Measles virus induction of human endothelial cell tissue factor procoagulant activity in vitro

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Measles virus infection of microvascular endothelium in vivo and ensuing endothelial cell activation may be important in the pathogenesis of subsequent inflammation in target organs. This study investigated the capacity of measles virus to induce procoagulant activity, in vitro, in endothelial cells isolated from human umbilical cord veins. Endothelial cells were infected with a clinical isolate of measles virus propagated in Vero cells. Cells were also incubated with bacterial lipopolysaccharide (10 μg/ml), herpes simplex virus type 1, cytomegalovirus or culture medium alone as positive and negative controls, respectively. Endothelial cell procoagulant activity was measured in a one-stage clotting assay. Measles virus stimulated both a time and dose-dependent endothelial cell procoagulant response by the induction of tissue factor synthesis, confirmed by both immunocytochemistry and its dependence on factor VII for activity. This activity was reduced by u.v.-irradiation of the virus. Infected cells were analysed by double immunofluorescent staining for both tissue factor and measles virus N-protein, and examined using confocal scanning laser microscopy. Cells expressing tissue factor were also positive for the measles virus N-protein. Low levels of interleukin-1 were detected in some viral inocula derived from measles virus-infected Vero cells, however neutralising antibody to interleukin-1 failed to inhibit the endothelial cell procoagulant response to measles virus, whereas it significantly reduced procoagulant activity induced in endothelial cells by recombinant interleukin-1. The capacity of measles virus to induce endothelial tissue factor in vitro, may be relevant to the thrombotic vasculopathy associated with measles virus infection in vivo.

Introduction

Measles virus has been identified in the microvascular endothelium during both acute and persistent phases of infection, and endothelial cell injury is thought to be important in the pathogenesis of measles-related disease (Norrby & Oxman, 1990). Measles virus antigen has been demonstrated within the dermal capillary endothelium (Kimura et al., 1975), and both the intestinal mucosal (Jirapinyo et al., 1990) and submucosal microvascular endothelium (Norrby & Oxman, 1990), in acute measles infection. In subacute sclerosing panencephalitis (SSPE), a disease that is associated with persistent measles virus infection, probes specific for measles virus nucleocapsid genomic RNA hybridized to human cerebral endothelium (Kirk et al., 1991).

Using transmission electron microscopy, we recently identified paramyxovirus-like particles in endothelial cells in foci of granulomatous microvascular inflammation in resected intestinal tissues from patients with Crohn's disease; both in situ hybridization and immunocytochemistry identified measles virus within the same cellular location (Wakefield et al., 1993). Crohn's disease is associated with widespread granulomatous and lymphocytic microvascular inflammation (Wakefield et al., 1991). Both tissue factor expression (More et al., 1993) and fibrin deposition in foci of endothelial cell injury, with thrombotic occlusion of inflamed blood vessels, are phenotypic features of this condition (Wakefield et al., 1989).

The endothelium plays a vital role in haemostasis: under normal circumstances it maintains an anticoagulant environment on its luminal surface by the production of factors such as heparan sulphate, prostacyclin, thrombomodulin and Protein S (Muller Berghaus, 1992). Endothelial cell procoagulant activity (PCA) is embodied principally in the inducible synthesis and cell-surface expression of tissue factor (TF), a glycoprotein that acts as a receptor for circulating factor VII. The resulting tissue factor–factor VIIa complex is a potent initiator of the extrinsic coagulation pathway.

This study investigated the capacity of measles virus to
Methods

Propagation of virus. A clinical isolate of measles virus, the HU-2 strain (gift from B. Rima, Queen’s University, Belfast), was used in all experiments. The virus was plaque-purified and propagated in Vero cells at a low m.o.i., in order to prevent the induction of interfering particles (Rima et al., 1977). Titration of virus stocks were performed on Vero cells according to the method described by Reed and Muench (Hoskins, 1967). In some experiments a laboratory strain of herpes simplex type 1 (HSV-1) and the AD 169 strain of cytomegalovirus (CMV) were used as a comparison, since these viruses have been shown to induce PCA in endothelial cells (Visser et al., 1988, Van Dam-Mieras et al., 1987). Both viruses were propagated and titrated in human embryonic lung cells.

Endothelial cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (Jaffe, 1973). Briefly, umbilical veins were cannulated at each end and washed through with PBS. The vessels were then incubated with collagenase (Boehringer Mannheim) at 200 U/ml in Medium 199 with Earl’s salts (M199) (ICN Flow) for 10 min at 37 °C. The cells were collected by washing through the veins with M199 and centrifuging the eluate at 500 g for 5 min. The cell pellet was resuspended in maintenance medium containing M199 supplemented with 10% fetal calf serum (ICN Flow), penicillin (100 U/ml) and streptomycin (100 μg/ml), and glutamine (3.2 mM) (ICN Flow). Secondary cultures were supplemented with endothelial growth factor/heparin (12.5 μg/ml and 62.5 μg/ml) (Boehringer Mannheim) and subsequently reseeded by brief exposure to trypsin–EDTA (0.1% to 0.02%) into 1% gelatin precoated (Sigma) 24-well tissue culture plates (Marathon Labs) for procoagulant studies, or into similarly treated chamber slides (Gibco) for immunocytochemical studies. Experiments were performed on HUVEC at passage 2 or 3 at an average density of 25000 cells/well. Endothelial cell purity was confirmed by immunostaining for thrombomodulin and factor VIII-related antigen, as well as by microscopic morphology analysis. Greater than 98% of cells were of endothelial origin based upon these assays (data not shown).

Infection of endothelial cell cultures with virus. Medium was removed from the monolayers, which were then incubated with the measles virus for 2 h at 37 °C. The cells were then washed with PBS and fresh medium was added. Control cells were incubated with medium alone, or with HSV-1 or CMV. In some experiments, measles virus was used at a range of m.o.i. from 0.02 to 20 as indicated.

Confirmation of endothelial cell infection. Measles virus antigen was visualized by using a fluorescein-conjugated primary antibody raised against the measles virus nucleocapsid (N) protein (Serolab). This was incubated with infected cells for 30 min at 37 °C, and the cells were then washed and examined under a fluorescence microscope. Negative controls were treated identically, except for the substitution of an irrelevant fluorescein isothiocyanate (FITC) conjugated antibody, anti-CD3 (Dako).

Harvest of endothelial cells for procoagulant studies. Supernatants were collected at intervals between 0 and 72 h following measles virus infection of endothelial cells, and from uninfected cells, and then frozen at —70 °C for later analysis. At the time points indicated, the cells were washed with PBS and lysed by the addition of 200 μl of water/well before freezing at —70 °C.

Endothelial cell PCA assay. Procoagulant activity was measured in triplicate wells using a modified one-stage clotting assay, in which 50 μl of the lysate of measles virus-infected cells was added to equal volumes of human citrated plasma and 25 mM-CaCl₂. The time taken for fibrin to form was recorded on a coagulometer (Heinrich Amelung, GmbH) and values were extrapolated from a standard curve generated from serial dilutions of a commercially available rabbit brain thromboplastin (Diagen). In order to demonstrate tissue factor activity, factor VII- and factor X-deficient plasma (Sigma) were substituted for normal citrated plasma in the PCA assay.

Ultraviolet irradiation of measles virus. To assess the requirement for viral replication in the endothelial cell PCA response, the virus was subjected to u.v.-irradiation from a 254 nm light source (San Gabriel model UVGL-58) for intervals ranging from 30 min to 2 h. The effect of irradiation on virus titre was determined by TCID₅₀.

Tissue factor immunocytochemistry. HUVEC cultured on chamber slides were fixed in methanol and acetone 1:1 at —70 °C for 8 min and washed with PBS, to prior an overnight incubation at 4 °C with the primary antibody specific for tissue factor. The cells were then washed for 10 min in PBS before blocking non-specific binding with 20% normal rabbit serum for 20 min at room temperature. A secondary rabbit anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate fluorochrome (Dako) was added at a 1:100 dilution in PBS, for 1 h at 37 °C. The tissue factor monoclonal antibodies were a gift from Dr T. S. Edington (Scripps Research Institute). A cocktail of three monoclonal antibodies was used, the production and characterization of which are reported elsewhere (Morrisey et al., 1988).

Double staining for tissue factor and measles virus N-protein. To study the possible cellular co-localization of tissue factor and measles virus N-protein, the tissue factor staining was performed as described above. Infected and control HUVEC were then washed in PBS, and unoccupied binding sites on the anti-mouse IgG conjugate were blocked by the addition of 20% normal mouse serum at room temperature for 20 min. This was followed by the addition of a FITC-conjugated antibody raised against measles virus N-protein, or a FITC-labelled irrelevant antibody of the same isotype and concentration. The slides were mounted using citifluor (Citifluor Ltd) and viewed under a fluorescence microscope (Zeiss) or a confocal scanning laser microscope (Bio-Rad MRC 600).

Analysis of viral inocula. Further studies were conducted in order to examine possible carry-over in the viral inocula of a soluble factor with known PCA-inducing activity. Levels of interleukin-1 (IL-1) α and β, and tumour necrosis factor (TNF), cytokines that possess this activity (Bevilacqua et al., 1984, 1986), were measured using ELISA (British Biotechnology). The lower limit of detection in these assays was 4.8 pg/ml for TNF and 3.9 pg/ml for both IL-1α and β.

Limulus assay for endotoxin. The possible presence of endotoxin contamination of reagents, media and cell culture supernatants that might affect measurements of PCA activity, was examined using the standard limulus assay (Sigma).

Comparison of endothelial cell PCA induction by measles virus and recombinant IL-1. In order to compare any PCA induction by measles virus with that induced by recombinant IL-1 (rIL-1), the endothelial cells were incubated either with increasing concentrations of rIL-1β (0.03 to 10 U/ml) diluted in 10% DMEM, or with viral inocula, for 2 h at 37 °C. All cells were then washed and incubated with maintenance media for a further 4 h prior to harvest.

Neutralization of IL-1β. In order to examine whether IL-1β activity was responsible for any PCA induction observed in measles virus-infected endothelial cells, experiments were designed firstly to neutralize...
Procoagulant activity induction by MV

Results

Measles virus infection of endothelial cells

Following infection with measles virus, the N-protein could be detected immunohistochemically in the cytoplasm of infected endothelial cells 6 h after the addition of virus. Its distribution was patchy, with clusters of positive cells adjacent to areas of apparently uninfected cells. At later time-points the intensity of fluorescence was increased, with some cells also exhibiting nuclear staining by 24 h. The percentage of cells infected was dependent on the virus to cell ratio; m.o.i.s of 0.02 resulted in only 2 to 5% positivity (Fig. 1), whereas m.o.i.s of 20 increased this value to 80%.

The c.p.e. of the virus was variable in different pools of HUVEC, and was not always seen in the cells infected with low m.o.i.s (i.e. 0.02), although measles virus N-protein could be detected by immunofluorescence at these virus doses. At the higher m.o.i.s most cultures demonstrated the appearance of multi-nucleated cells (Fig. 2), with a gradual destruction of the cell monolayer.

Induction of endothelial cell procoagulant activity by measles virus

Induction of PCA was detectable in measles virus-infected cells at all time-points examined. This activity was observed by 3 h post addition of virus, peaked at 6 h, and returned towards basal levels by 48 h. There were statistically significant differences in PCA levels between infected and control cells at 3, 6, 12 and 24 h ($P < 0.01$).
Fig. 2. Cytopathic effect of measles virus on HUVEC. Endothelial cells were infected with measles virus at a m.o.i. of 20. The multinucleate giant cells frequently observed at this viral dose can be seen. Bar marker represents 50 μm.

Fig. 3. Endothelial cell PCA induction by measles virus (■). Endothelial cell monolayers were infected with measles virus at an m.o.i. of 20 for 2 h at 37 °C. At 3, 6, 12, 24, 48 and 72 h post-addition of virus, cells were harvested and the amount of PCA induced per 2.5×10⁴ cells was recorded in a one-stage clotting assay. The mean ± standard error of the results from eight experiments is shown. Measles virus induced endothelial cell PCA at all time-points measured from 3 to 24 h, peaking at 6 h post infection and returning towards basal levels by 48 h *, P < 0.01 compared with uninfected HUVEC (■).

(Fig. 3). At 6 h post addition of virus, the mean PCA induction ± s.e.m. for infected cells was 341 ± 88, whereas the value for uninfected cells was 8 ± 5.

Fig. 4. Effect of dilution of measles virus inoculum on endothelial cell PCA. Endothelial cells were infected with measles virus at a range of m.o.i.s from 0.02 to 20. The cells were harvested as described in Fig. 3, 6 h post-addition of virus. There was a direct correlation between the m.o.i. and the amount of PCA induced. *, P = < 0.01. Decreasing viral titres resulted in diminishing levels of endothelial cell PCA.

The level of PCA induction correlated with the m.o.i.: decreasing virus titres resulted in diminished PCA (Fig. 4). Different cultures of endothelial cells varied in their PCA responsiveness to the same batch of measles virus from 89 to 250 mU PCA/25000 cells, but PCA was elevated significantly in all cultures as compared with
Fig. 5. Effect of u.v.-irradiation of measles virus on its capacity to induce endothelial cell PCA. Measles virus samples were exposed to u.v.-irradiation for various periods before being used to infect endothelial cells (EC) prior to the determination of PCA. Increased exposure time of the virus to u.v.-irradiation reduced its capacity to induce PCA.

uninfected controls \((P < 0.01)\) (data not shown). When a maximal PCA response was 250, PCA could be detected down to an m.o.i. of 0.03.

It was interesting to note that the virus also induced PCA in Vero cells. Although Vero cells have higher basal levels of PCA than endothelial cells (mean 147 ± 10 as compared with 8 ± 5), infection with measles virus increased these values by more than 200% (data not shown). Comparison of endothelial cell PCA induced by measles virus, HSV-1 and CMV showed that although HSV-1 was able to induce endothelial cell PCA (mean 46 ± 27), even with 100-fold higher viral inocula than that used for measles virus, HSV-1 was a far less potent stimulus for PCA induction. In contrast CMV, used at a m.o.i. of 20, did not induce PCA at any time-point measured from 6 h to 4 days (data not shown).

Ultraviolet irradiation of the virus reduced both the amount of replicating virus and the endothelial cell PCA induced in a dose-dependent fashion (Fig. 5), but it was not able to completely inhibit viral replication. Following 2 h irradiation the virus titre was reduced from \(10^{5.6}\) to \(10^{2}\) TCID_{50} units. Endothelial cells exposed to lipopolysaccharide (LPS) as a positive control also expressed PCA over a similar time course, although at lower levels than cells infected with measles virus (data not shown).

**Cytokine content of the viral inocula**

The viral inocula that were derived from measles virus-infected, lyzed Vero cells could have potentially contained cytokines capable of inducing PCA activity. The cytokine content of viral inocula was therefore assessed. TNF could not be detected in any viral stock tested \((n = 4)\). Low levels of IL-1β were detected in most inocula, with a mean concentration of 8 pg/ml (range 0 to 30), whereas IL-1α was detected in three out of eight samples tested, with a mean concentration of 6 pg/ml (range 0 to 20), suggesting that measles virus induced IL-1 production by Vero cells, since uninfected, lyzed Vero cells were negative for both of these cytokines. As endothelial cell PCA was induced by all viral inocula, this activity appeared to be independent of IL-1α at all levels that were detectable in the assay.
Figs 6 and 7. For legends see opposite.
Comparison of endothelial cell procoagulant activity induced by measles virus and rIL-1

A viral inoculum with a known concentration of IL-1β (4 pg/ml: 0.02 U/ml) was compared with increasing concentrations of rIL-1β, for its ability to induce endothelial cell PCA. The virus-induced levels of PCA were comparable with the highest concentration of rIL-1β used (1000 pg/ml: 5 U/ml) (Fig. 8). Concentrations of IL-1β similar to those found in viral inocula (0.02 U/ml) failed to induce significant PCA, suggesting that it was unlikely that the IL-1β contained in the viral inocula was responsible for the induction of PCA following infection.

Effect of addition of neutralizing antibody raised against IL-1β on PCA induced by measles virus

In order to provide further confirmation that the PCA induced by measles virus was unlikely to be due to the low levels of IL-1β in the viral inocula, the effects of the addition of a neutralizing antibody specific for IL-1β were studied. The viral inocula that was used for the experiments contained 4 pg/ml of IL-1β and no detectable IL-1α. The anti-IL-1β antibody, used at a concentration of 10 μg/ml reduced endothelial cell PCA induced by rIL-1β (1000 pg/ml i.e. 5 U/ml) by nearly 60%, but failed to inhibit virus-induced endothelial cell PCA (Fig. 9). The presence of anti-IL-1β antibody in the culture fluid of the measles virus-infected endothelial cells throughout the incubation period also failed to inhibit PCA induction using six different cultures of endothelial cells (mean ± S.E.M. without anti-IL-1β = 117 ± 16, with anti-IL-1β = 120 ± 14. P = > 0.5).

Limulus assay

It was possible that contamination with endotoxin might have been responsible for the induction of PCA by measles virus, since LPS is known to have this capacity (Colucci et al., 1983). However, LPS was not detected in the media or cell supernatants as determined by the limulus assay, which has a lower limit of detection of 4-2 pg/ml. Furthermore preincubation of the viral inocula with polymixin had no effect on the levels of PCA induced by the virus (data not shown).

Discussion

This study presents some novel observations on the capacity of measles virus to induce PCA in cultured human endothelium. This activity, the majority of which appeared to be due to tissue factor, was confined to the areas of virus-infected endothelium. The patchy distribution of the measles virus N-protein throughout the monolayer highlighted the observation that tissue factor expression was associated with foci of infected cells and was not seen in the smaller proportion of the monolayer negative for the measles virus N-protein. This is an important observation, and one which suggests either that tissue factor induction may be a direct consequence of infection, or that it is related to the induction of a local cytokine micro-environment created in response to infection. Although different pools of endothelial cells vary in their degree of PCA responsiveness, the observations that both the levels of PCA and the proportion of infected cells increased with increasing m.o.i. suggests that the dose-response effect on PCA was a function of the number of cells infected. The decrease in PCA induced by u.v.-irradiation of the virus suggests that viral replication may be important for the phenomenon. However, plaque forming assays revealed that although replicating virus was reduced by an order of 1000-fold by u.v.-irradiation, it was still present at low levels; thus further work is required to establish the requirement for viral replication. Although we were able to confirm that HSV-1 induced tissue factor, it was a far less potent stimulus than measles virus. This effect was not seen with CMV, although CMV has been reported to induce PCA in rat aortic endothelium (Van Dam-Mieras et al., 1987), suggesting that PCA-inducing capacity is related to the nature of the virus, rather than a phenomenon associated with viral infection in general. It is possible that cytokines present in the viral inocula contributed to PCA induction by measles virus. However, measles virus induced endothelial cell PCA in the absence of detectable TNF and IL-1α, and treatment of endothelial cells with levels
infected cells. Moreover, the lack of effect of anti IL-1β in infected cells suggests, but does not prove that PCA induction of endothelial cell PCA in measles virus-

A viral inoculum containing 4 pg/ml of IL-1β was incubated for 2 h at 37 °C. Following incubation, the cells were washed and incubated for a further 4 h with medium alone, after which time they were harvested and assayed for PCA. The results shown are the mean ± standard error of the results from three experiments. Measles virus inocula with an IL-1β content of 0.02 U/ml induced levels of PCA comparable with the highest concentration values of rIL-1β used, i.e. 5 U/ml rIL-1β.

![Graph showing comparison of endothelial cell PCA induced by measles virus and rIL-1β](image)

**Fig. 8.** Comparison of endothelial cell PCA induced by measles virus and rIL-1β. Endothelial cells were incubated with either recombinant IL-1β, at doses ranging from 0.03 to 5 U/ml, or with a viral inoculum with an IL-1β content of 0.02 U/ml, for 2 h at 37 °C. Following incubation, the cells were washed and incubated for a further 4 h with medium alone, after which time they were harvested and assayed for PCA. The results shown are the mean ± standard error of the results from three experiments. Measles virus inocula with an IL-1β concentration of 0.02 U/ml induced levels of PCA comparable with the highest concentration values of rIL-1β used, i.e. 5 U/ml rIL-1β.

![Graph showing neutralization of endothelial cell PCA with anti IL-1β antibody](image)

**Fig. 9.** Neutralization of endothelial cell PCA with anti IL-1β antibody. A viral inoculum containing 4 pg/ml of IL-1β was incubated for 2 h at 37 °C prior to the PCA assay with an anti IL-1β antibody (10 µg/ml), a concentration shown previously to inhibit PCA induction by 1000 pg/ml of rIL-1β. Data represent the mean of three experiments ± S.E.M. The anti IL-1β antibody was able to reduce PCA induced by rIL-1β by nearly 60%, but failed to inhibit virus-induced EC PCA.

of rIL-1β similar to those found in some virus stocks failed to induce PCA to the levels seen in infected endothelial cells. Furthermore, a neutralizing anti-IL-1β antibody produced no inhibitory effect in measles virus-infected cells, in contrast to its capacity to markedly inhibit the effect of recombinant IL-1β on the induction of PCA in parallel endothelial cell cultures. Together, these findings argue against a major role for IL-1 in the induction of endothelial cell PCA in measles virus-infected cells. Moreover, the lack of effect of anti IL-1β in infected cells suggests, but does not prove that PCA induction is not due to a synergistic effect occurring between measles virus and IL-1β present in viral inocula derived from Vero cells, or induced in endothelial cells. Nevertheless, the apparent ability of measles virus to induce IL-1 in Vero cells, which are often used to maintain and propagate viral stocks prior to experiments similar to those described in this study, is an important observation. The potential effects of such cytokines, derived from the cells in which the virus is propagated, should be excluded when interpreting data on the effects of viruses in *in vitro* systems. Similar studies in the past performed in other viral systems do not appear to have accounted for this possibility (Span et al., 1989; Zajac et al., 1988).

The limitations of this study are principally those of working with *in vitro* systems. Furthermore if, for example, a measles virus-like virus can be identified as a component cause of Crohn’s disease it is likely that (as in SSPE), a persistent form of this agent is responsible. It will be important to compare the effects of primary and persistent measles virus infections on the haemostatic properties of endothelial cells. Measles virus is likely to induce a number of additional effects in endothelial cells that may be relevant to the evolution of microangiopathic lesions during infection: these include the induction and expression of both cytokines and adhesion molecules for circulating inflammatory cells. Studies designed to examine these effects are underway in our laboratory.

How might our observations be relevant to measles virus pathology *in vivo*? Microvascular injury and necrosis of dependent tissues, presumably through an ischaemic process, was suggested as a mechanism for epithelial ulceration in measles virus infection (Kimura et al., 1975; Norrby & Oxman, 1990): this may provide the basis for the Kaposi spot that develops principally in the skin and the gut during acute infection. Endothelial cell infection by measles virus was observed in SSPE lesions (Kirk et al., 1991), and perturbation of the endothelium by this agent could lead to vessel-centred inflammatory lesions that are characteristic of the disease.

TF immunostaining was demonstrated in foci of vasculitis in the intestinal submucosal microvasculature of patients with Crohn’s disease (More et al., 1993): this suggests a shift from an anticoagulant to a procoagulant state of the endothelium in this disease. Spontaneous thrombosis occurs in some Crohn’s disease patients in association with exacerbations of the illness (Talbot et al., 1986), and we have reported evidence of persistent thrombogenesis in patients with Crohn’s disease in this condition (Hudson et al., 1992). Although the stimulus for thrombosis is not known, the identification of measles virus-like virus particles in the microvasculature endothelium of patients with Crohn’s disease may, on the
basis of the present study, provide one possible mechanism. The possible co-localization of measles virus and TF in Crohn’s disease tissue is also being investigated in our laboratory.

At the other end of the spectrum of thrombophilia, disseminated intravascular coagulation has been reported as a complication of measles virus (Davies et al., 1978; Malumo et al., 1978), a phenomenon that may occur following the systemic induction of both endothelial and monocyte procoagulant responses (Semeraro & Colucci, 1992).

In summary, we report that measles virus induces endothelial cell tissue factor synthesis and expression in vitro. This activity is restricted to foci of measles virus-infected cells and was not blocked by inhibition of IL-1, which was carried over in small amounts in viral inocula derived from Vero cells. These observations may be relevant to measles virus-induced microvascular lesions in vivo.

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