The partially purified virus was diluted in phosphate-buffered saline to a protein concentration of 10 mg/ml and plates were coated using 50 μl/well overnight at 4 °C. For detection of anti-CD13/APN antibodies, BBMVs in sucrose buffer were diluted in PBS to a protein concentration of 1.5 mg/ml, and 50 μl/well was used to coat plates overnight at 4 °C. Blocking of binding of rabbit antibody sc-15360 and rat monoclonal R3-242 by mouse sera demonstrated specificity of antibodies in sera of MCMV-infected mice for CD13/APN. Whole cell ELISAs were also performed to measure cell surface CD13 expression on the murine cell lines. Cells were plated at 10⁴ cells/well and incubated with R3-242 anti-CD13 antibody the next day after fixation with 2% glutaraldehyde. Horseradish peroxidase-conjugated secondary antibodies were used and detected by incubation with 1-Step ABTS (Pierce).

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