The \( \alpha v\beta 6 \) integrin receptor for *Foot-and-mouth disease virus* is expressed constitutively on the epithelial cells targeted in cattle

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Field strains of *Foot-and-mouth disease virus* (FMDV) use a number of \( \alpha v\)-integrins as receptors to initiate infection on cultured cells, and integrins are believed to be the receptors used to target epithelial cells in animals. In this study, immunofluorescence confocal microscopy and real-time RT-PCR were used to investigate expression of two of the integrin receptors of FMDV, \( \alpha v\beta 6 \) and \( \alpha v\beta 3 \), within various epithelia targeted by this virus in cattle. These studies show that \( \alpha v\beta 6 \) is expressed constitutively on the surfaces of epithelial cells at sites where infectious lesions occur during a natural infection, but not at sites where lesions are not normally formed.

Expression of \( \alpha v\beta 6 \) protein at these sites showed a good correlation with the relative abundance of \( \beta 6 \) mRNA. In contrast, \( \alpha v\beta 3 \) protein was only detected at low levels on the vasculature and not on the epithelial cells of any of the tissues investigated. Together, these data suggest that in cattle, \( \alpha v\beta 6 \), rather than \( \alpha v\beta 3 \), serves as the major receptor that determines the tropism of FMDV for the epithelia normally targeted by this virus.

**INTRODUCTION**

*Foot-and-mouth disease virus* (FMDV) is the aetiological agent responsible for foot-and-mouth disease, a severe vesicular disease of cloven-hoofed animals. FMDV shows a strong tropism for epithelial cells and, following a natural infection, initial virus uptake and/or replication is thought to take place in the pharynx and soft palate (Alexandersen et al., 2001, 2002a, c, 2003; Brown et al., 1996; Burrows et al., 1981; McVicar & Sutmoller, 1971, 1976; Oleksiewicz et al., 2001; Prato Murphy et al., 1994, 1999; Zhang & Kitching, 2000). During disease, virus is widely disseminated throughout the body and adult animals develop highly infectious lesions on the feet and mouth (Alexandersen et al., 2001, 2002b, 2003; Brown et al., 1995, 1996; Burrows et al., 1981). In cattle, goats and sheep (but not pigs) infection is commonly persistent (Alexandersen et al., 2002b, 2003; Burrows, 1966; McVicar & Sutmoller, 1969; Salt, 1993, 1998; Zhang & Alexandersen, 2003). The mechanisms of persistence are currently unknown, although it has been demonstrated that the dorsal soft-palate provide sites of virus persistence in infected animals (Alexandersen et al., 2002b, 2003; Prato Murphy et al., 1999; Woodbury et al., 1995; Zhang & Alexandersen, 2004; Zhang et al., 2004; Zhang & Kitching, 2001).

Several receptors for FMDV have been identified, these are the integrins (Jackson et al., 2003) and heparan sulfate proteoglycans (HSPGs) (Jackson et al., 1996). Currently there is no convincing evidence of a role for HSPG in cell entry by field viruses (Baranowski et al., 1998, 2000; Escarmis et al., 1998; Martinez et al., 1997; Neff et al., 1998; Sa-Carvalho et al., 1997). Instead, field viruses depend on integrins for infection *in vitro*, and integrins are believed to be the receptors used in animals (McKenna et al., 1995; Neff et al., 1998).

Integrins are a family of integral membrane receptors that function as cell adhesion molecules (Hynes, 2002). Each integrin is a heterodimer formed by the non-covalent association of \( \alpha \)- and \( \beta \)-subunits. In mammalian species, the integrin family consists of 24 different heterodimers, each of which has a distinct tissue distribution. A general property of integrins is that they exist in alternate active or inactive states (Hynes, 2002). Conversion between these states is regulated by reversible changes in the conformation of the extracellular domains in a process termed ‘integrin activation’ (Giancotti, 2003; Hynes, 2002; Kim et al., 2003).

In cell culture, FMDV has been shown to use four integrins, \( \alpha v\beta 1 \), \( \alpha v\beta 3 \), \( \alpha v\beta 6 \) and \( \alpha v\beta 8 \) as receptors to initiate infection.
Virus attachment to the integrin is mediated through an arginine–glycine–aspartic acid (RGD) tripeptide located on VP1 (Jackson et al., 2003; Logan et al., 1993). Several other integrins (αvβ5, α5β1, αβ1 and αⅡbβ3) are known to bind this motif; however, despite having an RGD, FMDV appears unable to use all of these integrins to initiate infection and evidence for αvβ5 and α5β1 as receptors has been consistently negative (Baranowski et al., 2000; Duque & Baxt, 2003; Jackson et al., 2000; Mason et al., 1993; Neff et al., 1998).

The integrin receptors of FMDV have been studied extensively in cell culture. However, the role(s) of the various integrins in determining the tissue tropism and pathogenesis of FMDV have yet to be established. For an integrin to function as a virus receptor it would appear reasonable to postulate that it should be expressed on the cell types that are normally targeted. Here, we present analyses, using immunofluorescence confocal microscopy and real-time RT-PCR, of αvβ3 and αvβ6 expression within the epithelia that are normally targeted by FMDV in cattle. These studies show that αvβ6 but not αvβ3 was expressed on the surfaces of the epithelial cells at the sites where FMDV is known to replicate at a high level during a natural infection. Expression of αvβ6 on the cells targeted by FMDV within the interdigital-skin was confirmed by showing the presence of this integrin on the surfaces of infected cells. Together, these data suggest that, in epithelia normally targeted by FMDV in cattle, (i) integrin αvβ6, but not αvβ3, serves as the major receptor that determines virus tropism, and (ii), αvβ6 is expressed constitutively at levels sufficient to allow initiation of infection.

**METHODS**

**Animals.** Eight Compton Holstein steers (UU66, UX10, UX11, VA16, VD38, VD39, V128 and V131) were housed in the bioscience facilities at the Institute for Animal Health, Pirbright, UK. Animals (V128 and V131) were infected with FMDV O UKG 34/2001, which causes a severe disease in cattle. Inoculation was by subepidermal injection of approximately 0.5 ml (6-9 log_{10} TCD_{50}) FMDV O UKG 34/2001 (Alexandersen et al., 2002c). The animals were euthanized and tissues collected from secondary lesions on the interdigital-skin at approximately 24 h post-injection.

**Antibodies.** mAb 23C6 (anti-αvβ3) was from Serotec. mAb B3A (anti-β3), mAb HB1.1 (anti-β1) and mAb 10D5 (anti-αvβ6) were from Chemicon. mAb 9EG7 was from Pharmingen. mAb 6.2A1 (anti-αvβ6) was from Biogen.

**mRNA extraction and real-time RT-PCR.** The details of the RNA extraction procedure, cDNA synthesis and real-time RT-PCR have been described previously (Alexandersen et al., 2002c; Oleksiewicz et al., 2001; Reid et al., 2002, 2003; Zhang & Alexandersen, 2003). Briefly, total cellular mRNA was extracted using the MagnaPure LC mRNA Extraction kit II (Roche) with an automated nucleic acid robotic workstation (Roche). RNA was reverse transcribed using the MultiScribe reverse transcriptase kit with random hexamers (Applied Biosystems). cDNA was then added to a TaqMan PCR mix (Applied Biosystems) containing 0.9 pmol each primer μl^{-1} and 0.2 pmol FAM-labelled probe μl^{-1}. PCR amplification was carried out as described previously (Zhang & Alexandersen, 2004). The sequences of the primers and probes for integrin amplification were: β6 forward primer, 5′-GAAGATGTGGTACAAATGCACAC-3′; β6 reverse primer, 5′-CAGAGACAAGAAAAAGCTATCC-3′; β6 probe, 5′-ACCATCAATGAAGAAACTCTGCAC-3′; β3 forward primer, 5′-GATGCGCGACGTGGTCT-3′; β3 reverse primer, 5′-GCGGAGGACACTTCTCACA-3′; β3 probe, 5′-TGTCACATTAGGACC-3′. Using β3 and β6 cDNA templates, the PCRs were shown to be specific for their target sequences as the β6 and β3 primer/probe sets were negative for the β3 and β6 templates, respectively. Similar, control reactions carried out using mRNA extracted from the tissues and not reverse transcribed were also negative in the real-time PCRs.

**Immunofluorescence microscopy.** Tissues were collected at post-mortem and dissected into ~0.5 cm^3 blocks, fixed in 4% paraformaldeyde for 1 h at room temperature and transferred to PBS at 4°C. Sections (70 μm) were prepared using a Leica vibrating microtome (Monaghan et al., 2001). For softer tissues (e.g. soft palate), sections were cut using a razor blade. Tissue sections were permeabilized in 0.1% Triton X-100 for 1 h at room temperature and blocked overnight in block buffer (PBS supplemented with 1 mM CaCl_2, 0.5 mM MgCl_2, 0.05% (w/v) sodium azide and 0.5% (w/v) BSA). Sections were incubated with the primary antibody (10 μg ml^{-1}) for 1.5 h at 37°C. Control sections were incubated either in the absence of the primary antibody or with an irrelevant matched isotype antibody. The sections were washed in PBS containing 1 mM CaCl_2, 0.5 mM MgCl_2 and incubated with a species-specific IgG conjugated to Alexa dyes (Molecular Probes) for 1.5 h at 37°C. After washing, the sections were incubated with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 30 min. The sections were then washed with water and mounted onto glass slides using Vectashield Mounting Medium for fluorescence (Vector Labs). Hand-cut tissues were mounted using Hardset Vectashield Mounting Medium (Vector Labs). Sections were viewed with a Leica TCS/SP2 confocal microscope.

**RESULTS**

**Integrin expression in uninfected epithelia**

We have investigated expression of the FMDV receptors, integrins αvβ3 and αvβ6, in the epithelium of the tongue, interdigital-skin, coronary band, flank-skin, and the dorsal and ventral soft-palate of cattle. For these studies, we have used immunofluorescence confocal microscopy and real-time RT-PCR. The β1 chain was also included in the immunofluorescence studies as β1 integrins are expressed on virtually all cell types and labelling for the β1 chain allowed the structure of the epithelium to be visualized. We also investigated expression of the β1 integrins using an antibody that binds only to the β1 chain in its active conformation.

**The β1 chain.** The β1 chain pairs with at least 12 different α chains (Hynes, 2002) and epithelial cells normally express at least three different β1 integrins, α2β1, α3β1 and α9β1. Expression of a fourth β1 integrin, α5β1 is rapidly induced by injury in most epithelia (Cavani et al., 1993; Juhasz et al., 1993; Larjava et al., 1993).

Fig. 1(a–c) shows tongue epithelium (UU66) labelled with a β1-specific antibody. The pattern of β1 expression was consistent with that reported for β1 integrins on normal
Fig. 1. Expression of the β1 and β3 chains in tongue epithelium. Tongue sections were prepared from animal UU66 (a–e) or UX10 (f) and labelled for immunofluorescence confocal microscopy. The integrins are shown in red and the cell nuclei in blue. (a and b) Labelling for β1 integrins is shown on epithelial cells of the basal layer (b) and stratum spinosum (s) and on blood vessels (arrowhead). (c) Labelling for β1 integrins is shown on the surfaces of the stratum spinosum cells. (d) Labelling for activated β1 integrins is shown on the basal surfaces of the basal cells and on blood vessels (arrowhead). β3 was detected on blood vessels (e, arrowhead) and inside the papillae (f). Bars, 100 μm (a), 50 μm (b), 10 μm (c), 20 μm (d, e) and 40 μm (f).
mammalian epithelia, β1 was detected at the highest level on the cells of the basal layer and at the edge of the papillae. Towards the outer keratinized layer, the level of integrin expression was reduced (Fig. 1a and b). Fig. 1(c) shows a higher magnification image of the cells of the stratum spinosum that lie adjacent to the basal cells. On these cells, β1 was located primarily at the cell surfaces with little or no β1 detected in the cytoplasm. The β1 chain was also detected on the vasculature including the large blood vessels of the dermis and on the capillaries located inside the papillae (Fig. 1b). The labelling shown in Fig. 1 was specific as a matched isotype antibody was negative (data not shown).

The pattern of β1 expression in tongue epithelium was similar to that shown in Fig. 1 for each of the animals investigated (VA16, UX10 and UX11; data not shown). The pattern of β1 expression on the epithelium of the interdigital-skin, coronary band, ventral soft-palate and normal flank-skin was similar to that on the tongue (data not shown). On the dorsal soft-palate, the pattern of β1 expression was also similar to the tongue, with the exception that this epithelium does not have an outer keratinized layer, and the stratum spinosum is relatively thin and does not contain papillae (Alexandersen et al., 2002b, 2003).

Fig. 1(d) shows tongue epithelium (UU66) labelled with an antibody (9EG7) that recognizes β1 integrins in their activated state. Expression of activated β1 was detected on the basal surfaces of the basal cells, and to a lesser extent on blood vessels. This pattern for activated β1 integrins was observed on all of the epithelia investigated.

The β3 chain. The β3 chain associates with two α chains, αv and αIIb, forming αvβ3 and αIIbβ3, respectively (Hynes, 2002). The integrin αIIbβ3 is expressed exclusively on platelets, whereas αvβ3 is normally expressed on endothelial cells and has been detected on certain blood vessels for several mammalian species (Damjanovich et al., 1992; Kubo et al., 2001; Mette et al., 1993; Singh et al., 2000, 2001).

Fig. 1(e and f) shows sections of tongue epithelium (UU66 and UX10) labelled with a β3-specific antibody. β3 expression was detected primarily on the large blood vessels of the dermis (Fig. 1e) and to a lesser extent on the capillaries located inside the papillae (Fig. 1f). β3 expression was not detected on the epithelial cells of the stratum spinosum. Fig. 1(e) shows that the β3 chain may be present on the basal cells of the tongue epithelium; however, the amount of β3 detected was low compared with the vasculature. Furthermore, β3 labelling on the basal cells was not detected on other tongue sections prepared from animal UU66, nor was it detected on the basal cells of the tongue epithelia of three other animals investigated. Similarly, β3 was not detected on the epithelial cells at the other sites investigated, including the interdigital-skin (UX10 and UX11), coronary band (UX10, UX11, VA16, VD38 and VD39), flank-skin (UU66, UX10 and UX11), and ventral and dorsal soft-palate (UU66, UX10, UX11, VA16, VD38 and VD39). We also failed to detect expression of αvβ3 on several of these epithelia using an αvβ3-specific antibody (mAb 23C6; data not shown).

Labelling for the β3 chain on the vasculature was predominantly weak, which may indicate low levels of protein expression. However, it is possible that the low level of β3 detection results from the anti-integrin antibodies being poorly cross-reactive for bovine integrins or from poor recognition of the integrin in paraformaldehyde-fixed tissues. Therefore, we labelled primary bovine thyroid cells with the antibodies used to detect αvβ3 (mAb 23C6) and the β3 chain (mAb B3A), following the protocols that were used to prepare the tissue for confocal microscopy. These experiments showed that both antibodies readily detected αvβ3 on the surfaces of paraformaldehyde-fixed cells (data not shown). This observation suggests that the failure to detect β3 expression on epithelial cells in fixed tissues most likely results from a low level of αvβ3 expression rather than from an artefact of tissue preparation.

The integrin αvβ6. The β6 chain pairs only with αv forming αvβ6 (Hynes, 2002). Fig. 2(a–c) shows tongue epithelium (VA16) labelled with an anti-αvβ6 antibody (mAb 10D5). In the tongue, αvβ6 was detected only on the epithelial cells and not on the vasculature, or the cells located in the dermis below the basement membrane. In contrast to the β1 chain, αvβ6 was expressed more strongly on the cells of the stratum spinosum than on the cells of the basal layer. Fig. 2(c) shows a higher magnification image of the cells in the stratum spinosum. As with the β1 chain, expression of αvβ6 was largely confined to the cell surfaces with little evidence of integrin expression in the cytoplasm. The same pattern of αvβ6 expression was observed on the tongue epithelium of the other animals investigated (UU66, UX10 and UX11). The labelling shown in Fig. 2 was specific, as labelling with a matched isotype antibody was negative for this tissue (data not shown).

The integrin αvβ6 was also detected on the epithelial cells of the interdigital-skin. The pattern of αvβ6 expression on the interdigital-skin (VA16) was similar to that seen in the tongue, with the greatest amount of integrin being detected on the cells of the stratum spinosum (Fig. 2d–f). The same pattern of αvβ6 expression was seen on the interdigital-skin epithelia of the other animals investigated (UX10 and UX11).

The integrin αvβ6 was also detected on the surfaces of the epithelial cells in the coronary band (Fig. 3a–c). The level of αvβ6 expression of the epithelial cells in the coronary band appeared to be lower compared with the tongue and interdigital-skin (compare Fig. 2 with Fig. 3) and furthermore, αvβ6 appeared to be expressed at similar levels on the cells of the stratum spinosum and basal layer. In addition, at this site, αvβ6 expression showed greater variation between animals and was detected within the coronary band of animals VD39, UX10 and VA16 (Fig. 3), but not within the coronary band of animal VD38. Expression of αvβ6 was also detected on the epithelial cells of the ventral soft-palate...
Fig. 2. Expression of αvβ6 in tongue and interdigital-skin epithelium. Tissue sections were prepared from VA16 and labelled for immunofluorescence confocal microscopy using mAb 10D5. The integrin is shown in red and the cell nuclei in blue. Tongue (a–c) and interdigital-skin (d–f). αvβ6 was detected only on epithelial cells and appeared more abundant within the stratum spinosum (s). Surface αvβ6 is shown on the stratum spinosum cells for tongue (c) and interdigital-skin (f). Bars, 60 μm (a), 25 μm (b), 10 μm (c), 50 μm (d, e) and 10 μm (f).
Fig. 3. Expression of αvβ6 on the coronary band and ventral soft-palate. Tissue sections were prepared from animal VA16 (a and d), UX10 (b), VD38 (f) and VD39 (c and e) and labelled for immunofluorescence confocal microscopy using mAb 10D5. The integrin is shown in red and the cell nuclei in blue. Coronary band (a–c) and ventral soft-palate (d–f). αvβ6 was not detected in the ventral soft-palate of animal VD38 (f). Bars, 50 μm (a), 20 μm (b), 50 μm (c), 50 μm (d), 40 μm (e) and 100 μm (f).
and its expression was also variable between animals. Integrin $\alpha_v\beta_6$ was detected within the ventral soft-palate of animals VA16 (Fig. 3d) and VD39 (Fig. 3e), but not within this tissue of animals UU66, UX10, UX11 and VD38 (Fig. 3f). In contrast to the above epithelia, $\alpha_v\beta_6$ was not detected within the dorsal soft-palate or flank-skin of any of the animals investigated (data not shown).

**Analysis of integrin mRNA.** A real-time RT-PCR was used to investigate expression of integrin $\beta_3$ and $\beta_6$ mRNA within several epithelia including the tongue (five animals), interdigital-skin (three animals), and the ventral (five animals) and dorsal soft-palate (five animals). The difference between the Ct values ($\Delta$Ct) was used to estimate the relative abundance of integrin $\beta_3$ and $\beta_6$ mRNA within each tissue. The Ct value is the cycle number at which the fluorescence of the PCR can be detected as positive, and for these experiments the positive threshold was set to intersect the exponential part of the amplification curve at 0.1. The Ct value is inversely proportional to the log amount of template in the PCR; and assuming a 100% effective PCR amplification, a difference of one Ct value corresponds to an approximate twofold difference in template amount. Using cDNA templates the efficiency of the PCR reactions for $\beta_3$ and $\beta_6$ were shown to be similar (data not shown) thus permitting the relative abundance of $\beta_3$ and $\beta_6$ mRNA to be estimated from the Ct values.

This analysis showed that the high levels of $\alpha_v\beta_6$ protein (compared with $\alpha_v\beta_3$) detected in the tongue and interdigital-skin by immunofluorescence confocal microscopy was supported by the PCR data as, at these sites, $\beta_6$ mRNA was more abundantly expressed than $\beta_3$ [$\Delta$Ct = $4.9 \pm 1.8$ (mean $\pm$ SD, n = 8)] where + indicates $\beta_6$ as the most abundant mRNA species. In contrast, at sites where little or no integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_6$) were detected (the ventral and dorsal soft-palate), $\beta_3$ and $\beta_6$ mRNAs were present at similar levels to each other ($\Delta$Ct = $0.3 \pm 1.2$, n = 10).

**Integrin expression in FMDV-infected epithelia**

The above studies show that, in cattle, $\alpha_v\beta_6$ is expressed on the epithelial cells normally targeted by FMDV. To confirm that the cells infected with FMDV express this integrin, we investigated $\alpha_v\beta_6$ expression within infected animals. Preliminary investigations indicated that the morphological structure of the tissue was not well preserved and the detection of cellular antigens was poor in advanced lesions (Monaghan *et al.*, 2005). Therefore, we used early lesion material collected from the interdigital-skin of cattle inoculated by injection in the tongue, as this route is the most effective way to establish infection in this species. This strategy enabled the appearance of early lesions on the feet to be predicted with greater accuracy.

To investigate $\alpha_v\beta_6$ expression in uninfected cattle, we used mAb 10D5. This mAb blocks the binding of natural ligands of $\alpha_v\beta_6$ and consequently also inhibits the binding of FMDV (Jackson *et al.*, 2000). Thus, we reasoned that, in animals with high viraemia, virus bound to the integrin might inhibit the binding of mAb 10D5, thereby preventing integrin detection. Therefore, to study integrin expression within infected tissues, we used a non-blocking anti-$\alpha_v\beta_6$ mAb 6.2A1 (Weinreb *et al.*, 2004).

Fig. 4 shows expression of $\alpha_v\beta_6$ within infected interdigital-skin epithelia. Fig. 4(a) shows the edge of an early lesion collected from animal V131. The cells are stained with DAPI (blue) and Alexa-488-conjugated phalloidin (green) to visualize the cell nuclei and actin cytoskeleton, respectively. The cells infected with FMDV (red) were detected using a rabbit polyclonal serum against type O FMDV, which recognizes viral capsid proteins. Infected cells were abundant throughout the stratum spinosum and the intensity of virus labelling was greatest towards the middle of the lesion compared with its edge. These observations suggest that the cells in the middle of the lesion may be at a more advanced stage of infection. It is worth recalling that in the interdigital-skin, the cells of the stratum spinosum showed the highest level of $\alpha_v\beta_6$ expression in uninfected animals. Fig. 4(c and d) shows a similar region of the early lesion from the same animal labelled for FMDV (red) and $\alpha_v\beta_6$ (green). At the edge of the lesion, $\alpha_v\beta_6$ protein was observed on the surfaces of both uninfected and infected cells. Towards the middle of the lesion, the infected cells appeared to express little or no $\alpha_v\beta_6$. At present we cannot be certain why these cells appear to express less surface $\alpha_v\beta_6$; however, it is likely that they are at a more advanced stage of infection than the cells at the edge of the lesion and the reduction in $\alpha_v\beta_6$ expression results from a combination of the poor morphology within infected samples, and the inhibition of both host-cell protein synthesis and protein secretory pathways that are characteristic of cells infected with FMDV *in vitro* (Belsham *et al.*, 2000; Moffat *et al.*, 2005).

Fig. 4(e and f) shows a similar early lesion prepared from the interdigital-skin of animal V128. At the edge of the lesion, the labelling for FMDV (red) and $\alpha_v\beta_6$ (green) were similar to that of animal V131.

The above experiments confirm that, in infected cattle, $\alpha_v\beta_6$ is expressed on the surfaces of the epithelial cells targeted by FMDV in the interdigital-skin. In contrast, using the anti-$\beta_3$ mAb we did not detect expression of the $\beta_3$ chain on the infected cells of the interdigital-skin of either animal investigated. Similarly, the $\beta_3$ chain was not detected on the uninfected cells immediately adjacent to the edge of the lesion.

We also investigated the activation status of the $\beta_1$ integrins in infected interdigital-skin epithelium. Fig. 4(b) shows that the labelling for activated $\beta_1$ integrins within the infected tissue was similar to that observed for uninfected animals. Labelling of activated $\beta_1$ integrins was detected primarily on blood vessels within the papillae and on the basal surfaces of the cells in contact with the basement membrane.
Fig. 4. Expression of $\alpha v \beta 6$ in FMDV-infected interdigital-skin. Sections were prepared from the edge of an early lesion for animals VI31 (a, c and d) and VI28 (b, e and f) and labelled for immunofluorescence confocal microscopy. The cell nuclei are shown as blue. (a) The actin cytoskeleton labelled with phalloidin (green) and infected cells (red) detected using a rabbit polyclonal serum against type O FMDV are shown. (b) Activated $\beta 1$ integrins in the epithelium of animal VI28 are shown. (c and e) $\alpha v \beta 6$ (green). (d and f) Panels (c) and (e) overlaid with the labelling for virus (red). Bars, 40 $\mu$m (a), 60 $\mu$m (b), 60 $\mu$m (d) and 40 $\mu$m (f).
DISCUSSION

The role(s) of the various integrin receptors in determining the tissue tropism and pathogenesis of FMDV have yet to be established. For an integrin to function as a receptor in an animal, we reasoned that it should be expressed on the cell types that are normally targeted. In this study, we have used immunofluorescence confocal microscopy and real-time RT-PCR to investigate the expression of integrins αvβ3 and αvβ6 within the epithelia that are normally targeted by FMDV in cattle.

In general, αvβ6 expression showed a good correlation with both the abundance of β6 mRNA and the sites of lesions in cattle. The tongue and interdigital-skin are sites where FMDV replicates to a high level and lesions normally form (Alexandersen et al., 2003). At these sites, αvβ6 protein was readily detected in the tissue and β6 mRNA was more abundantly expressed than that of β3. Within these epithelia, highest levels of αvβ6 were present on the surfaces of epithelial cells of the stratum spinosum. These are the cells targeted by FMDV in cattle (Fig. 4). Similarly, these cells are targeted in the tongue of FMDV-infected pigs (Monaghan et al., 2005).

The integrin αvβ6 was also detected on surfaces of epithelial cells within the coronary band and ventral soft-palate. However, at these sites, αvβ6 showed greater variation between animals and was detected on three of four and two of six animals for the coronary band and ventral soft-palate, respectively. These observations were reflected in the real-time RT-PCR data, as within the ventral soft-palate, β3 and β6 mRNAs were present at similar levels to each other. The variable expression of αvβ6 at these sites could result from differential integrin expression between cattle or, alternatively, from non-uniform expression of αvβ6 within the epithelium of individual animals. This latter scenario could result from upregulation of αvβ6 at sites of inflammation (see below). The variable expression of αvβ6 protein within the coronary band and ventral soft-palate is, however, consistent with the frequency of lesions at these sites. Lesions are often but not always present on the coronary band and rarely seen on the ventral soft-palate (S. Alexandersen, unpublished observation). Although it is likely that FMDV infection (and hence lesion formation) requires a number of cellular factors, it is interesting to speculate that lesion formation at these sites is dependent on expression of αvβ6.

The dorsal soft-palate and flank-skin are sites where lesions do not normally occur in FMDV-infected cattle, and at these sites αvβ6 protein was not detected. Consistent with these observations, β3 and β6 mRNAs appeared to be present at similar levels to each other within the dorsal soft-palate. However, despite being a site where lesions do not normally form, the dorsal soft-palate has attracted special interest because the epithelial cells have been shown to harbour viral RNA at early times post-infection (Alexandersen et al., 2003). Despite this evidence that the epithelial cells of the dorsal soft-palate are probably targeted by FMDV, it is puzzling why lesions do not develop at this site (Alexandersen et al., 2003). The mechanisms that underlie this apparent lack of cytopathology are unknown, but may be linked to the ability of FMDV to establish a persistent infection, as epithelial cells of the dorsal soft-palate have also been shown to harbour viral RNA in persistently infected cattle (Alexandersen et al., 2003; Prato Murphy et al., 1999; Zhang & Alexandersen, 2004; Zhang & Kitching, 2001). Although we cannot rule out the possibility that αvβ6 is expressed at low level at this site, our data suggest that infection within the dorsal soft-palate is not mediated by αvβ6 receptors.

Our studies confirmed that αvβ6 is expressed on the surfaces of cells infected with FMDV within the interdigital-skin. At the edge of the lesion, αvβ6 was seen on the surfaces of both uninfected and infected cells. Towards the middle of the lesion, the cells of the stratum spinosum showed the greatest intensity of labelling for viral proteins. Importantly, these cells had the most intense labelling for αvβ6 in uninfected tissues (Fig. 2). However, in the middle of the lesion, the infected cells appeared to express little or no αvβ6. At present we cannot be certain why these cells appear to express less surface αvβ6. However, it is likely that these cells are at a more advanced stage of infection than the cells at the edge of the lesion and consequently normal protein expression may be reduced as both host-cell protein synthesis and protein secretory pathways are inhibited by infection of FMDV in vitro (Belsham et al., 2000; Moffat et al., 2005).

Although shown to be a receptor for FMDV on cultured cells, αvβ3 was not detected on any of the epithelial cells investigated but was detected on the vasculature (Fig. 1). This observation is in agreement with studies of other mammalian species, which have concluded that αvβ3 normally predominates in endothelial rather than epithelial cells (Breuss et al., 1993, 1995; Clark et al., 1996; Damjanovich et al., 1992; Mette et al., 1993; Singh et al., 2000, 2001). However, although we did not detect expression of αvβ3 on epithelial cells, it should be acknowledged that expression of αvβ3 has been reported for epithelial cells lining the bile duct and small intestine of pig and cattle (Singh et al., 2001). With this in mind, we cannot rule out the possibility that αvβ3 is expressed on the epithelial cells investigated but at levels below that can be detected using our methods. Similarly, we cannot completely exclude a role for αvβ3 as a receptor at later stages of infection.

Integrin expression is upregulated during processes such as wound healing and inflammation (Clark et al., 1996; Gailit et al., 1994; Haapasalmi et al., 1995, 1996; Hakkinen et al., 2000; Larjava et al., 1993; Pilewski et al., 1997; Wang et al., 1996; Zambruno et al., 1995). This phenomenon suggests that integrin expression on epithelial cells could increase on FMDV infection. Although our data are limited to the interdigital-skin, we did not detect noticeable changes in the level of αvβ6 protein on the cells at the edge of the lesion, perhaps because within the interdigital-skin, αvβ6 is...
expressed constitutively at a high level. Similarly, we did not observe upregulation of \( \alpha v \beta 3 \) on the cells at the edge of the lesion. These observations suggest that within the interdigital-skin the integrins \( \alpha v \beta 3 \) and \( \alpha v \beta 6 \) are not upregulated on epithelial cells at sites of infection.

**In vivo**, many different integrin species are likely to be activated as a result of infection (Burns et al., 2001; Gonzalez-Amaro et al., 1998; Springer, 1990) and for viruses, such as FMDV, this activation could effectively ‘switch-on’ receptors for gaining entry into cells. Therefore, we investigated changes in \( \beta 1 \) integrin activation of infected interdigital-skin using mAb 9EG7. mAb 9EG7 recognizes \( \beta 1 \) integrins that are in their active state (Bazzoni et al., 1998). Using this mAb we did not detect noticeable changes in activated \( \beta 1 \) integrins on infection as, in both uninfected and infected cattle, activated \( \beta 1 \) integrins were detected primarily on the basal membrane of the basal cells and on blood vessels.

Two other \( \alpha v \) integrins, namely \( \alpha v \beta 1 \) and \( \alpha v \beta 8 \) (Jackson et al., 2002, 2004) have been shown to serve as receptors for FMDV *in vitro*. The integrin \( \alpha v \beta 8 \) has been shown to be expressed on human airway epithelial cells (Cambier et al., 2000; Fjellbirkeland et al., 2003). However, we have not been able to study this integrin in cattle, as cross-reactive antibodies for bovine \( \alpha v \beta 8 \) that can be used with fixed tissues have not been identified. Similarly, we could not study the tissue distribution of \( \alpha v \beta 1 \), since antibodies specific for the \( \alpha v \beta 1 \) heterodimer are currently not available.

At present we cannot be certain what role the various integrin receptors play in FMDV tropism and pathogenesis. However, our data show that \( \alpha v \beta 6 \) is expressed constitutively at high levels on the epithelial cells normally targeted by FMDV in cattle and suggest that \( \alpha v \beta 6 \), rather than \( \alpha v \beta 3 \), is the major receptor that determines the tropism of this virus.

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FMDV-receptor expression in bovine epithelia


