CHAPTER 2  

RAT CYTOMEGALOVIRUS INDUCES A TEMPORAL DOWNREGULATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I CELL SURFACE EXPRESSION

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Summary

Herpesviruses are known to influence expression of Major Histocompatibility Complex (MHC) class I molecules on the surface of infected cells using a large variety of mechanisms. Downregulation of MHC class I expression prohibits detection and elimination of infected cells by cytotoxic T lymphocytes. To investigate the effect of rat cytomegalovirus (RCMV) infection on MHC class I expression, we infected immortalized and primary rat fibroblasts with RCMV and monitored surface expression of MHC class I molecules at various time-points post infection. These experiments revealed a dramatic downregulation of MHC class I surface expression by RCMV, a phenomenon that has also been reported for human and murine CMV. However, in contrast to the other cytomegaloviruses, RCMV only causes a temporal downregulation of MHC class I, with a maximum decrease at 12 hrs post infection. Unlike murine and human CMV, RCMV does not induce proteolytic degradation of MHC class I molecules. In RCMV-infected cells, the MHC class I molecules are stable, but their exit from the ER is delayed.

Introduction

The rat cytomegalovirus (RCMV) is often used as a model to investigate the pathogenesis of human CMV (HCMV) infection. RCMV has proven to be a useful model to study CMV infection in, for example the context of transplant-associated arteriosclerosis and therapeutic interventions related to this disease \(^1\-^3\). Like HCMV infection in humans, RCMV infection does not result in clinical symptoms in immunocompetent rats. In immunosuppressed rats, however, RCMV causes a generalized infection with infectious virus being present in almost every organ. Eventually, RCMV establishes a latent infection \(^4\,^5\).

Elimination of virus-infected cells by cytotoxic T lymphocytes (CTL) relies on the recognition of antigenic peptides in the context of Major Histocompatibility Complex (MHC) class I molecules. The antigenic peptides result from proteolytic degradation of virus-encoded proteins by a multicatalytic, cytosolic proteasome \(^6\,^8\). The peptides are transported from the cytosol into the Endoplasmic Reticulum (ER) by the Transporter associated with Antigen Processing (TAP) \(^9\). In the ER, the trimolecular complex consisting of the MHC class I heavy chain, the light chain \(\beta_2\)-microglobulin (\(\beta_2\text{m}\)) and peptide is formed. Only those complexes that consist of a properly
folded heavy chain, β2m and peptide are stable; all others are degraded. The class I complex is transported from the ER to the cell membrane, where it presents its peptide content to cytotoxic T cells.

During a long co-evolution with their host, herpesviruses have developed effective mechanisms to prevent rapid clearance by the immune system. Studies with murine and human CMV have shown that these β-herpesviruses are capable of inhibiting the presentation of peptides by MHC class I molecules in numerous ways. To date, four genes affecting MHC class I-restricted antigen presentation have been identified in the HCMV genome. The HCMV US3 gene encodes a protein causing retention of MHC class I molecules in the ER. The US2 and US11 gene products mediate rapid degradation of MHC class I molecules by dislocating the heavy chains from the ER into the cytosol, where they are degraded by proteasomes. The protein encoded by US6 acts in yet another way, blocking translocation of peptides into the ER by TAP.

Different species of CMV have developed different strategies to elude the immune system. This is exemplified by the diversity of immune evasion strategies identified for murine and human CMV. MCMV interferes with the function of MHC class I molecules through three glycoproteins, none of which has homologs in HCMV. The protein encoded by m152 prevents export of class I complexes from the post-ER/early Golgi. The m06 gene product induces lysosomal degradation of the MHC complex after a transient interaction in the ER. A third gene product, the m04-encoded gp34, associates with properly folded MHC class I molecules in the ER. The resulting complex is transported to the cell membrane where it may modulate the function of NK cells. The MCMV-encoded immune evasion genes appear to counteract MHC class I restricted T cell activation in a cooperative fashion.

In addition to gene products that affect the expression of MHC class I molecules on the surface of infected cells, HCMV, MCMV and also RCMV encode homologs of MHC class I heavy chains. The HCMV and MCMV MHC class I homologs, encoded by UL18 and m144, respectively, inhibit activation of NK cells. In addition, the UL18 protein may interact with other leukocytes than NK cells, since the Leukocyte Immunoglobulin-like Receptor (LIR-1), identified as a UL18 receptor, is primarily expressed on B-lymphocytes and macrophages, but only on a small proportion of the NK cells.

As both HCMV and MCMV encode multiple gene products interfering with T cell recognition of infected cells, it is likely that the closely related RCMV also eludes host immunity. In view of the diversity of immune evasion mechanisms identified for HCMV and MCVM, the strategies employed by
RCMV may be completely different again. In this study, the integrity of the MHC class I antigen presentation pathway was evaluated in RCMV-infected cells using flow cytometry and biochemical assays. We present the first evidence that RCMV causes a temporal downregulation of MHC class I molecules at the surface of infected cells. This downregulation does not rely on degradation of MHC class I complexes, but involves a delayed maturation of class I molecules in the ER.

Results

**RCMV downregulates MHC class I expression at the cell surface.**

To obtain a first indication as to whether RCMV interferes with antigen presentation to cytotoxic T cells, the surface expression of MHC class I molecules was analyzed at various time points after RCMV infection. Primary rat embryo fibroblasts (REF) and immortalized rat fibroblasts (Rat2) were infected with RCMV and the expression of cellular and viral proteins was assessed using flow cytometry. Intracellular staining with the monoclonal antibody RCMV35, recognizing a 29-kDa RCMV early protein, revealed an infection efficiency of at least 75% (data not shown). The expression of MHC class I molecules was measured using the monoclonal antibody OX-18, recognizing a monomorphic determinant on RT1a molecules, the rat MHC.
class I products. Both, REF and Rat2 cells showed a temporary downregulation of MHC expression at the cell surface, with a maximum effect at 12 hrs post infection (figure 1A). The expression of MHC class I products was normalized or slightly increased at 24 hrs (figure 1A), and remained constant at 2 and 3 days post infection (figure 1C). To investigate whether RCMV infection caused a general reduction in the expression of cell surface molecules, we also analyzed the expression of the transferrin receptor (TfR). At 12 hrs post infection, staining of the plasma membrane with anti-TfR antibody revealed a similar fluorescence intensity to that of uninfected cells (figure 1B). At 24 hrs post infection, an increased intensity was detected.

These results indicate that RCMV causes a temporal decrease in MHC class I expression at the cell membrane. This decrease occurred in primary and immortalized rat fibroblasts.
The maturation of MHC class I molecules is delayed in RCMV-infected cells

To investigate the cause of the class I downregulation at the surface of RCMV-infected cells, the biosynthesis and intracellular trafficking of class I molecules was monitored at various time points after infection. The monoclonal antibody OX18 was used to immunoprecipitate class I molecules from metabolically labeled Rat2 cells (figure 2A). Two class I heavy chain species of 45 and 47 kDa, respectively, and β2m can be distinguished. Upon digestion with endoglycosidase H, a shift in mobility of the 45-kDa chains was observed, reflecting removal of the immature, high-mannose N-linked glycans. As the heavy chains containing the immature and mature N-linked glycans can be distinguished on the basis of their mobility in SDS-PAGE, endoglycosydase H treatment is not required to visualize maturation of the class I molecules in time (figure 2B). Immediately after labeling, the OX-18 antibody only precipitated the 45-kDa immature class I heavy chains, in addition to β2m. In the course of the chase, a proportion of the 45-kDa products shifted to 47-kDa, reflecting maturation of the class I heavy chains (figure 2B, left panel).

Rat2 cells were infected with RCMV and labeled with 35S-methionine/cysteine at 4, 6 and 8 hours post infection. The monoclonal antibody RCMV35 precipitated a 29-kDa early viral protein from the cell lysates after 4 hrs of infection, indicating successful virus infection (figure 2B, right panel). The synthesis of MHC class I heavy chains gradually increased in the infected cells. This increase was not only seen for the heavy chains, but also for β2m and transferrin receptor, suggesting a general enhancement of protein synthesis in RCMV-infected cells. The increase of class I heavy chains predominantly involved the lower, immature form. In agreement with this, a quantitative analysis revealed that the relative amount of mature class I heavy chains was reduced in infected cells (figure 2C). Whereas in mock-infected cells about 46% of the heavy chains had matured after 60 minutes of chase, in RCMV-infected cells only 37% of the total heavy chain pool was converted into the mature form after 60 minutes of chase. Thus, class I synthesis is enhanced in RCMV-infected cells, but the maturation of the class I molecules appears to be delayed. This delay in exit from the ER was even more pronounced when the experiment was performed at 24 hrs post infection (figure 3A and C). Note that in this experiment, the cells were chased for 3 hrs.

The delayed maturation of MHC class I molecules in RCMV infected cells is not caused by a reduction in the supply of peptides

In MCMV and HCMV-infected cells, MHC class I heavy chains are
targeted for degradation. The mo6 gene product induces migration of class I molecules to a lysosomal compartment, where degradation follows. HCMV US2 and US11 redirect the class I heavy chains back to the cytosol, where they are degraded by proteasomes. In RCMV-infected cells, no degradation of class I molecules is observed (figures 2B and 3A). In cells expressing HCMV US2 or US11, degradation of the class I molecules can be blocked using proteasome inhibitors. In that case, a deglycosylated heavy chain breakdown intermediate accumulates in the cytosol. The deglycosylated heavy chain becomes visible in the course of the chase as a discrete polypeptide band with increased mobility in SDS-PAGE. When RCMV-infected cells were treated with proteasome inhibitor, no deglycosylated heavy chains were visible (data not shown), which supports the conclusion that class I molecules are not degraded during RCMV infection.

The inhibition of proteasomal activity reduces the amount of peptides available for loading onto class I molecules. The heavy chain-2m complexes devoid of peptides are retained in the ER. In agreement with this, a complete
block of maturation was observed for class I heavy chains in Rat2 cells treated with ZL3H (figure 3B). Note that the mobility of the heavy chains was slightly increased at 180 minutes of chase. This presumably reflects trimming of the N-linked glycans in the ER. The class I migration pattern in uninfected control cells (figure 3B) is completely different from that observed in RCMV-infected cells, in which maturation is delayed, but not blocked (figures 2B and 3A). This indicates that the supply of peptides is not a limiting factor in RCMV-infected cells, implying that inhibition of TAP is highly unlikely. In this respect, RCMV resembles MCMV, for which no TAP inhibition has been observed, but behaves differently from HCMV, which blocks TAP through US6.

HCMV and MCMV both encode proteins that mediate retention of class I complexes in the ER. HCMV US3 inhibits tapasin-dependent peptide loading, thereby retaining those class I locus products that depend on tapasin for peptide loading. A homolog of US3 has not been found in RCMV. The MCMV-encoded m152 prohibits egress of class I complexes from the post-ER/early Golgi. RCMV encodes a close homolog of m152, r152. To investigate whether r152 fulfils a similar function as m152, a retroviral vector was constructed encoding the r152 gene upstream of a sequence encoding an internal ribosomal entry site (IRES) and EGFP, respectively. In Rat2 cells transduced with the resulting recombinant retrovirus, GFP-expression was observed, but no downregulation of class I surface staining (data not shown). This strongly suggests that r152 does not mediate intracellular retention of class I. R152-expression could not be confirmed due to the lack of an r152-specific antiserum. Therefore, this result should be interpreted with caution.

In conclusion, the results obtained indicate that intracellular trafficking of rat class I molecules is delayed in RCMV-infected cells. Class I heavy chain-β2m complexes are not degraded, but their maturation is retarded, most likely due to temporary retention in an ER/cis-Golgi compartment.

Discussion

Human, mouse and rat cytomegaloviruses all persist in their hosts for life, despite a fully competent immune system. This phenomenon is believed to involve specific evasion of host immunity, thus allowing these herpesviruses to replicate during primary infection, establish latency, and reactivate under circumstances profitable for these viruses. Immune escape by herpesviruses has been shown to involve very diverse mechanisms, even among members of the same virus family. MHC class I molecules appear to represent a universal target for herpesvirus immune evasion. Antigen
presentation via MHC class I molecules is inhibited by HCMV and MCMV through various strategies, including downregulation of class I complexes at the cell surface, alteration of the peptide array presented by MHC class I molecules, interference with MHC-T-cell receptor interaction, and modification of the response of immune effector cells triggered upon recognition of class I-peptide complexes. This is the first study to report on compromised MHC class I function in RCMV-infected cells. We found a temporal downregulation of class I molecules at the cell surface during the first 24 hours of RCMV infection. RCMV has been shown to resemble both HCMV and MCMV in genome organization as well as open reading frame homology. As both HCMV and MCMV downregulate class I expression at the cell surface at some point during infection it is not surprising to find that RCMV infection results in a similar phenotype. It should be emphasized, however, that HCMV and MCMV each use unique gene products and completely different strategies to achieve class I downregulation. Thus, although RCMV infection results in a similar phenotype, this may rely on gene products and mechanisms other than those identified for HCMV and MCMV.

Pulse-chase experiments revealed that the temporal downregulation of class I surface expression in RCMV-infected cells was not a result of degradation of heavy chains, but coincided with a delay in the maturation of class I molecules, most likely as a result of retention of the MHC class I complexes in an ER/cis-Golgi compartment. Surface expression increased again after 12-16 hours post infection. This is probably not due to reduced expression of the viral gene products responsible for MHC class I downregulation, since at 24 hours post infection, still only half of the heavy chains had matured after 180 minutes of chase. As heavy chain synthesis increased from 6 hours post infection and onward, it is more likely that the ultimate restoration of MHC class I surface expression relies on increased synthesis rates. The increased expression seems to be the result of a general increase in protein synthesis, as β2m and transferrin receptor expression was also elevated, in addition to a large number of unspecified proteins. Although IFNγ induction by the virus could explain the increase in MHC class I heavy chain levels, this is unlikely to be responsible for enhanced expression of β2m and TR.

The surface expression of class I recovered remarkably fast compared to HCMV and MCMV-infected cells, where heavy chain downregulation persisted for a much longer period. HCMV blocks the display of MHC class I-peptide complexes through immediate early (US3), early and late gene products (US2, US6, US10 and US11). In MCMV, downregulation seems to occur mostly during the early and late phase of infection. In RCMV, however,
MHC class I cell surface downregulation is observed during the first 16 hours only, which corresponds to the early phase of infection. At later time points during infection other immune evasion strategies, not involving class I downregulation, may be exploited by RCMV.

In an attempt to identify the RCMV protein responsible for the observed ER retention of class I, we searched the RCMV genome for homologs of HCMV and MCMV genes known to affect class I expression, including the herpes simplex virus (HSV) 1 and HSV 2-encoded ICP47, the HCMV US2, US3, US6, US10 and US11, and the MCMV m04/gp34, m06/gp48, and m152/gp40. Based on sequence similarity and genomic position, only a homolog of m152, r152, was encountered. Preliminary data suggest that expression of the r152 gene product in Rat2 cells did not influence MHC class I surface expression. Possibly, one of the other members of the r152 family may be responsible for impaired maturation of class I molecules. Alternatively, a completely unrelated gene product may be involved, analogous to what has been observed for TAP inhibition by HSV ICP47 and HCMV US6, or class I retention by HCMV US3 and MCMV m152/gp40, or degradation of heavy chains by MCMV m06/gp48 and HCMV US2 and US11.

In conclusion, we have shown that RCMV, like its relatives MCMV and HCMV, decreases MHC class I expression at the cells surface. Contrary to what has been found for MCMV and HCMV, this downregulation is only temporary and is not the result of proteolytic degradation of class I molecules. Instead, maturation of class I MHC products is delayed in RCMV-infected cells. Further experiments will have to be performed to uncover the gene product(s) responsible for this phenomenon.

Materials & Methods

Cell culture and virus
Primary rat embryo fibroblasts (REF) and the rat fibroblast cell line Rat2 were cultured as described previously. RCMV (Maastricht strain) was obtained by homogenization of salivary glands of acutely infected rats as described previously.

Antibodies
Mouse monoclonal antibodies recognizing RT-1A MHC class I molecules (OX18) and rat transferrin receptor (OX26) were purchased from Serotec (UK). Monoclonal antibody RCMV 35, directed against a 29-kDa RCMV protein, has been described previously. For flow cytometry experiments, either FITC-conjugated rabbit anti-mouse IgG (Dako A/S,
Denmark) or PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibody.

**Infection**

The cells were infected at 80-90% confluency, washed with PBS, placed on EMEM containing 2% FCS for 30 min, and washed again with PBS. The virus was diluted in EMEM with 2% NCS. For flow cytometry experiments, cells were infected with an m.o.i. of 1; for biochemical experiments, cells were infected with an m.o.i. of 3. To increase the efficiency of infection, cells were centrifuged at 700 g at 20 °C for 45 min and placed at 37 °C for another 10-15 min. The infection medium was replaced by culture medium containing 2% NCS. For biochemical experiments, RPMI was used for cell culture and infections.

**Flow cytometry**

Cell surface expression of MHC class I molecules, transferrin receptor and EGFP were analyzed by flow cytometry using FACSort equipment and Cell Quest software (Becton Dickinson, USA). Cells were stained in PBS containing 1% BSA and 10mM NaN₃.

**Metabolic labeling, immunoprecipitations and SDS-PAGE**

For pulse-chase experiments, cells were starved in medium lacking methionine and cysteine at 37 °C for 1 hr. The cells were labeled with 35S Promix (Amersham), and chased in medium with excess of L-cystine and L-methionine for the times indicated in the figures. Where indicated, media were supplemented with the proteasome inhibitor carboxybenzyl-leucyl-leucylleucinal (ZL3H). Cells were lysed in Nonidet-P40 lysis buffer containing leupeptin, AEBSF and ZL3H at 4 °C for 30 min. To remove cell debris, lysates were centrifugated at 10,000g for 10 min.

Prior to immunoprecipitation, lysates were precleared twice using normal rabbit and normal mouse sera precoupled to mixed (1:1) Protein A-Sepharose and Protein G-Sepharose beads. Immunoprecipitations were performed on precleared lysates at 4 °C for 2-4 h using specific antisera precoupled to Protein A/G Sepharose beads. Subsequently, the beads were washed with NET buffer [50 mM Tris/HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.5% (v/v) NP-40] supplemented with 0.1% SDS. The immunoprecipitates were boiled in sample buffer [40 mM Tris/HCl, pH 8.0/4 mM EDTA/8% (w/v) SDS/40% (v/v) glycerol/0.1% Bromophenol Blue] for 5 min and separated using SDS/PAGE. Gels were dried and exposed to a phosphor-imaging screen, which was scanned in a Personal Molecular Imager FX (Bio-Rad) and analyzed using Quantity One (Bio-Rad).
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