An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling

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Human cytomegalovirus (HCMV) is a frequent cause of major disease following primary infection or reactivation from latency in immunocompromised patients. Infection of non-permissive mononuclear cells is used for analyses of HCMV latency in vitro. Using this approach, it is shown here that repression of lytic gene expression following experimental infection of CD34+ cells, a site of HCMV latency in vivo, correlates with recruitment of repressive chromatin around the major immediate-early promoter (MIEP). Furthermore, long-term culture of CD34+ cells results in carriage of viral genomes in which the MIEP remains associated with transcriptionally repressive chromatin. Finally, specific differentiation of long-term cultures of infected CD34+ cells to mature dendritic cells results in acetylation of histones bound to the MIEP, concomitant loss of heterochromatin protein 1 and the reactivation of HCMV. These data are consistent with ex vivo analyses of latency and may provide a model for further analyses of the mechanisms involved during latency and reactivation.

Primary infection of healthy individuals with human cytomegalovirus (HCMV) is often asymptomatic and results in lifelong persistence of the virus within the host, a common feature of all herpesvirus infections. However, primary infection and reactivation of latent HCMV are responsible for serious disease in both immunosuppressed transplant recipients and during advanced human immunodeficiency virus infection (Adler, 1983; Rubin, 1990). The health threat posed by the reactivation of latent HCMV has resulted in considerable efforts from a number of laboratories to identify the sites of latency and potential mechanisms responsible for controlling the progression of HCMV latency to reactivation. These studies include the study of naturally infected cells ex vivo and experimentally infected model systems in vitro.

Analysis of peripheral blood mononuclear cells identified monocytes as one site of HCMV carriage in healthy individuals (Bevan et al., 1991; Taylor-Wiedeman et al., 1991). Monocytes represent a short-lived population of cells subject to constant renewal from myeloid precursors residing in the bone marrow. Analysis of these self-renewing CD34+ mononuclear haematopoietic progenitor cells showed that they also harboured HCMV genomes in naturally infected individuals (Kondo et al., 1996; Mendelson et al., 1996) and thus could represent an important reservoir of HCMV latency in vivo. Haematopoietic progenitors can differentiate into a number of cell types, including monocytes, macrophages, dendritic cells (DCs) and granulocytes, and in a recent analysis we showed that DCs generated ex vivo from healthy seropositive carriers reactivated HCMV and that latency and reactivation in these cells may be controlled by chromatin remodelling of the major immediate-early promoter (MIEP) to regulate lytic gene expression (Reeves et al., 2005). However, overall, the analysis of HCMV latency ex vivo has been complicated severely by the low frequency of genome-positive mononuclear cells, which is predicted to be around only 0·01% of the total population (Slobedman & Mocarski, 1999). Consequently, many studies have relied on experimental infection of primary cells and of cell lines to try to model the events that occur during latency and reactivation of HCMV in the myeloid lineage.

A number of these studies have shown that experimental infection of CD34+ cells is non-permissive and that a ‘latent infection’ is established during extended culture of these cells (Kondo et al., 1994; Minton et al., 1994; Movassaghi et al., 1996; Sindre et al., 1996). Such non-permissive infection is defined by the absence of lytic gene expression; however, it is worth noting that a small number of viral transcripts have been described in such experimentally infected cells, which have been suggested to be involved in the establishment or maintenance of latency (Jenkins et al.,
2004; Kondo et al., 1996), some of which have also been identified during latent infection in vivo (Jenkins et al., 2004; Kondo et al., 1996). However, the role for such transcripts in virus latency and reactivation in vivo, if any, is difficult to establish. Studies performed on experimentally infected fetal liver granulocyte/macrophage progenitors have shown that the carriage of HCMV genomes occurs with the absence of lytic gene expression in the precursors of the monocyte, dendritic and granulocyte populations of cells and that stimulation of these cells with pro-inflammatory cytokines can promote reactivation in vitro (Hahn et al., 1998).

Similarly, the terminal differentiation of experimentally infected bone-marrow CD34+ cells to macrophages can also result in the induction of lytic gene expression in these otherwise latently infected cells (Minton et al., 1994). Consequently, such in vitro data are consistent with analyses performed ex vivo on tissue isolated from naturally infected individuals (Mendelson et al., 1996; Söderberg-Nauclér et al., 1997; Taylor-Wiedeman et al., 1991, 1994).

CD34+ cells can also be differentiated into DCs (Strobl et al., 1997), which, like macrophages, are permissive for HCMV infection (Hertel et al., 2003). A previous study using experimentally infected CD34+ cells showed that HCMV genomes persist in DC precursors (Hahn et al., 1998) and, subsequently, analysis of myeloid DCs differentiated ex vivo from the mononuclear cells of healthy seropositive carriers showed that these cells are one site of HCMV reactivation (Reeves et al., 2005). Because of the inherent problems in the study of naturally acquired HCMV – specifically, the relatively low frequency of genome-positive cells and the availability of seropositive bone-marrow mononuclear cells from transplant programmes – we have attempted to generate and characterize a more tractable model in vitro system that will allow further analysis of the mechanisms of HCMV latency and reactivation.

Initially, we infected 5 × 10^6 granulocyte colony-stimulating factor-mobilized CD34+ cells with the HCMV strain TB40/E (m.o.i. of 1–2) for 24 h, thoroughly washed off non-adherent virus and then cultured these CD34+ cells in fresh X-vivo 15 medium (Biowhittaker) for 3 days (Fig. 1a). At 3 days post-infection, these infected CD34+ cells were split into two populations. Half of the CD34+ cells were cultured in X-vivo 15 medium for an additional 10 days in the absence of the cytokines needed to promote proliferation and differentiation (Fig. 1c). The remaining cells were cultured for 7 days in medium supplemented with granulocyte/macrophage colony-stimulating factor, fms-like tyrosine kinase-3 ligand, tumour necrosis factor alpha, transforming growth factor beta (all from Peprotech) and stem-cell factor, which promote differentiation to an immature DC phenotype (Fig. 1b) (Strobl et al., 1997). These immature CD34+ -derived DCs were then stimulated with lipopolysaccharide (LPS) at 50 ng ml^-1 (Sigma-Aldrich) to a mature phenotype for a further 3 days (Fig. 1b) (MacAry et al., 2001; Strobl et al., 1997). We then performed the same analysis on the unstimulated CD34+ cells (Fig. 1c), whereby

![Fig. 1. Differentiation of infected CD34+ cells to a mature DC phenotype is concomitant with the reactivation of infectious virus. (a) CD34+ cells were infected with HCMV strain TB40/E and then maintained in minimal medium for 3 days. Three days post-infection, 50% of the CD34+ cells were differentiated to mature DCs (b) or maintained in minimal medium (c). After a further 10 days, the CD34+ cells (c) were then subdivided and differentiated to mature DCs (d) or maintained in minimal medium as CD34+ cells (e). Following culture, samples of medium were used to infect fibroblasts to detect infectious virus, as shown by IE staining of infected HFs.](https://www.microbiologyresearch.org/)

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**Fig. 1.** Differentiation of infected CD34+ cells to a mature DC phenotype is concomitant with the reactivation of infectious virus. (a) CD34+ cells were infected with HCMV strain TB40/E and then maintained in minimal medium for 3 days. Three days post-infection, 50% of the CD34+ cells were differentiated to mature DCs (b) or maintained in minimal medium (c). After a further 10 days, the CD34+ cells (c) were then subdivided and differentiated to mature DCs (d) or maintained in minimal medium as CD34+ cells (e). Following culture, samples of medium were used to infect fibroblasts to detect infectious virus, as shown by IE staining of infected HFs.
we again split these cells into two populations. Half of the cells were differentiated to mature DCs as described before (Fig. 1d) and the remaining CD34+ cells were maintained in minimal medium as CD34+ cells (Fig. 1e). The differentiation of CD34+ cells to a DC phenotype was confirmed by both morphological changes and the expression of cell-surface markers. Briefly, differentiation resulted in an increase in cell size and the formation of proliferative clusters that are a characteristic of immature Langerhans DC growth in vitro. Furthermore, the formation of characteristic processes was seen following maturation of the DCs with LPS. These morphological changes were concomitant with the upregulation of major histocompatibility complex class I and class II surface expression and downregulation of CD34+ surface expression upon differentiation (data not shown).

We then tested for the presence of infectious virus during the course of this long-term culture of CD34+ cells and mature CD34+-derived DCs. To do this, samples of cell-free medium were taken and used to inoculate fresh human fibroblasts (HFs). Twenty-four hours post-infection, the HFs were permeabilized (70 % ethanol at −20 °C for 10 min) and stained for immediate-early (IE) protein expression in the nuclei by using a mouse anti-IE72/IE86 antibody (clone E13; Argene). Detection was performed by using a fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Sigma).

This analysis showed that no residual infectious virus was present in the culture medium of CD34+ cells 3 days post-infection (Fig. 1a). Similarly, no infectious virus was detected in the medium of the CD34+ cells cultured in the absence of cytokines that promote differentiation when assayed against fibroblasts (Fig. 1c). In contrast, infectious virus could be detected in the medium of the mature CD34+-derived DCs (Fig. 1b) 10 days post-maturation, suggesting that differentiation promoted reactivation of HCMV in the mature CD34+ derived DC population. Furthermore, subsequent differentiation of the long-term cultures of infected CD34+ cells previously maintained for 10 days in minimal medium (Fig. 1c) to a mature DC phenotype also resulted in the detection of infectious virus into the culture medium 14 days post-DC maturation (Fig. 1d). In contrast, no infectious virus was detected following long-term culture of the CD34+ cells in the absence of cytokines that promote DC differentiation (Fig. 1e). These experiments suggested that differentiation to a mature DC phenotype was concomitant with virus reactivation in vitro in this experimental system.

As well as analysing the release of infectious virus in long-term DC cultures, we also analysed the induction of IE gene expression in DCs 3 days post-maturation by immunofluorescent staining. We observed that IE gene expression was detected in around 5 % of DCs at this stage (data not shown). As infection of CD34+ cells with HCMV at an m.o.i. of 3–5 results in 10–15 % of cells carrying viral genomes (Goodrum et al., 2004; Movassagh et al., 1996), it would suggest that a number of the latent genomes were capable of reactivating following differentiation.

We have previously shown that chromatinization of the viral MIEP occurs following infection and may be important for the regulation of HCMV IE gene expression (Murphy et al., 2002). Specifically, following infection of non-permissive monocytes, the viral MIEP is associated with heterochromatin protein 1 (HP-1), a marker of transcriptional repression (Bannister et al., 2001; Lusser, 2002), whereas the infection of permissive monocyte-derived macrophages is concomitant with the association of the viral MIEP with acetylated histones, a marker of transcriptionally active regions of chromatin (Strahl & Allis, 2000), and not HP-1 (Murphy et al., 2002). However, we wanted to extend this analysis to determine whether the MIEP was chromatinized following experimental infection of CD34+ cells (an important site of HCMV latency) and, more importantly, whether the differentiation of long-term cultures of infected CD34+ cells, which results in reactivation of viral genomes, results in remodelling of the histones bound to the MIEP as the cells differentiate to mature DCs in vitro. Chromatin immunoprecipitation (ChIP) assays were performed on the long-term cultures of CD34+ cells and DCs from which the supernatants assayed for virus reactivation were taken (Fig.1a–d). ChIP assays were performed on 5 × 106 cells as described previously (Luo et al., 1998; Murphy et al., 2002). Briefly, formaldehyde-fixed cells were lysed, sonicated to fragment the DNA–protein complexes and then incubated with either control serum (1:200 dilution; Sigma-Aldrich), anti-acetylated histone H4 antiserum (1:200 dilution; Upstate Biotech) or anti-HP-1 antiserum (1:200 dilution; Serotec). The DNA bound to the isolated proteins was then purified and amplified in an MIEP-specific PCR and detected by Southern blotting using a radiolabelled DNA probe. The MIEP-PCR primers were designed to amplify a 285 bp fragment covering nucleotide positions −273 to +15 relative to the immediate-early gene (UL122/UL123) transcription start site (Davison et al., 2003).

These analyses show that, following experimental infection of CD34+ cells in vitro, the viral MIEP is associated with HP-1 protein (Fig. 2, lane 4) and not acetylated histones (Fig. 2, lane 3), which is consistent with CD34+ cells being non-permissive for HCMV IE gene expression. Furthermore, following the culture of experimentally infected CD34+ cells for 10 days in X-vivo 15 medium with no additional cytokines, from which we were unable to isolate infectious virus (Fig. 1c), the viral MIEP remained associated with HP-1 (Fig. 2, lane 12). In contrast, concurrent differentiation of the experimentally infected CD34+ cells to mature CD34+-derived DCs, which ultimately resulted in reactivation of infectious virus 10 days post-DC maturation (Fig. 1b), was preceded by chromatin remodelling of the MIEP, such that the viral MIEP was associated with acetylated histones (Fig. 2, lane 7) and was no longer bound by HP-1 (Fig. 2, lane 8) at 3 days post-maturation. Finally, the CD34+ cells that had previously been grown in X-vivo 15
medium for 10 days in the presence of infectious virus (Fig. 1c) were then cultured into mature CD34+ derived DCs in X-vivo 15 medium supplemented with cytokines that promote DC differentiation. Again, differentiation to a DC phenotype resulted in chromatin remodelling of the MIEP, such that the MIEP was associated predominantly with acetylated histones (Fig. 2, lane 15) and no longer with HP-1 (Fig. 2, lane 16) in mature CD34+ derived DCs.

Thus, our observations using this in vitro model of HCMV latency correlate strongly with our direct analyses of latency and reactivation ex vivo (Reeves et al., 2005).

The extremely low frequency of HCMV genome-positive mononuclear cells in vivo (Slobedman & Mocarski, 1999) has resulted in a number of studies using experimentally infected mononuclear cells as a tractable model for the study of HCMV latency (Hahn et al., 1998; Kondo et al., 1996; Maciejewski & St Jeor, 1999; Minton et al., 1994; Movassaghi et al., 1996). However, none of these studies has addressed reactivation upon specific differentiation of progenitor cells to DCs. A recent study that looked at the carriage of HCMV in mononuclear cells using a green fluorescent protein (GFP)-tagged virus also suggested that long-term culture can reactivate virus (Goodrum et al., 2004); however, this analysis is difficult to interpret, as the expression of GFP was under the control of an SV40 promoter – a promoter that may also be regulated differentially in undifferentiated and differentiated cell types (Taniguchi & Kitagawa, 1996).

In this study, we show that the repression of HCMV lytic gene expression following infection of CD34+ cells correlates with the recruitment of repressive chromatin markers (e.g. HP-1) to the MIEP. The exact mechanism by which recruitment of repressive chromatin occurs is unclear. However, it has been shown that Ets-2 repressor factor and Ying Yang-1, which can bind and repress the MIEP (Bain et al., 2003; Liu et al., 1994), also interact with histone deacetylases (Yang et al., 1996) and histone methyltransferases (Rezai-Zadeh et al., 2003; Wright et al., 2005). Similarly, the AML1b protein isoform, which can function as a transcriptional repressor by recruiting histone deacetylases (Lutterbach & Hiebert, 2000), has been shown to be upregulated upon infection of CD34+ cells with HCMV (Slobedman et al., 2004). Overall, this upregulation and recruitment of proteins involved in transcriptional repression may be a mechanism by which HCMV establishes latency following infection of bone-marrow mononuclear cells and maintains the MIEP in a transcriptionally repressed state in CD34+ cells.

In contrast, the reactivation of HCMV appears to be linked with the differentiation of latently infected myeloid cells both in vitro and in vivo (Hahn et al., 1998; Reeves et al., 2005; Sinclair & Sissons, 1996; Söderberg-Nauclér et al., 1997; Taylor-Wiedeman et al., 1994). As HCMV has been shown to remain latent in DC precursors in vitro (Hahn et al., 1998), we asked whether terminal differentiation to a

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**Fig. 2.** Differentiation of infected CD34+ cells to a mature DC phenotype is concomitant with a change in the chromatin structure of the MIEP. ChIP assays were performed on CD34+ cells infected with HCMV strain TB40/E (a) and then on infected CD34+ cells cultured for 10 days in cytokines that promote differentiation to mature CD34+ derived DCs (b), long-term cultures of infected CD34+ cells grown in the absence of cytokines that promote differentiation (c) or mature CD34+ derived DCs (d) derived from the long-term cultures of infected CD34+ cells (c). DNA was immunoprecipitated with anti-acetylated histone H4 antibody (lanes 3, 7, 11 and 15), anti-HP-1 antibody (lanes 4, 8, 12 and 16) or a normal serum control (lanes 2, 6, 10 and 14) and amplified by MIEP-PCR. Following electrophoresis and Southern blotting, MIEP sequences were detected by using a radiolabelled DNA probe. Inputs are also shown (lanes 1, 5, 9 and 13).
DC phenotype, known to be permissive for exogenous infection (Hertel et al., 2003), also supported reactivation in this in vitro model. Our data suggest that the terminal differentiation to a mature DC phenotype is consistent with the reactivation of infectious virus. Furthermore, the reactivation of HCMV, which would require the induction of lytic IE gene expression, is correlated directly with chromatin remodelling of the MIEP such that, as terminal differentiation occurs, the MIEP is no longer associated with HP-1, but with acetylated histones instead. These data are entirely consistent with our observations made ex vivo on natural HCMV latent infections (Reeves et al., 2005) and thus may provide a more tractable model for the analysis of HCMV latency in vitro. As chromatin remodelling of the MIEP was observed prior to the detection of infectious virus in the medium, this suggests that chromatin remodelling is occurring prior to reactivation. Indeed, one possibility is that this chromatin remodelling of the MIEP is linked intrinsically to the cell biology of DC differentiation. Inflammatory cytokines have been suggested to be important for reactivation of the MIEP in HCMV (Söderberg-Naucler & Nelson, 1999; Söderberg-Naucler et al., 1997) and murine CMV (Hummel & Abecassis, 2002; Simon et al., 2005) and, intriguingly, studies performed in monocyte-derived DCs have shown that LPS- or CD40L-induced maturation of DCs promotes p38-mediated phosphoacetylation of histone H3 on a subset of stimulus-induced cellular cytokine and chemokine genes that are NF-kB-responsive (Saccani et al., 2002). NF-kB upregulation has been shown to be important for transactivation of the MIEP (DeMeritt et al., 2004) following infection and it is possible that latent virus utilizes cellular pathways to control reactivation in differentiating myeloid cells, akin to specific cellular genes.

In conclusion, the results presented here show that the repression of HCMV IE gene expression following experimental infection of CD34+ cells is correlated with the recruitment of repressive chromatin to the MIEP. Furthermore, the long-term culture of CD34+ cells in the absence of stimuli that promote differentiation results in long-term carriage of viral genomes in which the MIEP remains associated with transcriptionally repressive chromatin. Finally, the differentiation of these long-term cultures of infected CD34+ cells to mature DCs results in the acetylation of histones bound to the MIEP, a concomitant loss of HP-1 and the subsequent detection of infectious HCMV in vitro. The correlation between these data and our analyses on naturally acquired HCMV and DCs (Reeves et al., 2005) suggests that this may provide a tractable model system for further analysis of HCMV latency and reactivation.

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References


