CD46 transgene expression in pig peripheral blood mononuclear cells does not alter their susceptibility to measles virus or their capacity to downregulate endogenous and transgenic CD46

Juergen Schneider-Schaulies,1 Michael J. Martin,2 John S. Logan,2 Ruth Firsching,1 Volker ter Meulen1 and Lisa E. Diamond2

1 Institut fur Virologie und Immunbiologie, Versbacher Str. 7, D-97078 Wu rzburg, Germany
2 Nextran Inc, 303B College Road East, Princeton Forrestal Center, Princeton, NJ 08540, USA

CD46 (or membrane cofactor protein) protects autologous cells from complement-mediated lysis and has been expressed as a transgene in pigs to overcome complement-mediated hyperacute rejection of porcine organs upon transplantation into primates. Since CD46 has been identified as a receptor for measles virus (MV), the susceptibility of CD46-transgenic (tg) pig peripheral blood mononuclear cells (PBMC) to infection with MV strains which do and do not use CD46 as receptor was investigated. Surprisingly, it was found that MV vaccine strains (e.g. Edmonston) bound to tg as well as non-tg pig PBMC. Phytohaemagglutinin-stimulated CD46-tg and non-tg pig PBMC were equally well infected with MV vaccine strains irrespective of CD46 expression. Upon infection, tg CD46 was downregulated from the cell surface. In contrast, the binding capacity for MV wild-type strains to pig and human PBMC was low, irrespective of CD46 expression. These MV strains did not infect tg or non-tg pig cells. Expression of endogenous pig CD46 was detected with polyclonal sera against human CD46. After infection of pig PBMC with MV strain Edmonston, endogenous pig CD46 was also downregulated. This suggests an interaction between MV Edmonston and pig CD46. However, polyclonal CD46 sera did not inhibit infection with MV Edmonston indicating that CD46 may not exclusively act as a receptor for MV on these cells. Interestingly, similar results were observed using human PBMC. Data suggest that CD46 downregulation after interaction with MV may also occur in porcine organs which express endogenous and/or human CD46 as a means of protection against complement-mediated damage.

Introduction

CD46, a member of the complement regulatory protein family, is a ubiquitously expressed membrane-bound glycoprotein in humans which acts as a cofactor for serum factor I cleavage of the C3b and C4b components of complement. Cell surface expression of CD46 protects cells from lysis by autologous complement (Johnstone et al., 1993; Liszewski et al., 1991; Loveland et al., 1993). Heterologous expression of human CD46 has been used experimentally to confer resistance of cells of non-human origin, as in CD46-transgenic (tg) mice, from lysis by human complement (Carrington et al., 1995; Seya et al., 1998; Thorley et al., 1997; White et al., 1995). We have shown that porcine organs expressing human complement regulatory proteins CD59 and decay accelerating factor (DAF, CD55) are protected from hyperacute rejection upon transplantation into non-human primates (Byrne et al., 1997; McCurry et al., 1995). This protection occurs at the level of regulation of primate complement activation. Subsequently, we generated tg pigs which express high levels of human CD46 in a cell- and tissue type-specific manner, and showed that hearts derived from these tg pigs can also overcome hyperacute rejection upon transplantation (Diamond et al., 1997).

In addition to its function in the inactivation of human complement, CD46 acts as a receptor mediating infection of cells with human herpesvirus-6 (HHV-6; Santoro et al., 1999)
and measles virus (MV) vaccine strains (Buchholz et al., 1996, 1997; Dörg et al., 1993; Gerlier et al., 1994; Hsu et al., 1997; Naniche et al., 1993a; Maisner et al., 1994; Manchester et al., 1994, 1995, 1997). Infection of cells with such MV strains or contact of cells with viral glycoproteins expressed on infected cells leads to downregulation of CD46 from the cell surface of the contacted cell (Naniche et al., 1993b; Krantic et al., 1995; Schneider-Schauslies et al., 1996) and renders these cells susceptible to lysis by complement in vitro (Schnorr et al., 1995). It is not clear what effect this may have in vivo, since most cells express other complement regulatory proteins, which will serve to protect the cells from complement-mediated lysis. However, it is formally possible in humans that MV infection may contribute to a rapid clearing of CD46-negative cells by complement, and thus to the attenuation of CD46-downregulating MV strains.

Not all MV strains have the capacity to downregulate CD46 and the degree of receptor modulation varies considerably between various MV strains which use CD46 as a receptor (Schneider-Schauslies et al., 1995a, b). A few defined amino acids in the MV haemagglutinin (H) protein determine the capacity to downregulate CD46 (Bartz et al., 1996; Lecouturier et al., 1996). Recently, we and others have found that a number of MV wild-type (WT) isolates appear to use receptors other than CD46 and these strains also do not downregulate human CD46 (Bartz et al., 1998; Hsu et al., 1998; Tanaka et al., 1998). Interestingly, it has been found that other morbilliviruses which are closely related to MV, such as rinderpest virus (RPV), can induce the downregulation of CD46 from the surface of cells, while not using CD46 as a receptor (Galbraith et al., 1998). Thus, receptor usage and CD46 downregulation appear to be independent processes. Recently, the pig homologue of human CD46 was cloned and found to share a similar structure and 42% amino acid identity with human CD46 (Seya et al., 1998; Toyomura et al., 1997; Van den Berg et al., 1997). It is not known whether pig CD46 interacts with any strain of MV.

In this publication, we compared the susceptibility of peripheral blood mononuclear cells (PBMC) from human CD46-tg and non-tg pigs to MV infection. Since human CD46 protects non-human cells from lysis by human complement, it is important to know under which conditions CD46 may be downregulated from the surface of xenotransplants. Furthermore, the possibility exists that expression of human CD46 on tg pig tissues may render these cells susceptible to MV infection. We found that PBMC from tg as well as non-tg pigs were susceptible to MV vaccine strains and that infection resulted in downregulation of both porcine and human CD46.

**Methods**

**Transgenic pigs.** A 60 kb genomic construct encompassing the human CD46 gene was isolated from a P1 phage library and microinjected into the male pronuclei of fertilized porcine oocytes to generate tg pigs using previously published techniques (Wall et al., 1985). The pigs are F2 outcrosses consisting of Landrace, Hampshire, Large White and Duroc. The frequency of obtaining tg animals was approximately 3%, consistent with prior experience (Logan & Martin, 1994). TG founders were identified by Southern blot analysis of DNA from tail biopsies. TG pigs used in this study were derived from one tg founder pig.

**Isolation and culture of PBMC.** PBMC from pigs and human blood donors were isolated by centrifugation of blood cells on a Percoll cushion (density 1.077 g/ml) for 20 min at 1000 g. Cells floating on the cushion were washed with PBS and frozen (1 x 10^7 per ml) in RPMI 1640 medium containing 50% foetal bovine serum (FBS) and 8% DMSO in liquid nitrogen. For the tests, cells were thawed and cultured in RPMI 1640 medium containing 10% FBS, penicillin and streptomycin. Pig PBMC were stimulated with 10 µg/ml phytohaemagglutinin (PHA) and human PBMC were stimulated with 5 µg/ml PHA.

**MV strains.** The MV strains genetically belonging to the vaccine group were Edmonston (Edm), Schwarz and Moraten. These strains were propagated using Vero cells and use CD46 as a receptor on human cells and downregulate CD46 after infection or contact with infected cells (Schneider-Schauslies et al., 1995a, b, 1996). WT strains TC5679, Wut671, W5404 and W5479 were isolated in 1996 from measles patients in Würzburg, Germany. The WT MV strains were isolated and propagated using the human B cell line BJAB, which was immortalized with Epstein–Barr virus (EBV) and does not produce EBV (Menezes et al., 1975).

**Antibodies.** The MAbs against CD46, MV H protein and MV nucleocapsid (N) protein (13/42, L77 and F227, respectively) were grown and purified over protein G columns in our laboratory, and used at a dilution of 5 µg/ml. FITC- or phycoerythrin-conjugated rabbit anti-mouse Ig, and goat anti-rabbit–FITC were purchased from Dako and used at 1:100 dilution. Polyclonal rabbit anti-CD46 sera were a generous gift from F. Wild, (Lyon, France) and G. Yeh (Cytomed Inc, USA).

**Flow cytometry.** For flow cytometry, PBMC (1 x 10^6 per tube) were harvested and washed with FACS buffer (calcium- and magnesium-free PBS containing 0.4% BSA and 0.01 M NaN3). After incubation with the first antibody diluted in FACS buffer on ice for 45 min, cells were washed twice with FACS buffer, incubated for 45 min with the second antibody, washed twice and analysed with a FACScan flow cytometer (Becton Dickinson).

**Virus binding assay.** Similar m.o.i. of virus preparations of the different MV strains were used in virus–cell binding assays as described previously (Bartz et al., 1998). The m.o.i. were determined according to titration of MV vaccine strains on Vero cells and MV WT strains on BJAB cells. PBMC (2 x 10^5) in 100 µl PBS were incubated at 4 °C for 1 h with virus at a given m.o.i., washed with FACS buffer, stained with anti-MV H protein MAb L77 and FITC-conjugated goat anti-mouse antibodies as described, and bound virus was determined by analysis with a FACScan (Becton Dickinson).

**Results**

**Expression of human CD46 on tg and non-tg pig PBMC**

The expression of the CD46 transgene in the pig PBMC was measured by flow cytometry using MAb 13/42 directed against the first domain of the human CD46 molecule. PBMC of three individual tg pigs expressed relatively high amounts of human CD46 compared with human PBMC. In contrast, PBMC from three non-tg pigs did not interact with MAb.
Infection of pig PBMC by measles virus

Infection of pig PBMC by measles virus

Fig. 1. Expression of human CD46 on the surface of PBMC from tg pigs. (a) PBMC from a CD46-tg pig (thick line) express high levels of CD46 detected with MAb 13/42 against the first complement control protein (CCP) domain of human CD46. The specificity of detection is shown by staining with isotype control antibody (neg.c). (b) Non-tg pig PBMC are not stained with MAb 13/42 against human CD46. (c) Human PBMC express slightly less CD46 than found on tg pig PBMC (a).

13/42. Representative examples for the detected signals are shown in Fig. 1. The endogenous pig CD46 homologue (Toyomura et al., 1997; Van den Berg et al., 1997) is not recognized by MAb 13/42.

**Binding of MV to tg and non-tg pig PBMC**

In order to test the function of human CD46 as an MV receptor on porcine cells, we first investigated the capacity of the pig PBMC to bind various MV strains. Virus binding assays were performed at 4 °C for 1 h, under conditions which have been successfully used for studies with human cells. The MV vaccine strains, such as Edm, bound in a dose-dependent manner equally well to pig and human PBMC (Fig. 2). Surprisingly, the human CD46-negative cells of non-tg pigs bound MV vaccine strains as well as both the CD46-positive tg pig cells and the human PBMC. In contrast, the capacity of the PBMC to bind to similar amounts of MV WT strains was low for both tg and non-tg pig cells, as well as for human PBMC. Thus, pig PBMC exert the same binding properties for MV strains as human PBMC, irrespective of the presence or absence of human CD46 on the pig cells.

**Infection of pig PBMC with MV vaccine and WT strains**

To assess the permissivity for MV, tg and non-tg pig PBMC were infected with the various MV strains for a single-step growth curve with an m.o.i. of 1. The increasing expression of viral proteins with time of infection was monitored by flow cytometry after 3, 5 and 7 days post-infection (p.i.; data not shown). Newly synthesized virus was titrated from infected cultures (cell-associated and cell-free virus) after 1, 3, 5 and 7 days p.i. Only the MV strain Edm replicated efficiently in pig PBMC, whereas MV WT strains
Fig. 3. Productive MV replication in CD46-tg and non-tg pigs and human PBMC. PBMC were stimulated with PHA and infected with MV Edm or MV WT (Wu5404) at an m.o.i. of 0.1. After 1, 3, 5 and 7 days p.i., cultures were frozen and cell-bound and -free virus were harvested together. Titres were then determined in an end-point titration using B95a cells.

Fig. 4. CD46 downregulation after infection of pig PBMC. CD46 protein levels on tg pig PBMC were reduced after infection of cells with MV Edm. An example of the CD46 signal detected by flow cytometry on PBMC of a representative tg pig is shown in (a). Non-tg pig PBMC did not show an altered signal by flow cytometric analyses with anti-CD46 MAb 13/42 (b). The mean fluorescence intensities of the CD46 signals of different CD46-positive (tg pig, human) and CD46-negative (non-tg pig) PBMC before and after infection of cells with MV Edm (m.o.i. = 0.1) for 3 days are presented in (c). As a control for the infection, staining of the same cells with anti-MV H protein MAb L77 is shown in (d). MV Edm infection is indicated by (+) and absence of infection is indicated by (−).

Fig. 5. Downregulation of endogenous pig CD46 after infection of pig PBMC by MV strain Edm. Rabbit polyclonal sera raised to human CD46 recognize an antigen on non-tg pig PBMC, which is most likely endogenous pig CD46 (a). The negative control staining was done with a rabbit preimmune serum (neg.c). The signals detected with two different polyclonal anti-CD46 sera (b, bars 2 and 4) were reduced (b, bars 3 and 5, respectively) when normal pig PBMC were infected with MV Edm. The mean fluorescence intensity of the control signal (normal rabbit serum) is given in (b) lane 1.

The infection of human PBMC with MV vaccine strains leads to the downregulation of CD46 from the surface of human cells (Naniche et al., 1993b; Schneider-Schaulies et al., 1995a, b). When tg pig PBMC were infected with MV Edm, the degree of CD46 downregulation from the surface of infected cells was similar to that found with human PBMC (Fig. 3). Interestingly, CD46-positive and -negative pig PBMC produced similar amounts of MV Edm as the human PBMC.

CD46 downregulation following infection

The infection of human PBMC with MV vaccine strains leads to the downregulation of CD46 from the surface of human cells (Naniche et al., 1993b; Schneider-Schaulies et al., 1995a, b). When tg pig PBMC were infected with MV Edm, the degree of CD46 downregulation from the surface of infected cells was similar to that found with human PBMC (Fig. 3). Using non-tg pig PBMC, the infection did not alter the
background signals detected with MAb 13/42 against human CD46.

Expression and downregulation of endogenous pig CD46

Since the non-tg pig PBMC were infected equally as well as the human CD46-positive tg pig PBMC, we hypothesized that the endogenous pig CD46 (Toyomura et al., 1997; Van den Berg et al., 1997) might serve as a receptor for MV vaccine strains. We therefore stained these cells with polyclonal antibodies raised against human CD46 which have the potential to cross-react with the porcine CD46 homologue. Two anti-CD46 rabbit sera from two different sources were used with similar results. The polyclonal sera detected a specific signal on the surface of non-tg pig PBMC (Fig. 5a). When these cells were infected with MV Edm, the signals for the endogenous pig CD46 were reduced (Fig. 5b, lanes 3 and 5, as compared to 2 and 4, respectively). Thus, it appears that MV Edm may interact with endogenous pig CD46 and induce its downregulation from the cell surface.

Does endogenous pig CD46 act as a receptor for MV Edm?

In order to investigate whether the pig CD46 may act as a receptor for MV and whether this interaction can be blocked by antibodies to CD46, we incubated non-tg pig PBMC with polyclonal anti-CD46 sera prior to infection with MV Edm (Fig. 6). As controls, human PBMC and Vero cells were pretreated with the anti-CD46 sera and the infection of cells was either measured by staining of viral surface proteins or, in case of the Vero cells, by observing the reduction of plaque formation. The infection of non-tg and tg pig PBMC with MV Edm was partially (approximately 17%) inhibited by the anti-CD46 serum in high concentrations (1:20). The infection of human PBMC with MV Edm was also only partially inhibited by approximately 28%. In contrast, the infection of Vero cells preincubated with similar dilutions of the polyclonal anti-CD46 sera was reduced by more than 95%. Thus, on pig as well as on human PBMC, MV Edm infection was inhibited only partially suggesting that MV Edm may use additional receptors other than CD46 on these cells.

Discussion

The binding of MV vaccine and WT strains, respectively, was similar to CD46-tg and non-tg pig, as well as human PBMC. WT strains had a considerably lower capacity to bind to target cells irrespective of origin, i.e. human or pig. Their binding to pig PBMC was not improved in the presence of tg human CD46. These results may reflect a selection for a high binding capacity of vaccine strains by long-term laboratory culture. We are aware of the fact that these binding studies were performed under experimental conditions where high m.o.i. of virus were incubated with target cells. Since one infectious particle per cell may be sufficient to infect the cells, the measured binding capacities cannot be correlated with the susceptibility of cells to infection. This is the case for human PBMC, which are fully permissive for MV WT strains, but demonstrate a low binding capacity for these viruses. Since, in contrast to human PBMC, the tg and non-tg pig PBMC were not susceptible to WT MV strains, it is not clear for these strains whether appropriate receptors or further unknown species-specific factors, which play a decisive role in permissivity, are missing. In CD46-tg rats intracellular factors restrict the replication of MV (strains Edmonton, Chicago 1 and CAM/RBH; Niewiesk et al., 1997). In CD46-tg mice, replication of MV was restricted (Horvat et al., 1996; Blixenkrone-Moller et al., 1998), but this restriction could be overcome eventually for unknown reasons (Oldstone et al., 1999), or by introducing a defect in the interferon-alpha/beta receptor in addition to the CD46 transgene (Mrkic et al., 1998). After polyclonal activation of lymphocytes, MV (strains Edmonston or Hallé) replication was found in a proportion of tg B and T cells in vitro (Horvat et al., 1996). These results appear to be similar to those found in this study with MV Edm in tg pig PBMC.

In addition to the fact that not all MV strains downregulate CD46, it was found that the H proteins of RPV and peste-des-petits-ruminants virus, closely related morbilliviruses, also induce the downregulation of CD46 from the surface of Vero and B95a cells, although CD46 did not appear to be the cellular receptor for these viruses (Galbraith et al., 1998). We also...
observed that on chimeric recombinants, the first domain of CD46 alone is sufficient to induce downregulation, while these molecules do not serve as receptors for MV (Buchholz et al., 1996; Firsching et al., 1999). This indicates that CD46 downregulation and receptor usage by different morbilliviruses are distinct processes. Interestingly, another virus that uses CD46 as receptor, HHV-6, also induces its downregulation from the surface of infected cells (Santoro et al., 1999).

Since CD46 acts in vivo to protect cells from autologous complement-mediated lysis, CD46 modulation may result in the interaction of complement cascade proteins with such cells and may induce their elimination. As we demonstrated, infection with MV can induce the downregulation of tg and endogenous CD46 from the surface of porcine cells. The pig homologue of human CD46 was recently cloned, and shares a similar structure and 42% amino acid identity with human CD46 (Seya et al., 1998; Toyomura et al., 1997; Van den Berg et al., 1997). Pig CD46 is expressed on all pig blood leukocytes and erythrocytes, and on endothelial and epithelial cell lines (Toyomura et al., 1997). Our data suggest that CD46 downregulation after interaction with MV may also occur in porcine organs which express endogenous and/or human CD46 as a means of protection against complement-mediated damage.

There is no doubt that pig PBMC, in the absence or presence of human CD46, can be infected with MV vaccine strains. The lack of a clear inhibition of MV infection by polyclonal antibodies to CD46, which also recognize endogenous pig CD46, did not resolve the question of receptor usage, since polyclonal sera to CD46 inhibit also the infection of human PBMC only partially. The MV vaccine strains therefore seem to recognize additional receptors that are not identical to CD46 on PBMC of human as well as porcine origin. This is in contrast to Vero cells, where anti-CD46 sera efficiently inhibit the infection. The uptake mechanism of MV by PBMC requires further investigation.

The use of pigs as potential organ donors for humans has drawn attention to the potential transmission of viruses from these xenografts to recipients (Patience et al., 1997). Additionally, the possibility formally exists that expression of human proteins in tg pigs may result in pigs which may now acquire infections to which they were previously immune. CD46 is also involved in the adherence of group A Streptococcus pyogenes, and Neisseria gonorrhoeae and Neisseria meningitidis to cells (Atkinson et al., 1994; Kallstrom et al., 1997; Okada et al., 1995). Another complement regulatory protein, CD55 (DAF), is a receptor for some strains of Escherichia coli (Nowicki et al., 1993) and for several enteroviruses including types of echovirus (Bergelson et al., 1994; Ward et al., 1994) and coxsackie B virus (Bergelson et al., 1995). Data presented here demonstrate that pigs are inherently susceptible to infection by MV vaccine strains, regardless of their tg status. Interestingly, MV WT strains, which have been described as using different receptors, did not infect pig PBMC regardless of whether they expressed human CD46. Therefore, in considering a potential xenograft recipient, it would appear unlikely that infection by WT MV would affect human CD46 expression on a tg porcine organ. Additionally, a high percentage of adult human recipients have a lifelong immunity against measles as result of vaccination or disease. Since expression of human CD46 on tg porcine cells does not alter their susceptibility to MV infection, it would therefore not appear to present an additional risk to a potential xenograft recipient in terms of MV infection.

We thank F. Dimpfel, C. Adams and E. Shanahan for excellent technical assistance and the Deutsche Forschungsgemeinschaft for financial support.

References


Infection of pig PBMC by measles virus


International Congress for Xenotransplantation, Nantes, France. Abstract O185.


Received 13 December 1999; Accepted 29 February 2000