Endothelial expression of the \( \alpha 6\beta 4 \) integrin is negatively regulated during angiogenesis

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Accepted 23 May 2003

Journal of Cell Science 116, 3771-3781 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00681

Summary

Development and homeostasis of the vascular system requires integrin-facilitated cellular adhesion, migration, proliferation and survival. A specific role for the \( \alpha 6\beta 4 \) integrin in the vasculature, however, has not been identified. Using immunohistochemistry, we observed \( \alpha 6\beta 4 \) expression on the dermal microvasculature of human foreskin. Analysis of individual cells isolated from trypsin-disrupted foreskin tissue indicated that \( \alpha 6\beta 4 \) was expressed by a subset of epithelial and endothelial cells, and not by smooth muscle cells. Expression of \( \alpha 6\beta 4 \) was also analyzed during new vessel growth using explants of human saphenous vein cultured in fibrinogen gels. The results indicate that \( \alpha 6\beta 4 \) is not expressed by outgrowing endothelial cells, and is downregulated by the original \( \alpha 6\beta 4 \)-positive endothelial cells of the explant. To determine whether \( \alpha 6\beta 4 \) is expressed during angiogenesis in vivo, the expression of the \( \beta 4 \) subunit was analyzed during the development of the mouse mystacial (whisker) pad.

Immunohistochemical staining of the whisker pad indicates that \( \beta 4 \) is expressed by the adult vasculature. To identify when and where \( \beta 4 \) is turned on in the vasculature, we examined the whisker pads from the developing embryo (E19.5 pc), and from postnatal days zero (P0), three (P3) and seven (P7) pups. The expression of \( \alpha 6\beta 4 \) was found to be turned on spatially and temporally from caudal to rostral regions and from the deep to superficial vasculature, correlating with the maturation of the whisker pad and its corresponding vasculature. Together, these findings suggest a potential role for \( \alpha 6\beta 4 \) as a negative component of the angiogenic switch, whereas expression of \( \alpha 6\beta 4 \) on the adult vasculature may indicate regions requiring additional adhesive mechanisms.

Key words: Endothelial cells, Integrins, \( \alpha 6\beta 4 \), Angiogenesis, Development, Explant cultures, Schwann cells

Introduction

The vascular system is responsible for the nutrient and oxygen supply of an organism. It develops initially via vasculogenesis, and subsequently through angiogenic remodeling (Flamme et al., 1997). Angiogenesis involves the sprouting of endothelial cells from the existing vasculature, by a process that requires the migration and proliferation of endothelial cells, resulting in new vessel formation (Carmeliet, 2000). Stromal cells destined to become smooth muscle cells are recruited, ensheathing vessels except the capillaries. The end product is a fully developed vasculature, in which the cells are differentiated and quiescent, with endothelial cells stably adhering to their underlying basement membrane (Carmeliet, 2000).

Throughout these processes, integrins play important roles in the development and maintenance of the vasculature (Hynes et al., 1999), by regulating cell adhesion, migration, proliferation and survival (Albelda et al., 1991; Giancotti and Ruoslahti, 1999; Pinter et al., 1997).

Integrins are \( \alpha \beta \) heterodimeric transmembrane proteins, and the specific combination of \( \alpha \) and \( \beta \) subunits determines ligand-binding specificity (Hynes, 1992). Endothelial cells express several different integrins, whose roles in regulating endothelial cell adhesion, migration, proliferation and apoptosis have been examined (Bazzoni et al., 1999; Hynes et al., 1999). The \( \alpha v \) integrins have received most attention, because they are upregulated during angiogenesis (Brooks et al., 1994; Clark et al., 1996) and blocking their function with antagonists can inhibit angiogenesis (Clark et al., 1996; Eliceiri and Cheresh, 1999). However, the role of \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \) is more complex than earlier thought, since mice lacking \( \alpha v \beta 3 \) or \( \alpha v \beta 5 \) integrins show normal vascular development and enhanced pathological angiogenesis, suggesting that the \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \) integrins may, in fact, negatively regulate the angiogenic process (Reynolds et al., 2002). The \( \alpha 5 \beta 1 \) integrin may play an important role in regulating new vessel growth, as demonstrated by \( \alpha 5 \)-null mice that die during embryogenesis as a result of defects in vascular development (Yang et al., 1993). Blocking the function of \( \alpha 5 \) integrins can also inhibit angiogenesis (Kim et al., 2000). Roles for other integrin heterodimers in new vessel growth have also been described (Bazzoni et al., 1999; Hynes et al., 1999; Senger et al., 1997; Senger et al., 2002). Additionally, several studies have reported the expression of \( \alpha 6\beta 4 \) in the vasculature (Enenstein and Kramer, 1994; Kennel et al., 1992; Koukoulis et al., 1991; Ryynanen et al., 1991); however, there have been conflicting reports as to whether it is expressed by endothelial cells or smooth muscle cells (Cremona et al., 1994).
unclear, its function in epithelia is well characterized. In keratinocytes, α6β4 is required for maintaining firm epithelial adhesion to the underlying dermis (Dowling et al., 1996; van der Neut et al., 1996) by connecting the laminin-containing basement membrane with the intracellular keratin intermediate filaments (Borradori and Sonnenberg, 1999). In some carcinoma cells, however, α6β4 promotes migration by activating specific signaling pathways and interacting with the actin cytoskeleton (Mercuro et al., 2001; Trusolino et al., 2001). The function of α6β4 in the vasculature may be analogous to its function in epithelial and carcinoma cells; α6β4 may confer a promigratory phenotype in response to angiogenic stimuli, and also contribute to stable adhesion in the mature vasculature.

In this study, we were interested in determining whether the α6β4 integrin functions in the vasculature to promote angiogenesis. In initial studies, we confirmed the expression of α6β4 on dermal microvascular endothelial cells in situ. We did not observe expression of α6β4 on smooth muscle cells. Additional experiments examined whether the expression of α6β4 was regulated during the formation of new vessels. Explant angiogenesis assays, using segments of human saphenous vein, demonstrated that endothelial cells downregulate α6β4 in the explant and that outgrowing endothelial cells also do not express α6β4. Additionally, we did not observe expression of α6β4 during early vascular development of the murine whisker pad, but α6β4 was expressed by the same vasculature of the adult animal. The developmental expression of α6β4 correlated spatially and temporally with the maturation of the whisker pad and its corresponding vasculature. Taken together, our studies indicate that α6β4 is not expressed during new vessel growth in our assays. This result implies the α6β4 integrin may be a negative component of the angiogenic switch.

**Materials and Methods**

**Antibodies**

The following antibodies were used: mouse monoclonal antibody (mAb) 3E1 (Chemicon International, Temecula, CA) and rat mAb 439-9B (BD Biosciences, San Diego, CA) to the human β4 subunit; rat mAb 346-11A to the mouse β4 subunit (Kennel et al., 1986) (a gift from Dr Stephen Kennel, Oak Ridge National Laboratory, TN); rabbit polyclonal antibody (pAb) to human von Willebrand Factor (Accurate Chemical & Scientific Corporation, Westbury, NY); rabbit pAb to human PECAM-1 (Albelda et al., 1991) (a gift from Dr Albrecht, University of Pennsylvania Medical Center, PA); rabbit anti-mouse PECAM-1 (Pinter et al., 1997) (a gift from Dr J. Madri, Yale University School of Medicine); rat mAb MEC13.3 to mouse PECAM-1 (BD Biosciences); mouse mAb IAF to human smooth muscle actin (Sigma, St Louis, MO); mouse mAb AE1/AE3 to human cytokeratin (Sigma); and rabbit pAb to human s100A and s100B (Serotec, Raleigh, NC). The secondary antibodies used were Alexa 488- or Alexa 546-conjugated goat anti-mouse, anti-rat and anti-rabbit IgG, CY3-conjugated donkey anti-rat IgG, FITC-conjugated goat anti-rat IgG adsorbed against mouse, TRITC-conjugated goat anti-mouse IgG adsorbed against rat (Jackson ImmunoResearch Laboratories, West Grove, PA), and the biotin-conjugated antibodies goat anti-rat IgG (Jackson ImmunoResearch Laboratories), goat anti-mouse IgG (Molecular Probes) and goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). When double staining with primary antibodies 3E1 (mouse IgG1) together with IAF (mouse IgG2a) the isotype-specific secondary antibodies used were Alexa 488- and Alexa 546-conjugated goat anti-mouse IgG1 and IgG2a, respectively (Molecular Probes, Eugene, OR).

**Isolation of primary cells from human neonatal foreskin**

Human neonatal foreskin, obtained under Albany Medical College IRB approved protocols, was cut into small pieces (~1×1 mm3) and placed in 0.25% Trypsin in PBS for 16-20 hours at 4°C. The supernatant was filtered through cheesecloth and centrifuged at 435 g at 4°C for 5 minutes. The cells were resuspended in PBS and plated into 6-well plates at 5-10×10⁴ cells/well. Each well contained coverslips precoated with poly-l-lysine (10 µg/ml in PBS for 2 hours at 37°C, washed 3× with PBS). The 6-well plates were centrifuged at 560 g for 5 minutes at 4°C, and the attached cells were fixed with 4% paraformaldehyde in PBS for 30 minutes. The cells were washed 3× with PBS, then immunostained.

**Explant cultures**

Explant cultures were prepared with slight modifications from a previously published method (Nicosia and Madri, 1987; Nicosia and Ottinetti, 1990). A segment of human saphenous vein, obtained under Albany Medical College IRB approved protocols, was cut into cross sections approximately 1 mm thick. The sections were washed 10× with PBS, and then placed onto a solidified gel of fibrinogen (Sigma) in 24-well plates. The gel was prepared by adding 3 mg of fibrinogen to 1 ml of EBM (Clonetics, San Diego, CA), and gelled by the addition of 1 unit/ml of thrombin (Sigma). The vein was positioned in the center of the well on top of the fibrinogen gel, and overlaid with additional fibrinogen to which thrombin had been added immediately prior to use. The gel was allowed to solidify, and EBM containing 15% serum, 100 units/ml penicillin/streptomycin, 1 µg/ml hydrocortisone (Sigma), 10 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA) was added on top. The culture medium was changed every other day with additions of the fibrinolytic inhibitor ε-amino-n-caproic acid (Sigma) for the first 4 days at 300 µg/ml, then subsequently at 50 µg/ml. Human neonatal foreskin explants were placed directly onto coverslips in 6-well plates, and medium changed as above. Saphenous vein explants were fixed (as above) for 4 hours at 4°C then washed 3× for 5 minutes with PBS, cryoprotected in 30% sucrose in PBS overnight at 4°C, dehydrated by passage through 75% ethanol and then 100% OCT for 24 hours each, and then cut into 10-12 µm sections. Human saphenous vein, tissue and explant, exhibited high intensity broad-spectrum autofluorescence and therefore we used immunoperoxidase staining, yielding colored reaction products. Human foreskin explants were fixed for 30 minutes, then immunostained.

**Preparation of mouse mystacial (whisker) pads**

Swiss Webster mice were purchased from Taconic. Adult, timed pregnancy mice and neonates were euthanized and the mystacial pads were removed and post-fixed and cryoprotected as for the explants. All procedures were in accordance with Albany Medical College IACUC approved protocols.

**Immunostaining**

Sectioned tissue was dehydrated in PBS, permeabilized with 0.5% Triton X-100 in PBS, blocked with 3% BSA-glycine, and then preincubated with 10% normal goat serum (Pierce, Rockford, IL) in PBS. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS containing 0.5% BSA, 10% goat serum and 0.1% Tween 20. Sections were washed and incubated with the appropriately labeled fluorescent secondary antibodies, and then washed and mounted in Anti-Fade (Molecular Probes). Cells on coverslips were permeabilized, blocked with BSA-glycine, then
incubated in primary antibody for 1 hour at room temperature, but otherwise treated as above. For immunoperoxidase staining, sections were rehydrated and endogenous peroxidase quenched by incubating in 3% 
$\text{H}_2\text{O}_2$ in $\text{H}_2\text{O}$ for 10 minutes, then rinsed 2× for 1 minute in distilled $\text{H}_2\text{O}$. Sections were permeabilized and incubated with primary antibody and washed as above, and then incubated with the appropriate biotinylated secondary antibodies for 30 minutes, followed by incubation with Vectastain ABC Elite reagents, and developed with the peroxidase substrate DAB reagents (Vector Laboratories, Burlingame, CA) as described by the manufacturer. Sections were dehydrated in a graded series of ethanol washes, cleared in xylene and mounted with Permount (Fisher, Pittsburgh, PA). Controls were as above. In some instances, tissue sections were rehydrated in PBS, incubated for 1 minute in Gills Hematoxylin No. 2 (Polysciences, Warrington, PA), followed by 3 minutes in tap water, 3 minutes in Scotts solution (24 mM NaHCO$_3$, 166 mM MgSO$_4$ in $\text{H}_2\text{O}$), 3 minutes in tap water, 10 seconds in Eosin-Y (Richard Allen Scientific, Kalamazoo, MI), 3 minutes in tap water, dehydrated and mounted as above. Immunostained material was visualized with either an Olympus BX60 microscope with an attached Spot camera and associated software, or with an Olympus AX70 with a Sony DKC-ST4 Digital Photo Camera using Northern Eclipse software. Immunofluorescently labeled sections were also visualized on a Noran Oz confocal laser scanning microscope interfaced with a Nikon Diaphot 200 inverted microscope equipped with a PlanApo ×60, 1.4 NA oil-immersion objective. Controls for all immunofluorescent staining in the absence of primary antibody showed no significant fluorescence over tissue or cellular autofluorescence.

Results
The $\alpha 6\beta 4$ integrin is expressed by microvascular endothelial cells

Reports from several laboratories have described the expression of the $\alpha 6\beta 4$ integrin on a subset of blood vessels; however, there have been conflicting reports as to whether the $\alpha 6\beta 4$ integrin is expressed by endothelial or vascular smooth muscle cells (Cremona et al., 1994; Enenstein and Kramer, 1994; Kennel et al., 1992; Koukoulis et al., 1991; Mechtersheimer et al., 1994; Ryynanen et al., 1991). In our initial studies, we used sections of human neonatal foreskin to examine the expression of $\alpha 6\beta 4$ in the dermal vasculature by fluorescent immunohistochemistry. As shown in Fig. 1, the $\beta 4$ subunit colocalized with the endothelial marker von Willebrand Factor (vWF) (Fig. 1C, yellow) and the pan-endothelial marker PECAM-1 (data not shown). Colocalization was also observed between $\beta 4$ and its only known integrin subunit partner $\alpha 6$ (data not shown) (Hynes, 1992). As expected, expression of $\alpha 6\beta 4$ was detected on the basal keratinocytes, which served as a positive internal control. In addition, the analysis of $\beta 4$ expression on cross sections of vessels, viewed at high magnification by confocal microscopy, suggested that $\alpha 6\beta 4$ localized at the basal surface of the endothelial cells, in contrast to vWF, which is mostly intracellular (Fig. 1D).

To more precisely characterize the expression of $\beta 4$ integrins on vascular cells, individual cells isolated from trypsin-disrupted human neonatal foreskin tissue were adhered to polylysine-coated coverslips and used for immunocytochemistry. The cell-type expression of $\alpha 6\beta 4$ was examined by determining whether $\beta 4$ was co-expressed with epithelial-, endothelial-, and/or smooth muscle cell-specific markers. These studies indicated that $\beta 4$ integrin was expressed by endothelial cells as identified either by PECAM-1 (Fig. 1E) or vWF expression (data not shown). Some endothelial cells did not show $\beta 4$ expression (not shown), which is consistent with published findings that $\alpha 6\beta 4$ is expressed by a subset of vessels (Mechtersheimer et al., 1994). We did not observe the co-expression of $\beta 4$ with smooth muscle actin (Fig. 1G), suggesting that $\beta 4$ may not be expressed by smooth muscle cells as previously reported (Cremona et al., 1994). As expected only a subset of cytokeratin-positive cells expressed...
β4 (Fig. 1I), since α6β4 is known to be expressed by basal keratinocytes and not by cells of the suprabasal layers (De Luca et al., 1990). Interestingly, the expression of β4 remained polarized in both endothelial cells and keratinocytes dissociated from foreskin tissue (Fig. 1E,I).

Fig. 2. β4 integrin is expressed by endothelial cells of the vasa vasorum but not by endothelial cells lining the lumen of human saphenous vein. (A) Frozen section of human saphenous vein stained with Hematoxylin and Eosin; the asterisk indicates the lumen of the saphenous vein. The dashed box shows an example of the region chosen for higher magnification immunohistochemical imaging in B-E. Cross sections of saphenous vein were stained with antibodies to vWF (B), marking endothelial cells lining the main lumen (arrow) and the vasa vasorum (arrowheads) and 3E1 to β4 (D), selectively staining endothelial cells of the vasa vasorum (arrowheads). (C,E) Control immunostaining in the absence of primary antibody, for B and D, respectively. Scale bars: (A) 500 μm; (B-E) 200 μm.

The α6β4 integrin is negatively regulated by angiogenic endothelial cells in explant culture

To begin to understand the function of α6β4 in the vasculature, we investigated whether the expression of α6β4 was regulated during new vessel growth. To determine this, we performed explant angiogenesis assays using segments of human saphenous vein (Kruger et al., 2000; Slomp et al.,...
The α6β4 integrin in the vasculature

1996) which have been successfully used in these types of assays. When we examined β4 expression in cross sections of saphenous vein, we found it was expressed by endothelial cells of the vasa vasorum, but not by endothelial cells lining the main lumen of the saphenous vein (Fig. 2A-E). To determine whether the expression of β4 integrins is regulated by outgrowing endothelial cells, human saphenous vein explants were cultured in fibrinogen gels as described by others (Kruger et al., 2000; Nicosia and Ottinetti, 1990; Slomp et al., 1996). Endothelial cell outgrowth was observed from the explant after 7 days with robust growth observed after 14 days. At day 14, most of the cells were arranged in tube-like structures that resembled vessels. Day-14 explants were immunostained to confirm endothelial outgrowth with vWF (Fig. 3A,B) and PECAM-1 (data not shown). Virtually all cells in the outgrowth stained positive with vWF and PECAM-1, with almost none of the outgrowth positive for smooth muscle actin (Fig. 3E,F). Interestingly, β4 expression was not observed on outgrowing endothelial cells, or on endothelial cells remaining in the vasa vasorum of the explant at day 14 (Fig. 3C,D). This was also true of α6 (data not shown).

To exclude the possibility that the outgrowing endothelial cells were originating only from the luminal endothelial cells of the explant as opposed to the β4-positive endothelial cells present in the vasa vasorum, the endothelial lining of the lumen was stripped by collagenase digestion, leaving the endothelial cells of the vasa vasorum intact as determined by PECAM-1 staining (Fig. 4A) and vWF (data not shown). Collagenase digestion did not affect the expression of β4 in the vasa vasorum (Fig. 4B). Explant assays were performed using collagenase-treated and untreated saphenous vein segments. A phase image of day-14 explants that had not been treated with collagenase showed normal outgrowth in the vessel lumen (Fig. 4C). In contrast, there was significantly diminished outgrowth into the lumen of the collagenase-treated explant (Fig. 4D), whereas the outgrowth of endothelial cells from the vasa vasorum of the vessel was unaffected (Fig. 4E). Immunostaining of collagenase-treated explants showed endothelial-positive staining for PECAM-1 (Fig. 4F,G) and vWF (data not shown). Similar to the outgrowth from untreated tissue, β4 expression was also downregulated both in the endothelial outgrowth and the original explant at day 14 (Fig. 4H,I). This was also true of α6 expression (data not shown). These results indicate that endothelial cells downregulate α6β4 expression during new vessel growth in culture.

To confirm that the loss of endothelial expression of β4 integrins was due to cell-type specific regulation and was not
an artifact of our outgrowth assay, explants of human neonatal foreskin tissue were similarly cultured, since migrating and proliferating basal keratinocytes are known to maintain β4 expression (Gipson et al., 1993; Larjava et al., 1993; Mercurio et al., 2001; Nguyen et al., 2000). The outgrowing cells maintained an epithelial appearance, and their identity as keratinocytes was confirmed by immunohistochemical staining with an epithelial cytokeratin marker (Fig. 4J). Dual labeling showed that the outgrowing keratinocytes maintained their expression of β4 integrins, which appeared in a punctate pattern typical of hemidesmosomes. This result indicates that outgrowing endothelial cells in explant culture down-regulate β4 expression in a cell-type specific manner.

Expression of α6β4 is temporally and spatially regulated during vascular development

Since the expression of some genes can be altered when cells are placed in culture (Antequera et al., 1990; St Croix et al., 2000) and this could potentially occur in a cell-type-dependent manner, we were interested in determining whether α6β4 was expressed by newly forming vessels in situ. To accomplish this,
we analyzed the expression of \( \alpha 6 \beta 4 \) in newly forming vasculature using the developing murine mystacial (whisker) pad as a model. This model was chosen because the whisker pad has a well described and predictable vascular architecture shown schematically in Fig. 5A (Fundin et al., 1997b). Initial studies demonstrated the expression of \( \beta 4 \) integrins in the vasculature of the adult whisker pad (Figs. 5, 8). Because \( \beta 4 \) may be expressed on endothelial cells, perineurial cells and Schwann cells within the dermis (Niessen et al., 1994) and because of limitations in the availability of immunological reagents for murine tissue, we analyzed the expression of \( \alpha 6 \beta 4 \) by double-immunofluorescence staining of neighboring sections, using either antibodies to PECAM-1 and to s100, a marker for Schwann cells (Kligman and Hilt, 1988; Zimmer et al., 1995), or antibodies to \( \beta 4 \) and s100. [Note that although s100 expression is concentrated in Schwann cells, it also labels perineurium, fat cells, and chondrocytes to varying degrees (Zimmer et al., 1995).] Endothelial staining with PECAM-1 antibodies was observed throughout the different vascular beds of the whisker follicle (Fig. 5B). This staining was distinct, compared to s100 expression. In the neighboring section, \( \beta 4 \) staining was prominent on the basal keratinocytes and epithelial invaginations of both hair and whisker follicles, which served as a positive control (Fig. 5C). \( \beta 4 \) was also expressed by the Schwann cells and perineurium of nerves that were identified by s100 labeling. \( \beta 4 \) was expressed on endothelial cells of most vessels supporting the various whisker pad sites (Fig. 5C,E,I). This was confirmed by double-label immunofluorescence showing the colocalization of \( \beta 4 \) and PECAM-1 (Fig. 8A-C). Interestingly, \( \beta 4 \) was lacking on a set of vessels affiliated with papillary muscle slings (Fig. 5G).

To examine \( \beta 4 \) expression during the development of the vasculature, the whisker pad from E19.5 embryos (where E20 corresponded with parturition) was analyzed similarly to the adult tissue shown in Fig. 5. In general, \( \beta 4 \) expression was not detected in the embryonic microvasculature when neighboring sections were stained with antibodies to PECAM-1 and \( \beta 4 \). However, some endothelial \( \beta 4 \) expression was observed in the caudal regions (Fig. 6A,B) and deep vasculature (Fig. 6C,D). Double labeling with \( \beta 4 \) and \( \alpha VWF \) supported this observation (data not shown). \( \beta 4 \) was also not coexpressed with s100, indicating that \( \beta 4 \) is not expressed by Schwann cells at this time during the development of the whisker pad (Fig. 6A,B).

Induction of \( \beta 4 \) expression in the whisker pad vasculature was analyzed at postnatal day (P), zero (P0, equivalent to E20), P3 and P7. At P0, \( \beta 4 \) expression could be seen on the vasculature in the caudal most region, and also in deeper regions of the tissue (Fig. 7B). However, at this time point, large regions of the vasculature still remained negative for \( \beta 4 \) expression, such as between all of the whisker follicles (Fig. 7A,B). At P3, \( \beta 4 \) expression was observed in the capillaries that lie between the facial muscles, again caudal and deep to the developing whisker pad (Fig. 7C,D). Using double-label immunofluorescence for \( \beta 4 \) and PECAM-1 expression, little or no detectable endothelial \( \beta 4 \) expression was observed on vessels between the whisker follicles (not shown) or between developing hair follicles in the upper dermis (Fig. 8D,E,F). By P7, the vasculature associated with the follicles of the whisker pad had started to express \( \beta 4 \) (Fig. 7E,F). It should also be noted that the expression of \( \beta 4 \) in the peripheral nervous system (perineurial sheaths and Schwann cells) and \( \beta 4 \) expression in the vasculature showed a similar pattern of progression from caudal to rostral and deep to superficial regions during the development of the whisker pad (Fig. 7B). Together these data suggest that the temporal and spatial progression of the \( \beta 4 \) integrin in the vasculature may correlate with vascular maturation in the whisker pad.

**Discussion**

Our studies focused on understanding the expression of the
The mechanisms regulating the expression of αβ4 in endothelial cells are poorly understood. Our data suggests that signals known to drive the angiogenic process, such as hypoxia, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) signaling (Carmeliet, 2000), may negatively impact on the expression of αβ4 in endothelial cells. In contrast, the expression of the αv, α1 and α2 integrins is positively regulated by angiogenic signals (Brooks et al., 1994; Senger et al., 1997). This is likely to be physiologically important because endothelial cell migration and invasion associated with angiogenesis is believed to occur through a collagen I-rich matrix (Senger et al., 1997) or a provisional matrix rich in fibronectin and vitronectin, which are ligands for these integrins (Senger, 1996). Interestingly, bovine adrenal cortex endothelial cells upregulate the expression of α6β4 in culture when stimulated with bFGF, suggesting that α6β4 may also promote angiogenesis in a tissue-specific manner (Klein et al., 1993). This is consistent with the observation that matrices rich in laminin 1, a known ligand for α6β4, induce the morphological differentiation of endothelial cells into capillary-like structures in culture (Grant and Kleinman, 1997).

Our data further suggests that signals promoting vessel maturation may positively regulate the expression of α6β4 by endothelial cells. For example, angiogenic vessels are known to undergo stabilization and maturation by mechanisms that require interaction between endothelial and mural cells, and the localized activation of TGFβ, resulting in the production of extracellular matrix components by endothelial cells (Folkman and D’Amore, 1996; Hellstrom et al., 2001; Hirschi and D’Amore, 1996; Neubauer et al., 1999; Nicosia and Madri, 1987; Shanker et al., 1999). Future studies in our laboratory will address these questions.

The expression of α6β4 by mature vessels, and its absence in newly developing vessels suggests that α6β4 may only be required after vessel maturation. This is supported by the fact that α6β4 integrin is a receptor for several laminin isoforms (Borradori and Sonnenberg, 1999; Lee et al., 1992), and although the isoforms expressed in the vasculature are not fully characterized, the endothelial basement membrane is known to be rich in laminins (Colognato and Yurchenco, 2000). Thus, α6β4 may be required for endothelial adhesion to the underlying basement membrane to promote the integrity of mature vessels. Consistent with this notion, previous studies from our laboratory suggest that α6β4 may uniquely contribute to endothelial cell adhesion by forming a transmembrane link between the basement membrane and the vimentin intermediate filament cytoskeleton (Homan et al., 1998).
integrins may negatively modulate angiogenic processes. This is consistent with recent reports that suggested that some needs to be downregulated at the onset of new vessel growth. Our findings that endothelial-specific downregulation of α6β4 during endothelial outgrowth contrasts with the known role of α6β4 in certain carcinoma cells where it promotes migration and invasion (Gambaletta et al., 2000; Mercurio et al., 2001; Trusolino et al., 2001). Basal keratinocytes also maintain expression of α6β4 during re-epithelialization (Gipson et al., 1993; Larjava et al., 1993; Mercurio et al., 2001; Nguyen et al., 2000). These differences in cell-type specific regulation of α6β4 may reflect the essential requirement of the epidermal layer to be firmly attached to the underlying basement membrane by the basal keratinocytes through their expression of α6β4. This is illustrated by the extensive detachment of the epidermal-dermal interface in neonatal β4 knockout mice (Dowling et al., 1996; van der Neut et al., 1996), and the microblistering phenotype observed in the skin of patients with Epidermolysis Bullosa, which is the result of mutations in β4 (Pulkkinen and Uitto, 1999). Together this strongly implies that even temporary downregulation of α6β4 expression by basal keratinocytes during re-epithelialization may compromise skin integrity, whereas remodeling vessels do not have this same requirement for maintained α6β4 expression.

Also, in contrast to keratinocytes, Schwann cells do not express β4 during in vitro migration except when they begin ensheathing/myelinating axons (Eiheber et al., 1993; Niessen et al., 1994; Previtali et al., 2001). Our data indicates that β4 is not expressed by Schwann cells during embryonic development of the whisker pad, but is detected on Schwann cells at P0, as reported by others (Feltri et al., 2002). This expression pattern of β4 on perineural cells and Schwann cells, appears similar temporally and spatially to that observed on endothelial cells. Thus, similar signaling and/or transcriptional mechanisms may regulate β4 expression in Schwann cells and endothelial cells. Our findings that α6β4 is not expressed on newly forming vessels is consistent with, and may in fact explain, the lack of a gross pathological phenotype in the vasculature of the β4-null mice (Dowling et al., 1996; van der Neut et al., 1996). Our results also suggest that endothelial expression of α6β4 may be a negative component of angiogenesis, and that its expression needs to be downregulated at the onset of new vessel growth. This is consistent with recent reports that suggested that some integrins may negatively modulate angiogenic processes (Reynolds et al., 2002; Stupack et al., 2001), perhaps by inhibiting endothelial growth and even triggering apoptosis when the appropriate ligands are unavailable.

Additional studies are needed to fully understand the role of α6β4 in the vasculature. A vascular-specific conditional knockout would be valuable in this regard. Even with this in hand, if α6β4 functions together with other adhesion molecules to promote vascular integrity, a vascular phenotype may only be observed after vascular challenge, such as intense cardiovascular activity or an inflammatory response. Based on our findings, we propose a novel function for α6β4 as a potential negative regulator of angiogenesis in some instances. Future experiments will test this hypothesis by determining whether forced vascular expression of β4 integrins significantly inhibits angiogenesis and/or vascular development. Additionally, it will be important to determine whether α6β4 is also negatively regulated during angiogenesis associated with wound healing and tumor vascularization, since normal and pathological angiogenesis can involve different mechanisms (Carmeliet et al., 2001; Carmeliet and Jain, 2000; St Croix et al., 2000). Finally, if expression of β4 can be anti-angiogenic, it will become important to determine the molecular mechanisms involved, since such studies may have potential therapeutic value in controlling pathological angiogenesis in specific tissue environments.

We thank Drs S. Kennel, S. Albelda, and J. Madri for generously providing antibodies to β4 and PECAM-1, Drs J. Sottile, C. M. DiPersio, L. Van De Water and A. Berrier for critically reading this manuscript, and Debbie Moran for her assistance during the preparation of this manuscript. This work was funded by grants from the American Heart Association/New York State Affiliate and the National Institute of General Medical Sciences to S.E.L., from the National Institute of Neurosciences (NS-34692) and the Albany Medical College Strategic Research Plan to F.L.R., and from the National Center for Research Resources (RR-12894-01A1) to J.E.M.

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Fig. 8. Confocal microscope images showing double immunostaining of microvascular endothelial cells for β4 (red) and PECAM-1 (green). Each image is a maximum intensity projection image of a sub-stack of six optical sections taken from a larger stack of images collected at 0.3 μm intervals. The images were collected in the region just below the epidermal-dermal junction from sections of skin from adult (A-C) and P3 (D-F) mice. In the adult, almost all the vessels in this region showed immunoreactivity for β4 and PECAM-1 (A-C, arrowheads). At P3, vessels expressing β4 were rare (D-E, solid arrowhead), most lacked β4 (open arrowheads). No vessels in this location showed β4 immunoreactivity at P0 (not shown). Arrows indicate β4 immunoreactivity on basal keratinocytes in the epidermis (e) and hair follicles (f). Scale bar: 25 μm.
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