Human parvovirus B19 experimental infection in human fibroblasts and endothelial cells cultures

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Abstract

With the aim to detect what kind of cells, in addition to erythroid progenitors, could be involved in the pathogenesis of B19 infection in some connective tissue diseases, primary cultures of human fibroblasts (HF) and endothelial cells (HUVEC) were exposed to a B19 positive serum (350 genome copies/cell). The presence of NS1 and VP1 mRNA, in both HF and HUVEC cultures 1, 2 and 6 days after the exposure, indicated infection by B19 virus. However, no significant increase of B19 DNA level in the infected HF and HUVEC cultures was detectable through the entire incubation period of 6 days. It is possible that HF and HUVEC are not permissive for B19 virus replication or, alternatively, that few cells only get infected by B19 virus. HF and HUVEC stimulation with different growth factors or cytokines could be required for a B19 productive infection to occur.
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1. Introduction

Human parvovirus B19 (B19 virus) is the etiologic agent of erythema infectiosum (Anderson et al., 1984) and of transient aplastic crisis (Pattison et al., 1981). It may cause also arthralgia and arthritis (Reid et al., 1985; White et al., 1985). Being able to cross the placenta it can cause hydrops fetalis and fetal loss (Brown et al., 1984; Knott et al., 1984). Additionally, B19 virus could be involved in an increasing spectrum of diseases, including rheumatoid arthritis and other connective tissue diseases (Takahashi et al., 1998; Kerr, 2000; Crowson et al., 2000; Lundqvist et al., 2005). Bone marrow (BM) is the main site of B19 virus replication; the erythroid progenitors are the target cells in which a productive, lytic, infection occurs (Ozawa et al., 1986). In vitro B19 parvovirus inhibits erythroid colony formation of normal human bone marrow cells (Mortimer et al., 1983) and human bone mar-

row primary cultures, in presence of erythropoietin, are able to support virus multiplication (Ozawa et al., 1986). Infected in vitro erythroblasts demonstrate characteristic morphological changes (Ozawa et al., 1987). Virus replication in bone marrow, and the consequent viremia, has a central role in the pathogenesis of the main clinical manifestations caused by B19 infection (Anderson et al., 1985). As supported by several observations, the tissue tropism of B19 virus is not restricted to BM cells: B19 DNA has been detected also in synovial tissue (Soderlund et al., 1997; Takahashi et al., 1998; Zakrzewska et al., 2001), in myocardium (Porter et al., 1988; Nigro et al., 2000), and in the skin biopsies (Vuorinen et al., 2002; Ferri et al., 2002; Hokynar et al., 2002) from individuals anti-B19 IgG positives, often in the absence of viremia and with not detectable anti-B19 IgM. B19 DNA has been detected in the skin of patients with systemic sclerosis (SSc) more frequently than in the skin of healthy controls (Ferri et al., 2000). Fibroblasts have been identified as target cells of B19 infection in the cutaneous tissue; in fact, B19 DNA was demonstrated in cultured skin fibroblasts from systemic

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sclerosis patients with a B19 DNA positive skin biopsy and it was still present even after several in vitro passages of the infected fibroblasts (Ferri et al., 2002). In addition to fibroblasts, B19 infection was demonstrated by RT in situ PCR in endothelial cells and in perivascular inflammatory cells in skin bioptic tissue from systemic sclerosis patients (Magro et al., 2004). Moreover, incubation with parvovirus B19-containing serum induced an invasive phenotype in normal human synovial fibroblasts (Ray et al., 2001). The assessment of the cell types infected by B19 virus, of the kind of the resulting virus-cell interaction and the effect of B19 infection on such cells is particularly important in order to establish the pathogenetic role of B19 infection in a number of clinical manifestations. With this aim we began to study B19 infection in two in vitro models: primary cultures of human cutaneous fibroblasts and human endothelial cells.

2. Materials and methods

2.1. Primary cell cultures

Human dermal fibroblasts (HF) were prepared according to a modified version of the Rheinwald and Green protocol (Rheinwald and Green, 1975). After epithelial sheet dispase removal, dermis was cut into small pieces and fibroblasts were isolated by sequential trypsin and collagenase digestion. The cells were then cultured in Dulbecco's minimum essential medium (DMEM) containing 10% foetal bovine serum (FBS) and antibiotics. Medium was changed twice a week and cells harvested by trypsin treatment. Human umbilical vein endothelial cells (HUVEC) were prepared according to a modified version (Tonello et al., 2003) of the Jaffe et al. (1973) protocol. Umbilical cords were obtained from the local obstetric and gynaecology division. Briefly, after perfusion with a metal gauge, the vein was rinsed twice with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. Endothelial cells were separated from the vein walls by collagenase type IA (Sigma) digestion (1 mg/ml, 300–400 U/mg) for 10 min at 37 °C in physiological solution. The reaction was terminated by addition of Medium199 (Seromed) supplemented with 20% FBS and the same medium was used for resuspension of the cell pellet after centrifugation for 10 min at 1100 rpm. The cells were seeded onto collagenated plastic flasks prepared by treating them overnight with solution of collagen type I (Sigma) (10 μg/ml) in PBS. For HUVEC passages, a split ratio 1:2 in Medium199 supplemented with 0.1 ng/ml human recombinant ECGF, 10 ng/ml human bFGF (Calbiochem) and 100 μg/ml porcine heparin (Seromed) was used.

2.2. FU and HUVEC infection

Serum S22 from a viremic patient with acute B19 infection screened in our laboratory was used for the experimental infection. Following a preliminary titration, S22 serum was added to 1×10^6 cultured primary cells at a dilution containing 350 genomes per cell. After 2 h of incubation at 37 °C, the monolayer was washed three times with PBS to remove the input virus and incubated in DMEM medium containing 10% FBS at 37 °C with 5% CO_2. Mock-infected cultures were included as negative control. Infection of UT7 Epo cells with S22 serum at a dilution containing 35 genome copies per cell was carried out as positive control. Two hours after the exposure to B19 positive serum and on day 1, 2, and 6 the monolayers were washed twice with DMEM medium and harvested by trypsinization. Total RNA and DNA were extracted by Trizol Reagent (GIBCO BRL) in accordance with the supplier's instructions.

2.3. PCR and RT PCR for B19 DNA and B19 mRNA detection

RT/PCR was used to detect spliced parvovirus mRNA species for the structural protein VP1, as described by Bostic (Bostic et al., 1999) with the following modifications: the antisense primer used both for transcription of cDNA and for PCR was (5′ CCA CGA TGC AGC TAC AAC TT 3′) and 45 cycles of amplification were carried out, using Taq-Gold DNA polymerase (Applied Biosystem). To detect the non-structural protein (NS1) mRNA, the total RNA was preliminarly treated with Dnase I, Rnase-free (Roche) for 10 min at 37 °C followed by 5 min at 95 °C. Then, reverse transcription with antisense outer primer P6 (1682-1659nt) (Durigon et al., 1993) was performed, followed by nested PCR, as previously described (Zakrzewska et al., 2001). The sensibility of RT-PCR assays was evaluated by amplification of ten fold dilutions of the RNA standard derived from in vitro transcription of target sequences cloned in pGEM-T vector (Promega). Both reactions demonstrated the same sensitivity limits.

B19 DNA was detected by nested PCR as described previously (Zakrzewska et al., 2001).

As control for reverse transcription and amplification, the detection of cellular β-actin mRNA was also performed (Bostic et al., 1999).

To compare the amount of viral DNA in infected cultures a Real-Time SYBR Green PCR was used (unpublished). The primer pair used was P1 (5′-ATA CAC TGT GGT TTT ATG GGC CG-3′, nts 1399-1422) and P6 (5′-CCA TTG CTG GTT ATA ACC ACA GG-3′, nts 1682-1704) (Durigon et al., 1993). Ten microliters of extracted DNA were used for real-time amplification in a final reaction volume of 50 μl. The PCR mixture consisted of: 5 μl of Gold buffer 10×, 5 μl of 87% glycerol, 2.5 μl of SYBR Green 10×, 0.4 μl of 1 μM Fluorescein, 1 μl of dNTPs/dUTPs 10/20 mM, 1 μl of each primer P1 and P6 (25 μM), 6 μl of 25 mM $MgCl_2$, 0.5 μl of Taq Gold (5U/μl) and 1 μl of UDG heat-lable (1U/μl); water was added to obtain a 40 μl volume. After initial incubation at 20 °C for 10 min and at 94 °C for 6 min to activate the Taq polymerase, the thermal conditions followed 2 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 40 cycles of 95 °C for 1 min, 55 °C for 30 s, 72 °C for 30 s. Fluorescence

was measured automatically during each PCR cycle through the entire amplification period. The reading was performed at 77 °C for 10 s (iCycler iQ real-time PCR system; Bio-Rad). The first PCR cycle when an increase of fluorescence associated with an exponential growth of a specific PCR product was detected was defined as the threshold cycle.

Melting curve analysis was performed to assess the specificity of the PCR products.

Serial dilutions of the PCR sequence P1-P6, 283 bp long, cloned in the p-GEM-T vector (Promega) were used for the calibration curve of the real-time PCR. The reliability of the PCR was also evaluated by the use of the working reagent B19-DNA ISS 0300 (Istituto Superiore di Sanità, Roma, Italy).

3. Results

The synthesis of B19 virus mRNAs was considered a marker of parvovirus infection in cultured cells (Bostic et al., 1999) and the results obtained are summarised in Table 1.

Transcripts for the main B19 non-structural protein, NS1, as well as mRNA for B19 capsid protein VP1 were present in both HF and HUVEC primary cultures at 24 h after virus exposure and were still present at day 6 post-infection. To determinate which of the two messengers species (NS1 mRNA or VP1 mRNA) was prevalent in infected fibroblasts and endothelial cells cultures we proceeded with end point titration of RT-PCR products. We did not find any significant difference in the levels of messengers for structural and non-structural proteins. The level of mRNA for both NS1 and VP1 synthesised in HF and HUVEC was compared to the mRNA level for both viral proteins obtained in UT7 Epo cells. By end-point titration RT-PCR, the levels of both NS1 mRNA and VP1 mRNA synthesised after exposure to B19 virus of 10^6 HF or HUVEC were approximately, 10 times lower than that obtained after exposure of the same number of UT 7 Epo cells to B19 virus.

Intracellular B19 DNA amount not varied significantly with the time after virus exposure neither in primary cultures nor in UT 7 Epo cells. The concentration of B19 in culture supernatants not increased significantly with the time after virus exposure (data not shown). However, it is to note that B19 DNA was still present in the supernatant of the cultures in spite of the repeated washes performed to remove the inoculum. No cytopathic effect was detectable in the infected

Table 2
B19 DNA and mRNA detection in sequential passages of HF infected culture

	No. of passage					
	1	2	3	4	5	6
B19 DNA	Pos	Pos	Neg	Neg	Neg	Neg
B19 mRNAs	Neg	Neg	Neg	Neg	Neg	Neg
β-Actin mRNA	Pos	Pos	Pos	Pos	Pos	Pos

cultures by direct microscopic observation of monolayers throughout the 6 days of incubation.

Six sequential passages of HF cell cultures, at split ratio 1:2, were performed at day 6 after B19 infection. No specific mRNAs were found in six subsequent sub-cultures and B19 DNA was detectable in the first two subcultures only, and not in the following passages. (Table 2).

4. Discussion

The presence of NS1 and VP1 mRNA in both HF and HUVEC primary cultures exposed to a B19 positive serum indicates infection by B19 virus. Approximately the same levels of the two messengers species (NS1 mRNA or VP1 mRNA) were synthesised in infected monolayers. This result shows that HF and HUVEC are susceptible to B19 infection. However, it do not necessarily support the conclusion that these cells are also permissive to B19 replication, as it is known that the presence of a full set of viral mRNAs may not be followed by the production of a full set of viral proteins (Gallinella et al., 2000). The same pattern was observed in UT7 Epo cells. At least tenfold higher amount of B19 virus was required to infect HF and HUVEC in comparison to UT7 EPO cells and lower levels of viral mRNA were expressed in the primary cell cultures in comparison to UT7 EPO cells. This suggests that, in the experimental conditions used in this study, lower percentage of HF or HUVEC cells gets infected in comparison to UT7 Epo cells. Such hypothesis could also be inferred by the rapid clearance of B19 DNA from the subcultures of B19 infected HF. The observation that B19 DNA was already undetectable in HF subcultures (split ratio 1:2) at the third passage seems indicate that only few cells get infected.

No significant variation of B19 DNA level in the infected HF and HUVEC cultures was detectable by the real-time PCR through the entire incubation period of 6 days. This

Table 1
Markers of B19 infection of HF and HUVEC

Time after inoculation	HF				HUVEC			
	B19 DNA[a]	β-Actin mRNA	VP1 mRNA	NS1 mRNA	B19 DNA[a]	β-Actin mRNA	VP1 mRNA	NS1 mRNA
2 h	1.2×10^7	Pos	Neg	Neg	9.6×10^5	Pos	Neg	Neg
1st day	9.1×10^6	Pos	Pos	Pos	2.8×10^6	Pos	Pos	Pos
2nd day	2.7×10^7	Pos	Pos	Pos	3.7×10^6	Pos	Pos	Pos
6th day	5.3×10^6	Pos	Pos	Pos	1.3×10^6	Pos	Pos	Pos

[a] Copy number.

observation could indicate that no viral replication occurs in these cells, that is to say HF and HUVEC are not permissive for B19 virus. Using different methods, Miki and Chantler (1992) arrived to a similar conclusion concerning the ability of cultured human synovial cells derived from synovial membrane and cartilage to support the replication of human parvovirus B19. On the other hand, it is possible that a large amount of virus particles are adsorbed to the cells during the first 2 h of incubation, but only few cells, depending on the phase of their cell cycle, are able to support virus entry and replication. B19 DNA synthesis in few cells could be insufficient to determine a significant variation in the total B19 DNA copy number, detectable by the quantitative PCR. Approximately 5% of UT7 EPO cells are able to support B19 infection (Shimomura et al., 1992) and the increase of B19 DNA following infection is not always demonstrable also in this model.

It is possible that in different experimental conditions of HF and HUVEC culturing, such as hypoxia or after stimulation of HF or HUVEC with different growth factors or cytokines, higher number of fibroblasts and HUVEC may be infected by B19 virus and may support viral replication. We wish to explore this possibility in the following study; in fact, in order to investigate the effect of B19 infection on HF and HUVEC cells a much higher number of infected cells is required.

Acknowledgements

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