Short Communication

Vascular endothelial and smooth muscle cells are unlikely to be major sites of latency of human cytomegalovirus *in vivo*

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Human cytomegalovirus (HCMV) is a frequent cause of major disease following primary infection or reactivation from latency in immunocompromised patients. It has also been suggested that there may be a link between HCMV and vascular disease. Both smooth muscle and endothelial cells are targets for primary infection with HCMV and have also been postulated as potential sites of HCMV latency. One of the most intensely studied sites of HCMV latency is the cells of the myeloid lineage; there is increasing evidence that the myeloid and endothelial lineages arise from a common precursor in the bone marrow, suggesting that endothelial cells could be another route of HCMV dissemination. However, using a highly sensitive PCR capable of detecting endogenous HCMV in myeloid cells, the HCMV genome in endothelial and smooth muscle cells isolated from the saphenous veins of seropositive patients was not detected. These data suggest that vascular endothelial and smooth muscle cells are unlikely to be important sites of HCMV latency *in vivo*.

Primary infection with the betaherpesvirus human cytomegalovirus (HCMV; human herpesvirus) results in lifelong persistence in the host, a classic characteristic of the *Herpesviridae* family (Ho, 1990). Although infection of healthy individuals is usually asymptomatic, HCMV infection produces symptomatic infection and serious disease in immunosuppressed transplant patients, immunocompromised patients and the immuno-naïve (Drew, 1988; Reinke *et al.*, 1999; Rubin, 1990).

Accumulating data suggest that HCMV remains latent in the bone marrow myeloid progenitor cells, which give rise to monocytes, macrophages and dendritic cells. Consistent with this, all these cell types have been shown to carry latent HCMV (Mendelson *et al.*, 1996; Taylor-Wiedeman *et al.*, 1991) with reactivation occurring only after differentiation of myeloid progenitors into macrophages or dendritic cells (Soderberg-Naucler *et al.*, 1997, 2001). However, bone marrow-derived cells also give rise to endothelial cells (EC) (Goodell *et al.*, 1996); so it is possible that some EC derived from bone marrow progenitors may also be sites of HCMV latency in healthy carriers, and the ability to detect circulating EC harbouring HCMV (Grefte *et al.*, 1993) could be due to reactivation of HCMV in these cell types.

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Consistent with this, analysis of post-mortem tissue from seropositive transplant recipients has shown the presence of HCMV in EC despite the absence of cytopathic effect (Myerson *et al.*, 1984). Also, it has been reported that the origin of EC may dictate the progression of HCMV infection *in vivo* (Fish *et al.*, 1998; Jarvis & Nelson, 2002). In contrast, EC isolated in lung and gastrointestinal tissue of individuals with HCMV disease are productively infected *in vivo* (Sinzger *et al.*, 1995), and *in vitro* infection of different types of EC has been reported to be dependent on the strain of HCMV rather than the vascular bed of origin of EC (Kahl *et al.*, 2000; Sinzger *et al.*, 2000).

The possibility that HCMV may also be carried in vascular smooth muscle cells (SMC) has also been raised. For instance there is circumstantial evidence to link HCMV and atherosclerosis, one of the major causes of morbidity in the developed world (Melnick *et al.*, 1993). It has been suggested that HCMV may be a causative agent of atherogenesis (Fabricant *et al.*, 1978; Melnick *et al.*, 1993). In a rat model, RCMV promotes smooth muscle proliferation following aortic grafts (Lemstrom *et al.*, 1993), which may result in restenosis in humans (Zhou *et al.*, 1996). However, a meta-analysis found that the published epidemiological evidence for an association between HCMV and coronary heart disease was inconclusive (Danesh *et al.*, 1997).

While there is good evidence that EC arise from the same

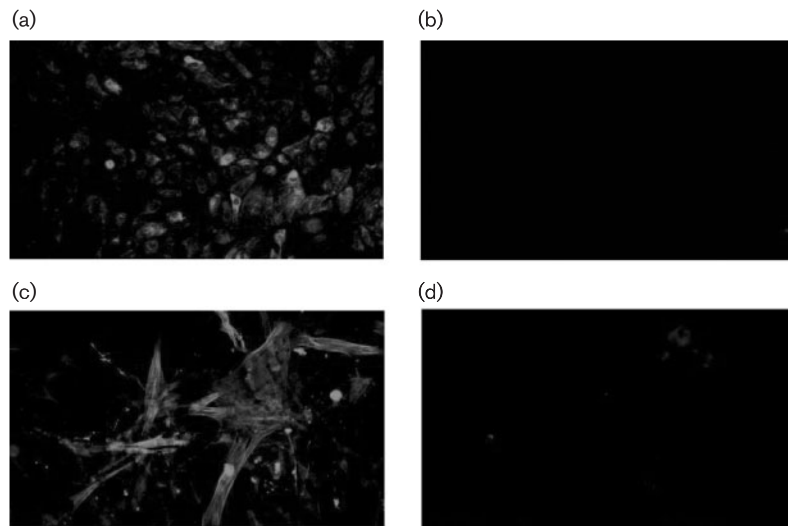

Fig. 1. Immunofluorescent staining of isolated cells. The identity of cultured cells was confirmed by morphology and staining for endothelium PAL-E protein or smooth muscle actin. (a) Cultured cells visualized by immunofluorescent staining with an anti-PAL-E antibody and then an FITC-conjugated secondary antibody. (c) Cultured cells visualized by immunofluorescent staining with an anti-actin antibody and then an FITC-conjugated secondary antibody. (b, d) No staining of cultured cells with isotype-matched controls.

progenitor as haematopoietic cells, the origin of SMC is not as well defined. A smooth muscle progenitor population has been identified in circulating blood (Shimizu *et al.*, 2001) and, perhaps more intriguingly, embryonic stem cells expressing the vascular endothelial growth factor receptor Flk-1, a protein also expressed by maturing EC (Drake & Fleming, 2000), can be differentiated into vascular SMC *in vitro* and *in vivo* (Yamashita *et al.*, 2000). These observations suggest that the precursors of EC may also serve as smooth muscle precursors (Sata *et al.*, 2002). Consequently, the likelihood that EC and SMC may be derived from a population of myeloid progenitors warrants a detailed analysis of whether these cell types may carry latent HCMV *in vivo*.

Consequently, we have asked specifically whether HCMV DNA can be detected in vascular SMC and EC of the microvasculature in normal, healthy, seropositive individuals under conditions that routinely detect HCMV in $CD34^+$ bone marrow progenitors. To perform our study, SMC and EC were isolated from saphenous vein tissue samples collected from patients who were undergoing cardiovascular surgery, and cultured *in vitro*. EC were isolated following collagenase digestion of the medial surface of the saphenous tissue and culture in Cs-C medium supplemented with endothelial growth factor and supplement (Sigma). The medial layer was nicked, allowing the upper vascular smooth muscle layer to be peeled off. Segmentation of the tissue and culture in a minimal medium facilitated the outgrowth of morphologically distinct SMC from the edges of the isolated tissue.

To test the identity of the cultured cells, eight-well slides were seeded with either putative EC or SMC, and stained for cell-specific markers or with an IgG isotype-matched control to confirm specificity. All primary antibodies were detected using an appropriate FITC-conjugated secondary antibody (Sigma). Fig. 1(a) shows that, following culture on plates coated with EC attachment factor (Sigma), adherent cells stained positively for endothelium protein (anti-Pal-E; Sera-lab) thus confirming their identity as EC. Similarly, the identity of the SMC was confirmed by staining positively for smooth muscle actin and also by their distinct morphology (Fig. 1c). The specificity of the staining was confirmed by using isotype-matched controls (Sigma) that showed no staining of the same cells (Fig. 1b and d).

Having determined that it was possible to culture both EC and SMC from saphenous vein tissue, a pilot study was performed to test whether endogenous HCMV DNA could be detected in these cell types. Using a highly sensitive PCR that amplifies a 308 bp fragment from the immediate-early (IE) gene region of HCMV, samples of EC and SMC DNA were screened for the presence of HCMV genomes. DNA was prepared, by the sodium perchlorate method, from EC and SMC derived from multiple saphenous vein tissue samples from donors of unknown serostatus ($n=13$). Dialysed DNA samples from multiple donors were then subjected to amplification by IE-PCR to determine the presence or absence of endogenous HCMV DNA. All samples were separated by gel electrophoresis, blotted onto nitrocellulose and probed for HCMV DNA using an IE-specific radiolabelled probe. This PCR protocol was able to detect routinely 10 copies of HCMV genome (based on plasmid reconstruction experiments and Southern blot analysis, Fig. 2a) in a background of cellular DNA from 10^6 cells. Using this highly sensitive IE-specific PCR, an amplified product of 308 bp was detected consistently in DNA from monocytes of healthy, seropositive individuals. To ensure that PCR products were specifically due to amplification of endogenous HCMV DNA, and not due to contamination, cells were cultured and DNAs isolated in a dedicated PCR suite that is rigorously maintained as a plasmid- and virus-free area. Multiple controls were also included in each assay. TE buffer, water and seronegative DNA controls were consistently negative for amplification of HCMV DNA by PCR. This confirmed that the IE-specific PCR employed was robust and sensitive enough specifically

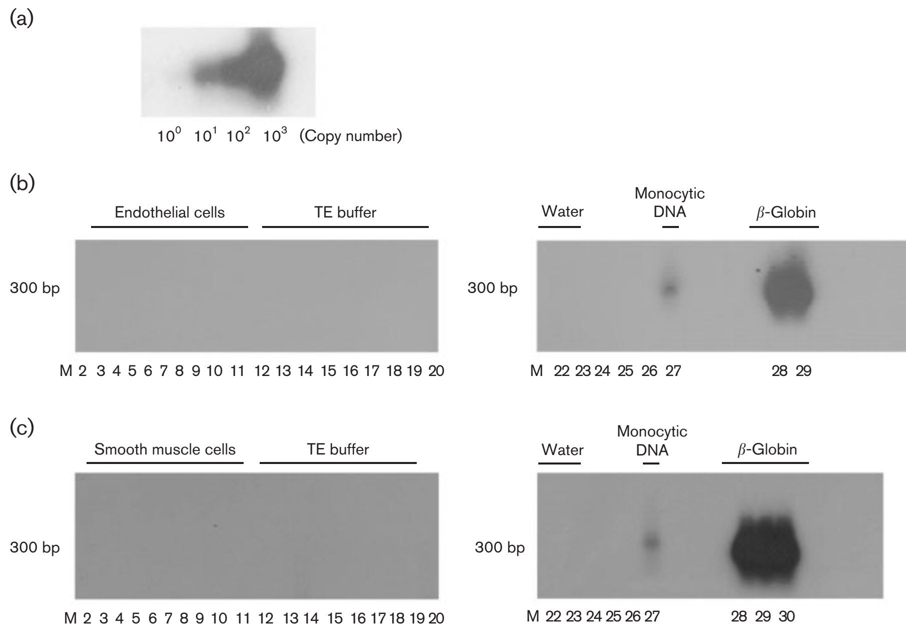

Fig. 2. (a) Sensitivity of IE-PCR. Tenfold dilutions of 1000–1 AD169 genomes were amplified in a background of 1 µg cellular DNA in an IE-PCR using sense primer (5′-CGTCCTTGACACGATGGAGT-3′) and anti-sense primer (5′-ATTCT-TCGGCCAACTCTGGA-3′). A DNA probe was generated using sense primer (5′-CCCTGATAATCCTGACGAGG-3′) and anti-sense primer (5′-CATAGTCTGCAGGAACGTCGT-3′) that amplified a 200 bp product. This probe was radiolabelled and used to detect amplification of HCMV DNA by IE-PCR. (b) Detection of HCMV DNA in EC. DNA from EC of a seropositive donor was amplified in an IE-PCR or β-globin PCR, blotted and probed sequentially with an IE-specific probe or β-globin probe. No amplified product from HCMV DNA could be detected in the EC samples (lanes 2–11). However, HCMV DNA was amplified from seropositive monocytic DNA (lane 27). The β-globin control confirms that the EC DNA extracted could be amplified by PCR (lanes 28 and 29). Control PCRs containing only TE buffer (lanes 12–20) or water (lanes 22–26) are shown. (c) Detection of HCMV DNA in SMC. DNA from SMC of a seropositive donor was amplified in an IE-PCR or β-globin PCR, blotted and probed sequentially with an IE-specific probe or β-globin probe. No amplified product from HCMV DNA could be detected in the SMC samples (lanes 2–11). However, HCMV DNA was amplified from seropositive monocytic DNA (lane 27). The β-globin control confirms that the SMC DNA extracted could be amplified by PCR (lanes 28–30). Control PCRs containing only TE buffer (lanes 12–20) or water (lanes 22–26) are shown.

to detect endogenous HCMV DNA in already established sites of HCMV latency.

Multiple analyses of DNA isolated from EC and SMC of 13 individuals undergoing triple bypass surgery did not give rise to an HCMV-specific PCR product (Table 1). The samples collected for this initial analysis did not include data on the HCMV serostatus of donors and, although a 50–60 % seropositive status would be predicted (Emery, 2001), we instigated a second study that included serotyping of all donors ($n=16$). In this second analysis, blood samples as well as saphenous vein tissue were, once again, obtained from all donors, and the HCMV serostatus of the donors was determined using an ELISA for detection of HCMV antibodies (Captia CMV-TA; Centocor). As before, multiple DNA samples from cultured EC and SMC of confirmed seropositive donors ($n=14$) were analysed for the presence of HCMV DNA using the IE-specific PCR. Fig. 2(b) shows a representative IE-specific PCR/Southern blot analysis of multiple EC DNA samples from one HCMV-seropositive donor. Ten aliquots, each containing approximately 1 µg EC DNA (roughly equivalent to 10^6 cells), were amplified using the IE-specific PCR. We were unable to detect HCMV DNA in any of the samples (lanes 2–11). We routinely carried out 10 PCR reactions on the DNA from each seropositive individual to ensure that any inability to detect viral genome was not due to low copy number of viral DNA such that any one subaliquot of cellular DNA did not include the viral genome. In contrast, samples of DNA from the monocytes of a seropositive donor routinely showed IE-specific amplification products (lane 27).

Additional controls confirmed that sample DNAs did not contain PCR inhibitors. First, 40 copies of AD169 DNA could be amplified routinely by the IE-specific PCR in the presence of 1 µg cellular DNA (data not shown). Second, a β-globin-specific PCR of predicted size 300 bp could be amplified consistently from subaliquots of sample cellular DNA (lanes 28 and 29). These observations were representative of multiple EC DNA samples from each of six

Table 1. Summary of PCR results for detection of HCMV DNA

Data generated following analysis of EC and SMC of saphenous veins for the presence of endogenous HCMV. ND, Not done; −, no HCMV DNA could be detected by IE-PCR.

Patient no.	Serostatus	HCMV DNA	
		SMC	EC
311418	ND	ND	−
943956	ND	−	ND
936671	ND	−	ND
946344	ND	−	ND
939232	ND	ND	−
933540	ND	−	−
916827	ND	−	ND
910313	ND	−	ND
934620	ND	−	ND
937419	ND	−	ND
941034	ND	−	ND
936224	ND	−	ND
921350	ND	ND	−
951628	Positive	−	ND
945521	Positive	−	−
950510	Positive	−	ND
950199	Negative	−	−
937827	Positive	−	−
338380	Positive	−	ND
951604	Negative	ND	−
951273	Positive	−	ND
953167	Positive	ND	−
957053	Positive	ND	−
987827	Positive	ND	−
950168	Positive	−	−
934927	Positive	−	ND
338380	Positive	−	ND
901573	Positive	−	ND
910510	Positive	−	ND

seropositive patients. The data obtained are summarized in Table 1.

Fig. 2(c) shows a similar analysis of DNA isolated from SMC, showing one representative seropositive individual. SMC DNA was amplified in an IE-specific PCR, and again no amplified product could be detected following PCR/Southern blot analysis of 10 subaliquots each containing 1 μg total cellular DNA (lanes 2–11). Multiple controls showed that DNA from monocytes of a seropositive donor under identical conditions showed IE-specific amplification products (lane 27). Finally, a 300 bp product of the β-globin-specific PCR could be amplified from multiple subaliquots of the SMC DNA (lanes 28–30). These observations were representative of multiple SMC DNA samples from each of the 11 seropositive individuals. The data obtained are summarized in Table 1.

The data presented here show that endogenous HCMV DNA cannot be detected routinely in EC or SMC populations isolated from the saphenous vein of seropositive individuals using a highly sensitive, IE-specific PCR that routinely detects HCMV DNA in myeloid cells, a well established site of HCMV latency. Previous analyses of post-mortem tissue have identified HCMV nucleic acids in the arterial wall tissue of seropositive individuals in the absence of atherosclerosis (Hendrix *et al.*, 1991; Melnick *et al.*, 1994). However, the exact cell type was undefined, and it is possible that detection of HCMV DNA is the result of contamination with blood cells, such as monocytes, previously shown to carry HCMV genomes (Taylor-Wiedeman *et al.*, 1991). Also, other studies that have analysed post-mortem tissue suggest that reactivation of HCMV may occur upon death. Thus, the widespread detection of HCMV nucleic acid in a variety of tissues post-mortem may be the result of stress-related virus reactivation rather than detection of latent HCMV genomes (Toorkey & Carrigan, 1989).

We therefore believe that EC or SMC are unlikely to be a major site of latency of HCMV in normal carriers, despite evidence suggesting that vascular cells could ultimately be derived from myeloid progenitors – a progenitor cell type that has been shown to carry latent HCMV *in vivo* (Mendelson *et al.*, 1996). Although we cannot formally rule out the possibility that the culture conditions used for EC and SMC may lead to a loss of latent viral genomes, we note that HCMV genomic DNA can be detected routinely, even after long-term culture of monocytes from healthy, seropositive individuals (Taylor-Wiedeman *et al.*, 1991).

We also recognize that the results presented here are from vascular EC and SMC from saphenous vein; aortic EC and SMC (which are much more difficult to obtain *ex vivo* from human subjects) are not represented in this analysis. However, as vascular EC and SMC appear to originate from the same progenitors as aortic EC and SMC (Sata *et al.*, 2002), it is unlikely that aortic cells will differ substantially from vascular EC and SMC with respect to potential carriage of HCMV *in vivo*.

Consequently, the failure to detect HCMV DNA in EC and SMC cultured from the saphenous veins of seropositive patients suggests that these cells, as well as aortic cells, are unlikely to represent major sites of HCMV latency *in vivo*.

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