Pathogenesis of coxsackievirus A9 in mice: role of the viral arginine-glycine-aspartic acid motif

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Coxsackievirus A9 (CAV9) contains an arginine-glycine-aspartic acid (RGD) motif which participates in cell entry. Mutants with alterations in the RGD-containing region were utilized to explore the importance of the tripeptide in the pathogenesis of CAV9 in mice. Using in situ hybridization, the parental CAV9 strain was observed to infect skeletal muscle (intercostal, platysma, lingual and thigh muscles) of newborn mice, whereas the RGD-less mutants were detectable only in platysma and lingual muscles. In addition, newborn mice infected with the mutants survived longer than CAV9-infected mice. In adult mice, the parental strain of CAV9, but not the mutants, achieved moderately high titres in the pancreas. These results suggest that the RGD motif has a significant role in the pathogenesis of CAV9 in mice but also that RGD-independent entry routes can be utilized in the infection of murine tissue.

Coxsackievirus A9 (CAV9) is a non-enveloped RNA virus, a member of the genus Enterovirus in the family Picornaviridae. Enteroviruses are responsible for a wide spectrum of diseases, such as respiratory infections, rashes, myocarditis and infections of the central nervous system, and they have been associated also with chronic dilated cardiomyopathy and type I diabetes. CAV9 is among the most frequent pathogens causing aseptic meningitis (Grist & Reid, 1988; Hovi et al., 1996). Typically, many enteroviruses have similar clinical manifestations in humans and, in addition, the same serotype can cause diverse symptoms (Grist & Reid, 1988), thus making them difficult to investigate. However, insights into pathogenicity and tissue tropism can be gained through the analysis of a mouse model (Hyypia¨ et al., 1993; Harvala et al., 2002).

Coxsackieviruses are divided into subgroups A and B, according to pathogenicity in newborn mice: CAVs cause flaccid paralysis; in contrast, CBVs induce spastic paralysis. Accordingly, CAVs affect striated muscle, while CBVs replicate in several tissues, including the central nervous system, pancreas and liver (Hyypia¨ et al., 1993). CAV9, despite its CAV-like pathogenicity in newborn mice, is genetically more closely related to CBVs than to other CAVs. However, when compared to CBVs, the CAV9 capsid protein VP1 has an apparent insertion of approximately 15 amino acids at its C terminus (Chang et al., 1989). This insertion contains an arginine-glycine-aspartic acid (RGD) tripeptide, which has been shown to be fully conserved among clinical CAV9 strains isolated from different geographical regions over the past five decades but has not been found in other CAVs (Chang et al., 1992; Santti et al., 2000). The RGD motif mediates attachment of CAV9 to the cell surface integrin αvβ3 but the virus is able also to utilize alternative pathway(s) in cell entry, since deletion or mutation of the RGD motif does not destroy infectivity completely (Roivainen et al., 1991, 1994; Hughes et al., 1995). The C-terminal region of the VP1 capsid protein has been shown also to be antigenic by peptide scanning but it was found that the RGD motif itself was poorly immunogenic, whereas antibody-binding sites were located at both sides of the motif (Pulli et al., 1998a, b).

To analyse the effect of the RGD motif on tissue tropism and pathogenicity in vivo, BALB/c mice were infected with the parental (CAV9) or mutant (CAV9RGE, CAV9d4 and CAV9d12) viruses described previously (Chang et al., 1989; Hughes et al., 1995). The substitution mutant (CAV9RGE) contains an RGE (arginine-glycine-glutamic acid) motif instead of an RGD motif, whereas in the genomes of deletion mutants, the region deleted included the RGD motif alone (CAV9d4) or the RGD motif and eight additional amino acids located at both sides of the motif (CAV9d12). Transfection of rhabdomyosarcoma (RD) cells with RNA transcripts, generated from the parental and three mutant plasmids, resulted in complete CPE within 3 days. Examination of the growth curves of mutant viruses showed similar production of infectious virus when compared to parental CAV9 (data not shown).
Groups of newborn BALB/c mice (Animal Center of the University of Turku), aged between 8 and 24 h, were infected intraperitoneally with 2 × 10⁴ p.f.u. of the mutant or parental viruses in 50 µl PBS. The size of groups varied from 10 to 18. The litters within groups were maintained separately and observed daily. Following inoculation, one to six mice from each group were sacrificed after 1 to 5 days and fixed in 10% formalin. Transverse sections of the head, upper and lower abdomen, and lower limb were embedded in paraffin and used to analyze the presence of viral RNA by in situ hybridization using a radiolabeled CAV9 cDNA probe, as described previously (Harvala et al., 2002). Both uninfected mouse tissue and a plasmid probe were used to control the specificity of the hybridization reactions.

All of the newborn mice infected with CAV9 died between 3 and 5 days post-infection (p.i.), whereas the CAV9-mutants appeared to be less virulent: some mice survived until day 5 p.i. (Fig. 1). After parental CAV9 infection of newborn mice, viral RNA was detectable by in situ hybridization in skeletal muscle (intercostal, platysma, lingual and thigh muscles), whereas CAV9RGE and CAV9d4 RNA was seen only in lingual (CAV9RGE and CAV9d4) or platysma (CAV9RGE) muscle. CAV9d12 mutant RNA was not detected in any tissue (Fig. 2A–H). The signal obtained 3 days after mutant virus infection was much weaker than that observed in CAV9 infection. Surprisingly, the genome of parental CAV9 was also detectable in the exocrine part of the pancreas, in addition to muscle tissue, 3 days after infection (Fig. 2D). All other tissues studied were negative for viral RNA (data not shown).

To study further the in vivo pathogenicity of the RGD mutant viruses, 4- to 6-week-old adult male BALB/c mice were infected intraperitoneally with 2 × 10⁵ p.f.u. of the mutant or parental viruses in 100 µl PBS. Three mice from each group were sacrificed using CO₂ anaesthesia on days 1, 3, 5, 7 and 12 after infection. Fresh frozen tissues from diverse organs (heart, pancreas, liver, spleen, limb muscle and spinal cord) were homogenized in 1 ml PBS and the titres of parental and mutant viruses in tissue and blood samples were determined on monolayers of RD cells by plaque assay.

None of the adult mice, infected with either the parental or the mutant CAV9 strains, died during the follow-up period. The parental CAV9 strain replicates to high titres in the pancreas, whereas the RGD mutant viruses did not (Fig. 3). CAV9 was seen to replicate in the pancreas at day 1 p.i. (the tissue of the mouse that was positive contained 1·3 × 10⁶ p.f.u. g⁻¹), whereas maximal virus titres in the pancreas were detected at 3 days p.i. (mean of three mice, 6·5 × 10⁶ p.f.u. g⁻¹; range of titres, 3·2 × 10⁶ p.f.u. g⁻¹ to 8·8 × 10⁶ p.f.u. g⁻¹). Virus replication in the other tissues and blood remained undetectable. The plaque assay used has a sensitivity of about 100 p.f.u. g⁻¹ and it is possible that small amounts of virus, below this level of detection, were present in these tissues.

Previous investigations have provided somewhat conflicting information about the role of the VP1 RGD motif in CAV9 replication and pathogenesis. The fully conserved nature of the RGD sequences among CAV9 isolates indicates its importance in the replication of CAV9 in humans; the ability of CAV9 to use an RGD-independent entry pathway in RD cells argues against an essential function of this tripeptide during infection (Roivainen et al., 1991; Chang et al., 1992; Hughes et al., 1995; Santti et al., 2000). The role of RGD-dependent cellular interactions of CAV9 in murine infections has not been characterized previously. In the present study, the hybridization signal in skeletal muscle following inoculation of newborn mice with wild-type CAV9 was found to be more extensive than that obtained after infection with CAV9RGE or CAV9d4 mutant viruses, and newborn mice infected with the CAV9 mutants also survived longer than CAV9-infected mice. Parental or mutant CAV9 strains did not give a detectable signal in the muscle tissue of adult mice. However, active virus replication of CAV9, but not the mutants, could be demonstrated in the pancreas. These results suggest that the RGD motif is a significant factor that affects the pathogenicity of CAV9 in mice. The data are partly consistent with work done on echovirus type 9 (EV-9, strain Barty), the other enterovirus with an RGD motif. This showed that the replication of the EV-9 strain lacking the corresponding RGD region was not detectable in skeletal muscle of newborn mice, while the virus containing an RGD motif replicated to high titres in skeletal muscle (Nelsen-Salz et al., 1999). In common with CAV9, the RGD tripeptide is also known to mediate EV-9 binding to the αvβ3 integrin.
integrins are involved in terminal muscle cell differentiation (Menko & Boettiger, 1987) and the downregulation of $\alpha V \beta 3$ integrin is part of the myogenic differentiation programme of human skeletal muscle (Blaschuk et al., 1997), the reduced pathogenicity of CAV9 and EV-9 in older mice could be due to the lower expression of $\alpha V \beta 3$ integrin (Nelsen-Salz et al., 1999).

Two CAV9 mutants lacking the RGD motif (CAV9RGE and CAV9d4) were able to infect platysma and lingual muscles in newborn mice, which may be due to the reported capability of CAV9 lacking the RGD motif to interact with the $\alpha V \beta 3$ integrin (Triantafilou et al., 2000). An alternative explanation could be the use of another, presumably RGD-independent, receptor pathway by the CAV9 mutant viruses. The limited tropism and lower signal, indicative of a reduced ability to infect the tissue, could be because RGD-mediated integrin clustering and signalling may be needed for efficient virus internalization, as has been reported for

Fig. 2. Localization of viral RNA in skeletal muscle and pancreas of newborn mice in bright-field pictures by in situ hybridization. Tissue samples were taken at 3 days after infection. Intercostal (A, bottom of section), platysma (A, top of section), lingual (B) and thigh (C) muscles appeared to be strongly positive after parental CAV9 infection, but CAV9 genomes were also detected in the exocrine part of the pancreas (D). The CAV9RGE mutant replicated only in lingual (E) and platysma (F) muscles. The CAV9d4 strain replicated only in lingual muscle (G). The lingual muscle of an animal infected with CAV9d12 remained negative (H). (A), (D) and (F) Original magnification, $\times 52$; (C) original magnification, $\times 80$; (B), (E), (G) and (H) original magnification, $\times 51$.

Fig. 3. Replication capability of the parental and mutant viruses in the pancreas of adult mice. The titres of parental and mutant viruses in different tissues (heart, pancreas, liver, spleen, limb muscle and spinal cord) and blood samples were determined on monolayers of RD cells using plaque titration. Parental CAV9 was able to replicate to high titres in the pancreas (mean of three mice, $6.5 \times 10^4 \pm 2.9 \times 10^4$ p.f.u. g$^{-1}$), whereas the CAV9 mutants were not able to replicate to detectable levels. Other tissue and blood samples examined remained negative.

(Zimmermann et al., 1997; Nelsen-Salz et al., 1999). Because integrins are involved in terminal muscle cell differentiation (Menko & Boettiger, 1987) and the downregulation of $\alpha V \beta 3$ integrin is part of the myogenic differentiation programme of human skeletal muscle (Blaschuk et al., 1997), the reduced pathogenicity of CAV9 and EV-9 in older mice could be due to the lower expression of $\alpha V \beta 3$ integrin (Nelsen-Salz et al., 1999).
adenoviruses (Chiu et al., 1999). In addition, the interaction of integrins with RGD-containing peptides can also activate caspases and lead to apoptosis (Buckley et al., 1999; Ruoslahti & Reed, 1999), although it is not known whether viruses themselves can induce such signalling through interaction with integrin. If apoptosis does play a role in CAV9 infection, it may be the case that RGD-less mutants fail to replicate efficiently because they do not induce apoptosis.

It is interesting that, although CAV9RGE and CAV9d4 were able to infect platysma and lingual muscles in newborn mice, the mutant CAV9d12 could not be detected in these tissues. This mutant grows as efficiently in RD cells as the two less radical mutants, suggesting that differences in pathogenicity are not due to markedly diminished particle stability or altered polyprotein processing (data not shown). It is possible that the extra eight amino acids deleted in this mutant may themselves contain determinants that contribute to pathogenicity.

It is well known that CBVs can replicate to high titres in the mouse pancreas and cause acinar cell destruction but, until recently, the ability of CAV9 to replicate in pancreatic tissue has not been documented. In our current work, however, the parental CAV9 strain was seen to replicate in the pancreas, in both newborn and adult mice, while the CAV9 mutants were not detectable in this tissue. The hybridization signal obtained in the pancreas after inoculation of newborn mice with parental CAV9 was relatively weak and virus titres in the pancreas of the CAV9-infected adult mice remained low compared to, for instance, those observed in CBV3-infected mice (data not shown). In addition, our earlier findings suggest that non-capsid determinants contribute to pancreotropism in newborn mice, since a recombinant containing the 5′NCR from CBV3 and the rest of the genome from CAV9 showed substantially greater replication in the pancreas when compared to parental CAV9 infection (Harvala et al., 2002). Nonetheless, the data support the importance of the RGD motif in mouse pancreotropism of CAV9. It is not clear if this observation has implications for human disease, since it is not known whether CAV9 can infect the human pancreas. However, a strain of EV-9, the other enterovirus with an RGD motif in VP1, was shown to be cytolytic for pancreatic cells (Acharya et al., 1999; Forss et al., 1984; Hyypiä et al., 1992; Zimmermann et al., 1996). According to mutagenesis studies, the RGD motif plays a critical role in HPEV-1 infection, since viruses carrying an RGE sequence are not viable and transfection of mutant RNA resulted only in production of revertant viruses with a restored RGD motif (Boonyakiat et al., 2001). The substitution of the RGD motif by an RGE tripeptide or deletion of the RGD motif does not abolish the infectivity of EV-9 strain in green monkey kidney cells but the presence of an RGD motif correlates with EV-9 pathogenesis in mice (Zimmermann et al., 1997). Viable FMDV mutants lacking the RGD motif have been characterized also (Baranowski et al., 2000) but FMDV strains virulent for cattle appeared to utilize the RGD-mediated integrin interaction (Neff et al., 1998). CAV9, EV-9 and FMDV mutants lacking the RGD tripeptide can be viable in certain cell lines (Baranowski et al., 2000; Hughes et al., 1995; Zimmermann et al., 1997) but the almost ubiquitous occurrence of this motif in isolates suggests again that it is required for natural infections by these viruses. This is consistent with the observations described here, in that the RGD motif influences the tropism and pathogenicity of CAV9 in mice.

**ACKNOWLEDGEMENTS**

We thank Mrs Liisa Lempiainen, Mrs Marja-Liisa Mattila and Mrs Seija Linqvist for technical assistance. This study was supported by grants from the Academy of Finland, the Turku University Foundation, the Finnish Cultural Foundation, the Finnish Medical Foundation, the Research and Science Foundation of Farmos, Turku Graduate School of Biomedical Sciences and by the EVO research fund (no. 13475) of Turku University Hospital.

**REFERENCES**


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