Journal of Cell Science 108, 2655-2661 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

An antagonist of integrin $\alpha_v\beta_3$ prevents maturation of blood vessels during embryonic neovascularization

Christopher J. Drake[1], David A. Cheresh[2] and Charles D. Little[1,*]

[1]The Department of Cell Biology and the Cardiovascular Developmental Biology Center, The Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA
[2]Departments of Immunology and Vascular Biology, The Scripps Research Institute, LaJolla, CA, USA

*Author for correspondence (e-mail: Charles_Little@smtpgw.musc.edu)

SUMMARY

Experimental data in this study demonstrate that integrin $\alpha_v\beta_3$ is fundamentally involved in the maturation of blood vessels during embryonic neovascularization (vasculogenesis). Integrin $\alpha_v\beta_3$ was specifically expressed on the surface of angioblasts during vessel development in quail embryos and vitronectin, a ligand for $\alpha_v\beta_3$, localized to the basal surface of these cells. More importantly, microinjection of the anti-$\alpha_v\beta_3$ monoclonal antibody, LM609, disrupted the normal pattern of vascular development. After exposure to LM609 the angioblasts in experimental embryos appeared as clusters of rounded cells lacking normal cellular protrusions. This led to disruption of lumen formation and abnormal vessel patterning. These findings demonstrate that during vasculogenesis ligation of integrin $\alpha_v\beta_3$ on the surface of primordial endothelial cells is critical for the differentiation and maturation of blood vessels. Similar studies on chicken chorioallantoic membrane showed that LM609 blocks angiogenesis. Together the two studies suggest that integrin $\alpha_v\beta_3$ plays a role in neovascularization of tissues.

Key words: integrin, $\alpha_v\beta_3$ integrin, vasculogenesis, neovascularization, angioblast, embryonic blood vessel, quail embryo, QH1 antibody

INTRODUCTION

Blood vessels are established by one of two processes, vasculogenesis or angiogenesis. Vasculogenesis involves the development of blood vessels derived from mesodermal stem cells known as angioblasts, whereas angiogenesis depends on the expansion of cells sprouting from preexisting vessels. While these events are initiated somewhat differently, it is likely that certain aspects of these two biological processes are identical. Both processes involve endothelial cell invasion (motility), proliferation, and differentiation leading to lumen formation (Folkman, 1984; D'Amore and Thompson, 1987; Coffin and Poole, 1988; Noden, 1989).

Evidence suggests that angiogenesis depends on endothelial cell adhesive events (Ingber, 1991). Recently integrin $\alpha_v\beta_3$ was shown to be preferentially expressed by endothelial cells undergoing angiogenesis in human wounds and in the chicken chorioallantoic membrane. Importantly, in the same study antagonists of this receptor were found to be capable of blocking angiogenesis initiated by various cytokines or tumor fragments, resulting in the regression of these tumors (Brooks et al., 1994a,b). These investigations suggested that the role of $\alpha_v\beta_3$ during angiogenesis may involve a late cellular process, downstream from multiple angiogenic stimuli. While $\alpha_v\beta_3$ integrin was originally thought to be a vitronectin receptor it is now known to recognize a variety of extracellular matrix components (Hynes, 1992). Thus, one or more of these adhesive proteins may be involved in ligation of integrin $\alpha_v\beta_3$, providing an important signal during the growth of new blood vessels.

To understand more precisely the role of integrin $\alpha_v\beta_3$ in the formation of blood vessels we examined vascular development in the quail early embryo. This embryonic model is particularly useful for analysis of vascular development because quail angioblasts and endothelial cells express a distinctive marker antigen, QH1 (Pardanaud et al., 1987). This allows one to identify and monitor angioblasts during the entire assembly of primordial vessels in the quail (Coffin and Poole, 1988). In fact, during an 8 hour period the process of vasculogenesis gives rise to the major vessels of the early embryo. Formation of blood vessels in the embryo requires angioblast differentiation, cell shape change/motility, formation of lumens, and fusion of vessels. Evidence is provided that integrin $\alpha_v\beta_3$ is highly expressed on angioblasts prior to and during vessel formation. Moreover, the adhesive protein, vitronectin, the major ligand for $\alpha_v\beta_3$, was associated with the basal surface of these cells. Most important, a direct role for $\alpha_v\beta_3$ in vasculogenesis was demonstrated since an $\alpha_v\beta_3$ antagonist, LM609, prevents the maturation of primordial endothelial cells into blood vessels. Therefore, ligation of integrin $\alpha_v\beta_3$ is fundamentally involved in blood vessel development during both vasculogenesis and angiogenesis.

MATERIALS AND METHODS

Immunoprecipitation

The anti-human $\alpha_v\beta_3$ monoclonal antibody LM609 recognizes the $\alpha_v\beta_3$ receptor in midgestation chicken embryos (Ross et al., 1993). To confirm that early quail embryos also synthesize $\alpha_v\beta_3$, specimens were labeled overnight with [^{35}S]methionine/cysteine (Amersham, Inc.) and subjected to immunoprecipitation, and electrophoresis as described earlier (Gallagher et al., 1993). The LM609 antibody specifically precipitated two polypeptides (M_r 150,000 and M_r 97,000) with the migration positions of α_v and β_3 integrin chains. Controls showed that neither an irrelevant mouse monoclonal IgG, nor a monoclonal IgG raised against avian β_1 integrins, CSAT (Neff et al., 1982), precipitated α_v or β_3 integrin chains (data not shown).

Embryo microinjection

Preparation of early stage Japanese quail embryos (*Coturnix coturnix japonica*, Manchester Farms, Dalzell, SC) for microinjection has been described (Drake et al., 1991; Drake and Little, 1991). Delivery of test reagents was accomplished by positioning the micropipette (18 μM bore) in the interstitial space lying between the endoderm and the splanchnic mesoderm, a site of active vasculogenesis. This was accomplished using a Leitz micromanipulator coupled to a Narishige hydraulic drive micromanipulator. A 25 nl volume was delivered using the pneumatically driven Pico-Injector (Medical Systems Corp.). Calibration was accomplished by collecting ten (10) ejections in a 1 μl micropipette (Bolab Inc.), determining this volume and averaging.

LM609 injection

In initial screening assays, a total of 50 embryos (1-7 somites) were injected with LM609 (10-125 ng/nl) and then incubated for various times (1-8 hours) in order to establish the experimental conditions required for a robust morphological effect (not shown). Based on the preliminary work 28, 3-4 somite embryos were injected with LM609 (125 ng in 25 nl), and incubated for 4 or 6 hours.

Control-cultured embryos

Microinjection controls from this and earlier studies consist of the following: PBS (n=10); human serum albumin (n=25); hybridoma 'G' (n=18) a mouse monoclonal antibody against avian β_1 integrins that does not affect ligand binding (see Drake et al., 1991, 1992); nonimmune immunoglobulins of the subclass, IgG2b (n=11). Additionally, immunoglobulins of the same subclass as LM609, IgG1 were injected (n=25, data not shown). Thus, in no case, from a total of 54 embryos did microinjection of control immunoglobulins ranging in concentration from 1-5 ng/nl, in volume from 10-50 nl, cause any alteration to either normal embryonic development or vascular development (Drake et al., 1991, 1992; Drake and Little, 1995; Drake and Little, unpublished observations).

On the basis of experience with several hundred embryos, we have no evidence that microinjection of immunoglobulins, per se, causes any developmental malformations. A far more critical aspect of normal development is the quality of the embryos used. In order to control for this variable, stage-matched normal embryos were routinely cultured alongside experimentally microinjected embryos. The control-cultured embryos served as a measure of both the general health of a particular clutch of embryos and the quality of the culture conditions.

Embryo culture

Live embryos were mounted on paper rings attached to the vitelline membrane and removed from the yolk (Drake et al., 1991). The resulting embryo/ring assemblies were subjected to the appropriate experimental procedure, transferred to a 35 mm Petri dish containing an egg supernatant/agar mix (Packard and Jacobson, 1976), and placed ventral side down. Embryo-containing 35 mm dishes were then placed in a 150 mm × 20 mm dish containing water-saturated paper towels, and incubated for 4 or 6 hours at 37°C, in a humidified CO_2/air mixture (10%/90%). Embryos exhibiting normal morphology after culture were fixed in 3% paraformaldehyde.

Whole-mount immunofluorescence labeling

Immunofluorescence data in Figs 2, 3 and 4 were obtained using whole-mounted quail embryos still attached to the paper rings used for culture and microinjection. The specimens were immunolabeled using the monoclonal antibody QH1 according to a method described by Poole and colleagues (Coffin and Poole, 1988). The QH1 hybridoma was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Whole embryos were also immunolabeled using a polyclonal rabbit antibody directed against chicken vitronectin, and LM609. The images shown in Fig. 1 were obtained by microinjecting antibodies exactly as described above for LM609 perturbation studies; in this case, however, 25 nl of: anti-vitronectin serum (a gift from Dr L. Reichardt, UCSF; see Neugebauer et al., 1991, for specificity data); QH1 (10 μg/ml); or LM609 (5 ng/nl) were injected, and incubated for 45-60 minutes. Specimens were fixed and then processed as previously described (Drake et al., 1992). Except for embryos that were cross-sectioned after immunolabeling (Fig. 2), the ring/embryo assemblies were mounted under a no. 1 coverslip using an anti-bleaching mounting medium, 5% *n*-propyl gallate, 0.25% 1,4-diaza-biocyclo-(2,2,2)-ortene, and 0.0025% paraphenylenediamine in glycerol (Giloh and Sedat, 1982), and viewed on a MRC-1000 Bio-Rad™ laser scanning confocal microscope (LSCM).

Negative controls for immunolabeling included nonimmune polyclonal rabbit antibodies and two irrelevant mouse monoclonal antibodies of the same subclass as LM609 (IgG1), none of which showed staining above background levels. With respect to $\alpha_v\beta_3$ immunostaining, internal positive controls included comparisons between embryos labeled with monoclonal LM609 (Fig. 1A) and the standard marker monoclonal QH1 (Fig. 1C). Also, in recent studies using similar conditions immunolabeling with antibodies to avian β_1 integrins was used (Drake et al., 1991, 1992).

Cross-sectional analysis

After examination using LSCM some embryos were removed from the paper rings and embedded in 15% polyacrylamide, according to Germroth et al. (1994). Cross-sections of 200 μm thickness, were prepared on a vibratome, mounted on microscope slides and further sectioned on the LSCM (similar to Fig. 2).

Laser scanning confocal microscopy

Whole-mounted embryos were scanned in a plane parallel to the embryonic plate. Sequential optical planes were acquired in 5 μm steps along the *z* axis through the full thickness of a stage 8-9 quail embryo. This typically required 18 image planes to completely section an embryo from the dorsal to the ventral surface. The 18 stored graphics files were then collapsed to a single virtual image using the manufacturer's proprietary software (Bio-Rad, Inc.). This processing resulted in a QH1-derived fluorescence map of all the blood vessels and angioblasts in the quail embryo. The vascular map graphics file was imported into Adobe Photoshop™ for further image processing. Similar operations were used with the acrylamide-embedded transverse sections, except that fewer optical sections were required (2 scans, at 2 μm thickness) to construct the final image.

RESULTS

$\alpha_v\beta_3$ and vitronectin are expressed during vasculogenesis

Recent evidence suggests that integrin $\alpha_v\beta_3$ is preferentially

expressed on angiogenic vascular cells in vivo (Brooks et al., 1994a). To determine whether $\alpha_v\beta_3$ is also expressed on primordial endothelial cells, vasculogenic stage quail embryos (9 somites) were immunolabeled using the LM609 antibody. Bright $\alpha_v\beta_3$ fluorescence localized to the surface of the aortic endothelial cells (Fig. 1A, arrowheads). This laser scanning confocal microscope (LSCM) image depicts the full thickness of a segment of dorsal aorta viewed en face. This image demonstrates that $\alpha_v\beta_3$ is expressed at the surface of early quail endothelial cells.

Vitronectin is the major ligand for $\alpha_v\beta_3$. Therefore, additional stage 9 quail embryos were probed with antibodies to chicken vitronectin (Neugebauer et al., 1991; Little et al., unpublished data) to determine if a known $\alpha_v\beta_3$ ligand was distributed near developing vessels. The LSCM immunofluorescence image in Fig. 1B is an optical section that passes through the lumen of a dorsal aorta. The image shows distinct extracellular labeling juxtaposed to the newly formed vessel. Note that the intense staining is closely associated with the basal surface of the primordial endothelial cells, while the lumen of the vessel (da) is negative. This image depicts a 2 μm thick optical section viewed en face.

As a positive immunofluorescence control for the LM609 labeling in Fig. 1A, 9-somite embryos were immunolabeled with the endothelial marker QH1 (Fig. 1C). Bright QH1 immunofluorescence decorates the external (basal) surface of embryonic quail blood vessels. The QH1 labeling also serves as an internal control for the specificity of the $\alpha_v\beta_3$ antibody (Fig. 1A), as both are mouse monoclonal antibodies. Non-immune controls revealed no staining above background (not shown).

As an additional control for LM609 specificity in the quail, immunoprecipitation from radiolabeled extracts of cultured whole embryos yielded two polypeptide bands at the expected migration position for avian integrin α_v and β_3 chains (not shown). Taken together, these immunological data show that stage 9 quail embryos express $\alpha_v\beta_3$ integrins at the surface of primordial endothelial cells, and that the integrin codistributes with extracellular vitronectin (Fig. 1A and B).

Antagonists of $\alpha_v\beta_3$ integrin inhibit maturation of early blood vessels

To assess the function of integrin $\alpha_v\beta_3$ during vessel formation we examined whether the LM609 antibody would influence the formation of early quail embryo blood vessels. LM609 (125 ng in 25 nl) was injected directly into the region of vessel formation and subsequent vessel morphology was monitored with QH1 immunostaining and the LSCM. Twenty-eight embryos (3-4 somites) were injected with LM609, and incubated for either 4 or 6 hours. Twenty-six out of 28 embryos (93%) exhibited conspicuous vascular malformations (described below). In the case of the two embryos that failed

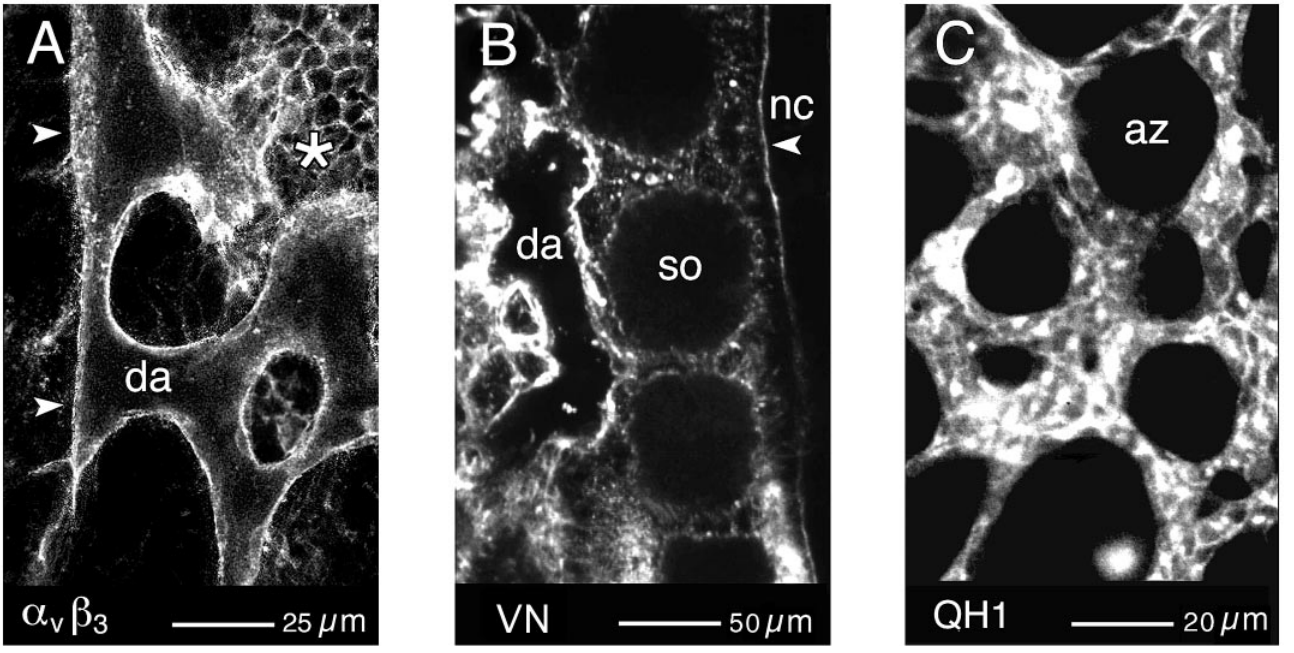

Fig. 1. (A) LM609 binds the surface of embryonic endothelial cells (arrowheads). When viewed from above, whole-mounted embryos present bright integrin $\alpha_v\beta_3$ immunofluorescence along the dorsal aorta (da). This LSCM image is constructed from multiple optical sections representing approximately 10 μm of vertical space. Staining is also associated with endodermal cells (asterisk). ×400. (B) Vitronectin is a constituent of the ECM adjacent to the embryonic aortae. This LSCM image is a relatively thin optical section (≈2 μm), which passes through the lumen of the dorsal aorta (da). Intense rabbit anti-chicken vitronectin immunofluorescence delineates the boundaries of the embryonic endothelial cells. Less intense immunostaining is associated with the spaces between the somites (so), and the notochordal basement membrane (nc). ×200. (C) This image serves a positive immunological control for A, as both specimens were prepared in identical fashion, using mouse monoclonal antibodies. Brightly labeled QH1-positive primordial blood vessels surround dark avascular zones (az). This is also an en face view. ×300. All three images are of the para-aortic region of individual, 9 somite, whole-mounted embryos. The perspective in each image is from above looking down onto the embryo - these are *not* transverse sections. The images were selected to demonstrate the distinct staining patterns for each antigen and to underscore antibody specificity. Irrelevant mouse monoclonal antibodies, and non-immune rabbit antibodies did not produce fluorescence above background (not shown). Additional immunological controls are described in Materials and Methods.

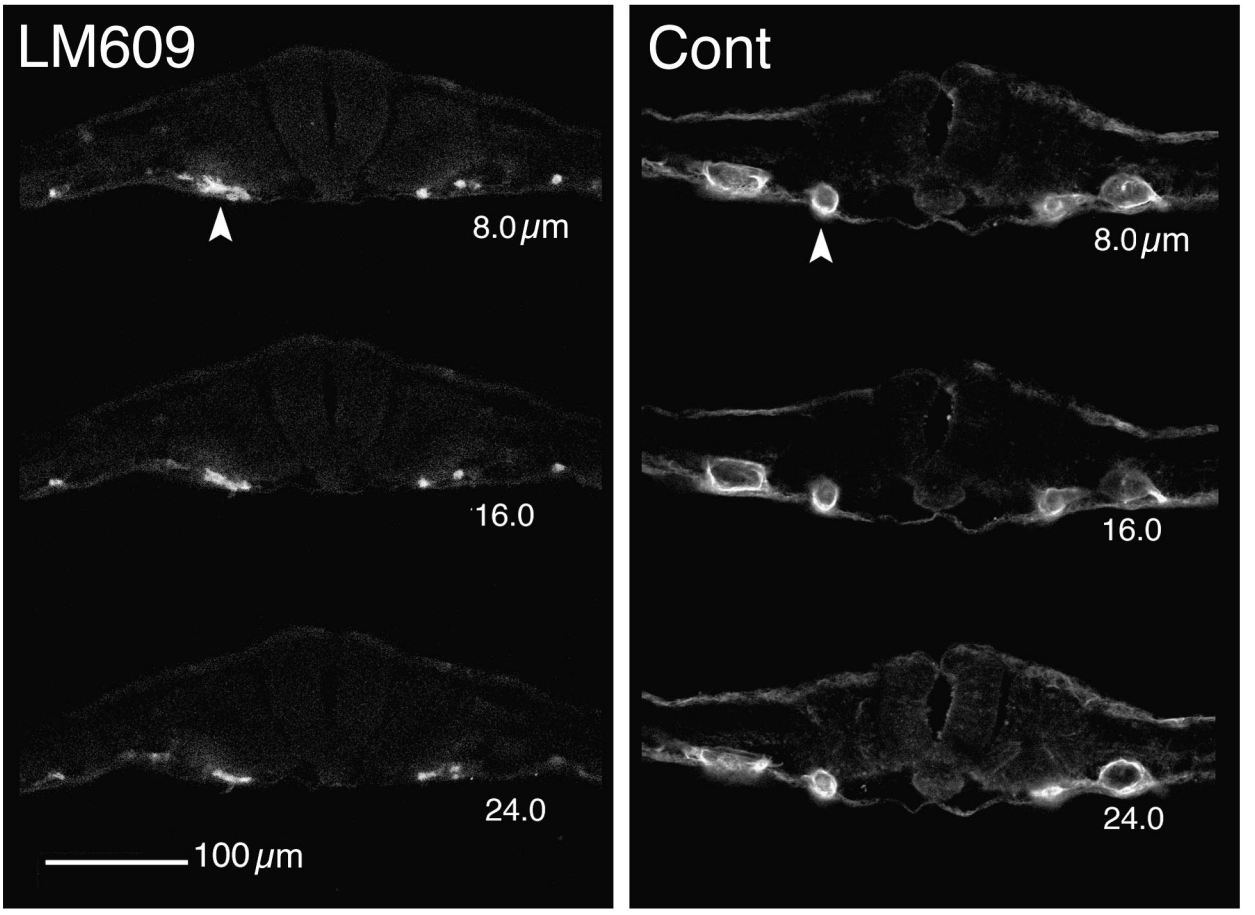

Fig. 2. LM609 inhibition of $\alpha_v\beta_3$ activity altered the formation of vessel lumens (arrowhead). In contrast, the control-cultured, stage-matched embryos, showed aortae with prominent lumens (Cont; arrowhead). To produce these images, whole mounted 9 somite quail embryos were immunolabeled with the QH1 marker, embedded in polyacrylamide and sectioned in the transverse plane. Thick acrylamide sections (200 μm), from the level of the second somite, were optically sectioned (8 μm) at three sequential positions using the LSCM. ×130.

to manifest vascular abnormalities, it is likely that the LM609 antibodies leaked out of the injection site.

Fifteen staged-matched embryos served as culture controls; of these, one embryo showed mild vascular malformations. Extensive microinjection controls, including mouse monoclonal antibodies against other avian integrins and irrelevant (nonimmune) mouse monoclonal antibodies have been previously described (Drake et al., 1991, 1992; Drake and Little, 1994). For example, injecting QH1 (unpublished observations) or non-inhibitory integrin antibodies (Jaffredo et al., 1988), has no detectable effect on embryonic blood vessel formation after 6 hours. (See Materials and Methods for additional details regarding culture and injection controls.)

LM609 perturbs vessel lumen formation

Formation of a lumen is a critical element of vessel morphogenesis. When analyzed by LSCM and QH1 staining 6 hours after LM609 injection, cross-sections showed vessel segments that had failed to organize lumens (Fig. 2, LM609). In contrast micrographs of stage-matched control embryos demonstrate prominently labeled angioblasts surrounding well-formed vascular lumens on each side of the central axis (Fig. 2, Cont). These data strongly suggest that $\alpha_v\beta_3$ integrins actively participate in morphogenesis of patent endothelial tubes.

The pattern of neovascularization in the quail embryo is perturbed by LM609

Vascular development requires that the pattern of blood vessels be carefully regulated. To monitor blood vessel patterns, whole-mounted embryos were immunolabeled with the QH1 endothelial marker and examined en face. Each embryo was viewed in a series of frontal planes from the dorsal to the ventral surface using the LSCM. The resulting series of QH1 images was collapsed into a single “roadmap-like” view of all the blood vessels within the embryo.

The entire vascular map of a *normal* embryo is shown in Fig. 3. Most prominent are the dorsal aortae (da) on each side of the axis. At this stage (9 somites), a normal dorsal aorta (da) is a continuous vessel from its cranial to its caudal extent and presents a relatively smooth medial border. Smaller vessels connect the aortae to the lateral vascular networks. The box outlines the region examined in detail (below).

In contrast to a normal pattern, embryos examined 4 hours after injection with LM609 showed abnormal vascular patterns (Fig. 4). Malformations included fragmented aortae with irregular medial borders (Fig. 4B, arrows) and abnormal anastomotic connections to thin, poorly formed lateral vessels (Fig. 4B, arrowheads). Control-cultured embryos, however, contain continuous dorsal aortae with smooth medial borders (Fig. 4A,

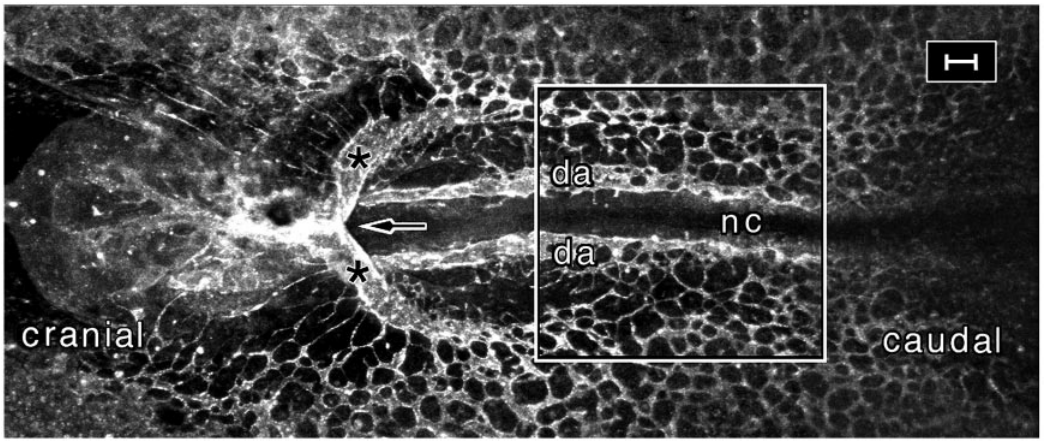

Fig. 3. The LSCM and QH1 antibodies were used to construct a roadmap-like image of all the vessels in a whole-mounted quail embryo. These vascular maps can be used to monitor pattern formation. Important features of the vasculature of normal 9 somite embryo include elongated dorsal aortae (da) lying parallel to the notochordal axis (nc), and the two cardiac endothelial tubes (asterisks) that are fusing at the midline cranial to the anterior intestinal portal (arrow). Lateral vascular networks, which appear as fluorescent polygons surrounding non-labeled, dark, avascular centers, lie on each side of the embryo. At this stage the entire vascular network lies in a plane approximately 80 μm thick. The LSCM optical assay was used to monitor the effects of LM609. The perspective is from above. The absolute size of a 9 somite quail embryo (3.5 mm) is indicated by the white bar inserted at the upper right. ×40.

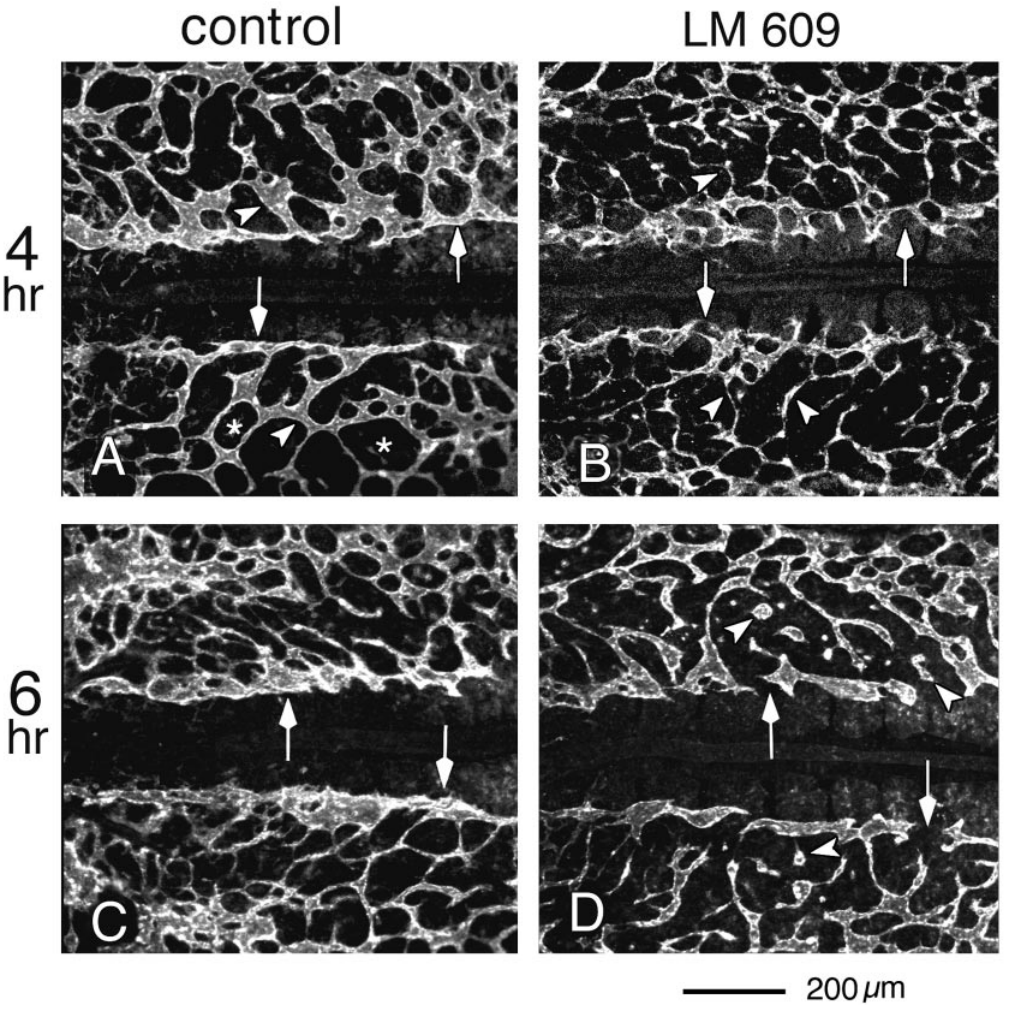

Fig. 4. Experimental quail embryos were injected at the 3-4 somite stage with LM609 (25 nl, 125 ng), incubated for 4 or 6 hours, then processed as described above. LM609 causes vascular abnormalities that are detectable 4 hours after injection. Malformations included disrupted, irregular dorsal aortae (arrows, B) and thin poorly organized lateral vascular networks (arrowheads, B). In distinct contrast, 4 hour control-cultured embryos exhibited smooth aortic borders (arrows, A) and smooth well spread para-aortic vessels (arrowheads, A). Also, evident in control-cultured embryos are well formed polygonal networks that define avascular spaces (asterisks, A). For the most part these lateral vascular patterns are absent in treated embryos (B). Extending the post-injection incubation time from 4 to 6 hours increased the severity of vascular malformations. Stage-matched (9 somite) control embryos incubated for 6 hours show well formed aortae that are continuous along their entire length (arrows, C). In contrast, LM609-injected embryos show distinctly malformed aortae that are disrupted at several positions along the length of the vessel (arrows D). Also, highly abnormal islands or clusters of endothelial cells are present lateral to the dorsal aortae in LM609-injected embryos (arrowheads, D); whereas, isolated clusters of endothelial cells are not present in control embryos (C). Each panel in this figure corresponds to the area denoted by the rectangle in Fig. 3. ×70.

arrows), and distinct anastomotic connections to well established lateral vascular networks (Fig. 4A, arrowheads). Also, lateral vessels in control embryos, exhibited broad, smooth contours, which formed polygons surrounding avascular spaces (Fig. 4A, asterisks). In sharp contrast, few polygons are present in treated embryos (Fig. 4B); instead, small arrays of

poorly spread angioblasts are observed (Fig. 4B, arrowheads). Six hours after LM609 injection the embryonic vessels were more severely malformed than at the 4 hour time point. Most conspicuous is the fact that untreated embryos display continuous, relatively wide aortae (Fig. 4C, arrows), while LM609-injected embryos (Fig. 4D, arrows) exhibited discontinuous aortae with interruptions along the longitudinal axis of the vessel (arrows). In addition, 6 hour control vessels formed a robust lateral vascular network, whereas LM609-treated vessels did not. Equally abnormal are the rounded clusters of angioblasts present in LM609-injected embryos (Fig. 4D, arrowheads) that are absent in control-cultured embryos. The abnormal cellular clusters appear to be assemblies of angioblasts that had aborted their normal protrusive and spreading activity. The data in Figs 2 and 4 strongly suggest that $\alpha_v\beta_3$-treated angioblasts are compromised in their ability to extend protrusions and to change shape - a type of cellular behavior fundamental to neovascularization. Indeed, the vessels in LM609-injected embryos show few if any fine cellular protrusions compared to controls, strongly suggesting altered cell-ECM adhesion.

In summary, the results presented here show that neutralizing the $\alpha_v\beta_3$ integrin in vivo strongly impacts the behavior of primordial endothelial cells. Specifically, the data suggest that $\alpha_v\beta_3$ mediates local cell spreading activity, and assists cells in the formation of protrusions. Moreover, a candidate ligand, vitronectin, was shown to surround primordial vessels. These findings are consistent with a role for $\alpha_v\beta_3$ in the maturation of newly forming blood vessels.

DISCUSSION

Neovascularization is a complex biological process that is required for embryonic development. The process is highly regulated and depends on the differentiation of primordial endothelial cells known as angioblasts. Similarly, in the adult organism neovascularization or angiogenesis depends on specific cytokines that stimulate the formation of cells analogous to angioblasts. These are cells that emerge from pre-existing fully mature blood vessels near sites of inflammation, wounds or tumors. It has become increasingly clear that there must be considerable mechanistic overlap between vasculogenesis and angiogenesis. This is especially true with respect to the assembly of the new endothelial tubes. In fact, most descriptions of angiogenesis include a step whereby preexisting vessels give rise to “sprouts” of endothelial cells. Such cells are described as having lost their epithelial integrity and having acquired a spindle shape (Folkman, 1984; D’Amore and Thompson, 1987). Thus, it is conceivable that the emigrant endothelial “sprouts” of angiogenesis correspond to the small assemblies of angioblasts in the embryo (see Fig. 3). Recent studies have shown that the adhesion receptor, $\alpha_v\beta_3$, plays a critical role during angiogenesis induced by cytokines and human tumor fragments. Specifically, the mouse monoclonal antibody LM609 directed to $\alpha_v\beta_3$ integrin blocked new vessel growth into human tumors grafted to chicken chorioallantoic membrane (Brooks et al., 1994a,b). Evidence was provided that antibody LM609 disrupts a late event in the angiogenic process by promoting apoptosis of vascular sprouts (Brooks et al., 1994b).

We have now investigated the potential role of integrin $\alpha_v\beta_3$ during embryonic neovascularization, in vivo. In this case we used an early quail embryo experimental system, since it provides several advantages for the study of neovascularization. First, vasculogenesis is rapid. Once angioblasts are detected at the 2 somite stage, the first vessels form within 7 hours. Thus, embryos (3-4 somite stage) could be injected just as vessel maturation begins. Second, embryonic vessels form in a synchronous, highly patterned manner, in contrast to angiogenic models such as chorioallantoic membranes, tumors or wounds. Third, early avian embryos are highly planar and allow in situ monitoring of the entire vascular network.

In this study we directly tested the possibility that mAb LM609 would block embryonic neovascularization. Embryos were microinjected at the 4 somite stage, a time of intense vessel morphogenesis and maturation; important events include lumen formation and the organization of a tightly regulated pattern of vessels. LM609 had an unambiguous impact on vascular development; specifically, the primordial endothelial cells of injected embryos failed to form continuous lumens and were unable to extend normal cellular protrusions. These results are supported by considerable morphological data establishing that cellular extension and protrusive activity are necessary for small pre-vessel segments to fuse into complete vessels (Poole and Coffin, 1989; Vernon et al., 1994). Therefore we propose that $\alpha_v\beta_3$ is an ECM adhesion receptor active during primordial endothelial cell shape change and extension of protrusions.

The findings reported here are consistent with the studies on the chorioallantoic membrane-induced angiogenesis. In the case of the CAM assays, LM609 specifically promoted the apoptosis of vascular cells previously stimulated by bFGF (Brooks et al., 1994b). At present, it is not clear whether the quail endothelial cells undergo a similar fate after exposure to LM609. However, close examination of embryos exposed to this antibody for 4-6 hours reveals QH1 positive cells that have a retracted morphology (Fig. 4B,D) consistent with cells beginning apoptosis. The survival of cells is known to be partially dependent on correct ECM interactions (Meredith et al., 1993). It is possible, therefore, that the LM609-treated embryonic endothelial cells might also undergo premature cell death. In this regard, recent work has shown that ECM adhesions directly influence the behavior and signaling pathways of angiogenic cells in vitro (Yamada and Kleinman, 1992; Dameron et al., 1994; Vernon et al., 1994).

Integrin $\alpha_v\beta_3$ recognizes a wide variety of extracellular matrix proteins (Hynes, 1992). Of these proteins, vitronectin is the primary ligand (Cheresh, 1987; Smith and Cheresh, 1988). In this report we localized vitronectin to the surface of primordial endothelial cells at the same developmental stage at which LM609 had its impact on vascularization. These findings suggest that vitronectin may be a relevant ligand for $\alpha_v\beta_3$ during the process of embryonic blood vessel maturation. However, at present we cannot rule out the possibility that other $\alpha_v\beta_3$ ligands might contribute to this response.

A role for integrins in avian vessel development has been established in this article and previous studies using the β_1 antagonist CSAT (Drake et al., 1992). It is important to point out that there are clear differences between the abnormalities induced by LM609 and CSAT. For example, injection of the monoclonal antibody CSAT resulted in abnormal somitogen-

esis in addition to altered vasculogenesis (Drake et al., 1991). In contrast, LM609 had no effect on somites, or other developmental processes. Moreover, CSAT-induced vascular malformations much more quickly, within 1 hour; whereas, LM609 manifested maximum effects at 6 hours. Also, the morphologies of affected vessels were different. Angioblasts in CSAT-injected embryos formed compact, continuous cord-like assemblies; while LM609 induced flattened, non-patent vessel segments, but not compact cords. Finally, LM609, unlike CSAT, caused overt discontinuities along the aortae (arrows, Fig. 4D).

Due to its broad range of recognition (all β_1 family members), it is extremely likely that CSAT interfered with multiple integrins, and thus had widespread effects compared to LM609. We speculate that CSAT caused considerable disruption of $\beta_1$integrin-mediated, cytoplasmic signaling pathways. This may have provoked angioblasts to lose most, if not all, integrin-mediated adhesions. LM609, on the other hand, interfered with a single integrin, $\alpha_v\beta_3$, and elicited less severe, more discrete effects on angioblast behavior.

Taken together the data in this article strongly suggest that embryonic neovascularization in vivo depends on integrin $\alpha_v\beta_3$. This receptor appears to be important for vessel maturation, since microinjection of an antibody to integrin $\alpha_v\beta_3$ perturbed angioblasts while the cells were in the process of vessel patterning and lumen formation. These findings, together with the chorioallantoic membrane results of Cheresh and colleagues (Brooks et al., 1994a,b), suggest that embryonic neovascularization and angiogenesis share common biological mechanisms. In particular, both processes depend on $\alpha_v\beta_3$-mediated endothelial cell adhesion activity downstream of the initial signal for commitment to a neovascular fate.

This work was supported by awards NIH R01 HL37709, R01 HL45348 and P01 HL52813 to C.D.L.; and by NIH R01 CA45726, R01 CA50268 to D.A.C., who is also the recipient of a Faculty Research Award from the American Cancer Society.

REFERENCES

Brooks, P. C., Clark, R. A. and Cheresh, D. A. (1994a). Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* **264**, 569-571.

Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., G., K. and Cheresh, D. A. (1994 b). Integrin $\alpha_v\beta_3$ antagonist promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79**, 1157-1164.

Cheresh, D. A. (1987). Human endothelial cells synthesize and express an Arg-Glu-Asp directed adhesion receptor involved in attachment to fibrinogin and von Willebrand factor. *Proc. Nat. Acad. Sci. USA* **84**, 6471-6475.

Coffin, J. D. and Poole, T. J. (1988). Embryonic vascular development: Immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. *Development* **102**, 735-748.

Dameron, K. M., Volpert, O. V., Tainsky, M. A. and Bouck, N. (1994). Control of angiogenesis in fibroblasts by p53 regulation of thrombosondin-1. *Science* **265**, 1582-1584.

D'Amore, P. A. and Thompson, R. W. (1987). Mechanisms of angiogenesis. *Annu. Rev. Physiol.* **49**, 453-464.

Drake, C. J., Davis, L. A., Hungerford, J. E. and Little, C. D. (1991). Perturbation of β_1 integrin-mediated adhesions results in altered somite cell shape and behavior. *Dev. Biol.* **149**, 327-338.

Drake, C. J. and Little, C. D. (1991). Integrins play an essential role in somite adhesion to the embryonic axis. *Dev. Biol.* **143**, 418-421.

Drake, C. J., Davis, L. A. and Little, C. D. (1992). Antibodies to β_1 integrins cause alterations of aortic vasculogenesis, in vivo. *Dev. Dynam.* **193**, 83-91.

Drake, C. J. and Little, C. D. (1995). Exogenous VEGF induces malformed and hyperfused vessels during embryonic neovascularization. *Proc. Nat. Acad. Sci. USA* (in press).

Folkman, J. (1984). Angiogenesis. In *Biology of Endothelial Cells* (ed. E. A. Jaffe), pp. 412-428. Boston: Martinus Nijhoff Publishers.

Gallagher, B. C., Sakai, L. Y. and Little, C. D. (1993). Fibrillin delineates the primary axis of the early avian embryo. *Dev. Dynam.* **196**, 70-78.

Germroth, P. G., Gourdie, G. and Thompson, R. P. (1995). Confocal microscopy of thick sections from acrylamide gel embedded embryos. *Microsc. Res. Technique* **30**, 513-520.

Giloh, H. and Sedat, J. W. (1982). Fluorescence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propylgallate. *Science* **217**, 1252-1255.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Ingber, D. (1991). Extracellular matrix and cell shape: potential control points for inhibition of angiogenesis. *J. Cell. Biochem.* **47**, 236-241.

Jaffredo, T., Horwitz, A. F., Buck, C. A., Rong, P. M. and Dieteerlen-Lievre, F. (1988). Myoblast migration specifically inhibited in the chick embryo by grafted CSAT hybridoma cell secreting an anti-integrin antibody. *Development* **103**, 431-446.

Meredith, J. E., Fazeli, B. and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953-961.

Neff, N. T., Lowrey, C., Decker, C., Tovar, A., Damsky, C., Buck, C. and Horwitz, A. F. (1982). A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. *J. Cell Biol.* **95**, 654-666.

Neugebauer, K. M., Emmet, C. J., Venstrom, K. A. and Reichardt, L. F. (1991). Vitronectin and thrombospondin promote retinal neurite outgrowth: developmental regulation and role of integrins. *Neuron* **6**, 345-358.

Noden, D. (1989). Embryonic origins and assembly of blood vessels. *Am. Rev. Resp. Dis.* **140**, 1097-1103.

Packard, D. S. J. and Jacobson, A. G. (1976). The influence of axial structures on chick somite formation. *Dev. Biol.* **53**, 36-48.

Pardanaud, L., Altmann, C., Kitos, P., Dieterien-Lievre, F. and Buck, C. A. (1987). Vasculogenesis in the early quail bastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* **100**, 339-349.

Poole, T. J. and Coffin, J. D. (1989). Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. *J. Exp. Zool.* **251**, 224-231.

Ross, F. P., Chappel, J., Alvarez, J. I., D., S., W. T., B., Farach-Carson, M. C., Mintz, K. A., Robey, P. G., Teitelbaum, S. L. and Cheresh, D. A. (1993). Interaction between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin $\alpha_v\beta_3$ potentiate bone resorption. *J. Biol. Chem.* **268** (May 5), 9901-9907.

Smith, J. W. and Cheresh, D. A. (1988). The Arg-Gly-Asp binding domain of the vitronectin receptor. *J. Biol. Chem.* **263**, 18726-18731.

Vernon, R. B., Lara, S. L., Drake, C. J., Iruela-Arispe, M. L., Angello, J. C., Little, C. D., Wight, T. N. and Sage, E. H. (1995). Organized type I collagen influences endothelial patterns during "spontaneous angiogenesis in vitro": planar cultures as models of vascular development. *In Vitro Cell Dev. Biol.* **31**, 120-131.

Yamada, Y. and Kleinman, H. K. (1992). Functional domains of cell adhesion molecules. *Curr. Opin. Cell Biol.* **4**, 819-823.

(Received 23 February 1995 - Accepted 17 April 1995)