THE ROLE OF $\beta_1$ INTEGRINS IN DORSAL AORTA VASCULOGENESIS IN THE
CHICKEN EMBRYO

by

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We accept this thesis as conforming
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ABSTRACT

Vasculogenesis is the *de novo* formation of blood vessels from endothelial cell precursors known as angioblasts. In the chicken embryo, the dorsal aorta is formed by this process. Adhesion molecules such as integrins and their extracellular matrix ligands are believed to mediate vasculogenesis.

Immunohistochemistry was used to determine the temporal and spatial distribution of the \( \alpha_5 \), \( \alpha_6 \) and \( \beta_1 \) integrin subunits, the extracellular matrix proteins, fibronectin and laminin, and the endothelial cell marker, thrombomucin, in the chicken embryo from stages 8 through 14. The \( \beta_1 \) integrin subunit and fibronectin were shown to be localized to the site of dorsal aorta vasculogenesis prior to lumen formation. The \( \alpha_5 \) integrin subunit and laminin were shown to be localized to the site of dorsal aorta vasculogenesis after lumen formation had commenced. The \( \alpha_6 \) subunit was not detected on cells of the dorsal aorta at the stages examined but was detected in control tissues.

In order to investigate the role of \( \beta_1 \) integrins in dorsal aorta vasculogenesis, integrin blocking antibodies and peptides containing integrin recognition sequences were injected at the site of dorsal aorta vasculogenesis *in ovo* to disrupt receptor-ligand interactions. The \( \beta_1 \) integrin blocking antibodies, CSAT and W1B10, prevented dorsal aorta lumen formation and induced lateral displacement of somites while non-specific antibodies had no effect. None of the \( \alpha_5\beta_1 \), \( \alpha_6\beta_1 \), or \( \alpha_5\beta_1 \) blocking peptides (RGD, DGEA, and YIGSR, respectively) or the control peptide, RGES, induced dorsal aorta malformations.

The dorsal aorta malformations induced by blocking the \( \beta_1 \) integrin subunit were proposed...
to be mediated by integrins other than α5β1, and α6β1 due to the absence of the α5 and α6 subunits
during lumen formation. For the same reason, the ligands of the β1 integrins involved in dorsal
aorta vasculogenesis would not include laminin; however, fibronectin remained as a possible
ligand. Conversely, the localization of the β1 subunit along cell-cell contacts of angioblasts
suggests that the ligand may be a cell membrane-bound molecule rather than an ECM component.
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<td>BAEC</td>
<td>Bovine aortic endothelial cell</td>
</tr>
<tr>
<td>BMEC</td>
<td>Bovine microvascular endothelial cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorio-allantoic membrane</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>DGEA</td>
<td>Peptide sequence Asp-Gly-Glu-Ala</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Ln</td>
<td>Laminin</td>
</tr>
<tr>
<td>MEC</td>
<td>Microvascular endothelial cells</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet-endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RGD</td>
<td>Peptide sequence Arg-Gly-Asp</td>
</tr>
<tr>
<td>RGES</td>
<td>Peptide sequence Arg-Gly-Glu-Ser</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SIKVAV</td>
<td>Peptide sequence Ser-Ile-Lys-Val-Ala-Val</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic, rich in cystine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase-type plasminogen activator</td>
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<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
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<td>Vn</td>
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INTRODUCTION

The vascular endothelium is a mesodermally-derived tissue that covers the entire inner surface of the cardiovascular system. It has been suggested that vascular tissue is produced by two processes: vasculogenesis, the formation of vessels by direct differentiation of mesoderm, and angiogenesis, the production of vessels by sprouting of pre-existing vessels (Risau and Lemmon, 1988). These processes involve a common series of events: migration and proliferation of endothelial cells, junction formation, extracellular matrix (ECM) production and lumen formation. Vasculogenesis is most commonly studied in the developing yolk sac while angiogenesis has been studied during vascularization of the embryonic limb, brain and chorioallantoic membrane, and during wound healing. Angiogenesis can also be experimentally induced in adult cornea and rat mesentery.

The production of vessels is a normal process in the vascularization of tissue during development and wound healing but can be unregulated in certain diseases such as arthritis, diabetic retinopathy, retinopathy of prematurity and atherosclerotic plaque vascularization. In tumours, vascularization promotes growth and the potential for metastasis (Folkman and Shing, 1992). Understanding the molecular changes that occur during endothelial cell differentiation and the potential points of control will provide a foundation for the development of future strategies for treatment of diseases involving inappropriate blood vessel formation.

Vasculogenesis

Vasculogenesis is the differentiation of mesoderm to endothelial cells which form vascular
structures in the developing embryo. Blood vessel formation in the avian yolk sac, the most widely studied model of vasculogenesis, and dorsal aorta formation will be described herein. Presumptive endothelial cells originate in the epiblast of the bilaminar embryo and migrate through the primitive groove to become mesoderm. These mesodermal cells migrate between the ectoderm and the endoderm in a rostra-lateral direction. In quail embryos, the endothelial marker QH-1 is first seen in isolated cells of the area opaca mesoderm during the headfold stage (stage 6; Hamburger and Hamilton, 1951) and these cells have been termed hemangioblasts (Pardanaud et al., 1987). At stage 7 hemangioblasts are seen to interconnect in the area opaca and QH-1 positive cells can be seen in the adjacent area pellucida mesoderm of quail embryos (Pardanaud et al., 1987). TEM also demonstrates the appearance of cell aggregates at this time in the yolk sac (Gonzalez-Crussi, 1971). The splanchnic and somatic mesoderm separate by stage 8 and it is in the splanchnic mesoderm that the blood islands develop (Murphy and Carlson, 1978). Isolated clusters with junctions are now seen by TEM (Murphy and Carlson, 1978) and immunohistochemistry demonstrates a thin thread-like pattern of QH-1 positive cells in the area pellucida (Pardanaud et al., 1987). The anlagen of the vitelline veins, which will form the inflow tract to the yolk sac, appear as cords of angioblasts by SEM from a convergence of the developing yolk sac plexus (Hirakow and Hiruma, 1981). Within cell aggregates of the yolk sac the peripheral cells separate from the central core of cells beginning from stage 8+ (Gonzalez-Crussi, 1971) to stage 10 (Murphy and Carlson, 1978) and are termed angioblasts and hemoblasts, respectively. At stage 9- each dorsal aorta anlage is seen by SEM as a cord of angioblasts (Hirakow and Hiruma, 1981) and by LM and TEM as individual or pairs of angioblasts (Hirakow and Hiruma, 1983) ventral to the somites. At stage 9 this cord of cells begins to form a lumen
surrounded incompletely by at least two angioblasts (Hirakow and Hiruma, 1983) and develops into an endothelial tube by stage 9+ as seen by SEM (Hirakow and Hiruma, 1981). There are no specific junctional complexes formed at this stage but plasma membrane thickenings are present along endothelial contacts (Hirakow and Hiruma, 1983). The clefts in the endothelial tube are seen as late as stage 10- (Hirakow and Hiruma, 1983). The anlagen of the cardinal veins appear by stage 10- at the dorsa-lateral side of the somites (Hirakow and Hiruma, 1981). At stage 11, ECM material can be seen by TEM between splanchnic mesoderm and the endothelial cells of the blood islands (Murphy and Carlson, 1978) and fibronectin (Fn) can be immunolocalized surrounding the blood islands. Free erythroblasts are observed in the blood islands of stage 15 embryos and endothelial cells are joined by tight junctions. Components of the basal lamina also appear and organize into a basement membrane by stage 16 (Murphy and Carlson, 1978). By stage 19 to 20, splanchnic mesoderm surrounding vessels appears smooth muscle-like (Gonzalez-Crussi, 1971; Murphy and Carlson, 1978) and collagen fibrils are seen between mesoderm and endothelium which now appears morphologically mature (Murphy and Carlson, 1978).

**Angiogenesis**

In the process of angiogenesis, the endothelial cells of a vessel (usually a venule but may include others) are stimulated by a soluble factor from a source (tumour, ischemic tissue, growing structure, implant, etc.). The cells become activated, displaying an enlarged nucleus which bulges into the lumen (Cliff, 1963; Clark et al., 1982), an increase in rough endoplasmic reticulum (Cliff, 1963) and increased DNA synthesis (Ausprunk and Folkman, 1977), and the vessel dilates (McCracken et al., 1979; Burger et al., 1983; Sholly et al., 1984). Endothelial cells produce
matrix-degrading enzymes such as collagenase which breaks down the basal lamina (Moscatelli and Rifkin, 1988). Where the basal lamina is absent, endothelial cells may enter the extracellular space (Cliff, 1963) and this is often seen as a bicellular process (Ausprunk and Folkman, 1977; Paku and Paweletz, 1991). Two endothelial cells protrude while maintaining contact with each other and have junctions at their tips and a narrow slit-like lumen between them (Bar and Wolff, 1973; Wakui, 1988; Paku and Paweletz, 1991). Thus vessel integrity is maintained while the sprout forms. Many sprouts will form from the existing vessels in response to the signal from the source (Burger et al., 1983). This ensures that there will be a functional system of distributing vessels carrying blood from the arterial system to the capillary plexus and out to the venous system. Adjacent sprouts may come into contact with each other and junctions may form between cells at the sprout tips. Fusion of their lumens may result in formation of a capillary loop (Cliff, 1963). Further sprouting may occur from the apices of these loops (McCracken et al., 1979). Once the endothelial cells migrate out from the parent vessel, they undergo mitosis; mitotic activity is observed in ECs proximal to those forming the tip of the sprout (Cliff, 1963; McCracken et al., 1979; Sholly et al., 1984). A dense anastomosing network is formed with some vessels forming an inflow system and others forming an outflow system (Burger et al., 1983). Sometime during the process the arterial system begins feeding the plexus but it is not known if sprouting occurs from arterioles. From the vessels supplying and draining the plexus, there is a selection of vessels based on blood flow dynamics: those vessels with greater flow will develop and mature to become more arteriole-like or venule-like while others that do not have sufficient blood flow will regress resulting in fewer, larger vessels supplying the capillary network (McCracken et al., 1979; Burger et al., 1983; Sholly et al., 1984). Further sprouting from the
plexus will advance the plexus toward the stimulus with concomitant regression and maturation of vessels as before. The plexus eventually reaches the source of the stimulus and infiltrates or surrounds it (Burger et al., 1983). Maturation of vessels is indicated by the appearance of a basal lamina (Bar and Wolff, 1972) and production of components such as laminin (Ln) and collagen IV and a decrease in Fn (Clark et al., 1982). This is first observed at the site of origin of the new vessels and progresses distally as the sprout elongates. Pericytes will also migrate up to the tips of sprouts and envelop the vessels (Burger et al., 1983; Paku and Paweletz, 1991; Bar and Wolff, 1973). If the angiogenic stimulus is removed then there may be regression of the capillary plexus although the newly formed arterioles and venules may persist (Ausprunk and Folkman, 1978; Burger et al., 1983).

During embryonic development vascularization of structures and organs may occur by angiogenesis or vasculogenesis, the latter being further classified as type I vasculogenesis, formation of vessels in situ by differentiating angioblasts, and type II vasculogenesis, vessel formation by angioblasts that have migrated from their site of differentiation (Poole and Coffin, 1991). The first vessels to form in the embryo arise by the process of vasculogenesis. The yolk sac vessels and the dorsal aortae form by type I vasculogenesis while the heart endocardium, the vessels of the head (Noden, 1991) and intersomitic arteries are formed by type II vasculogenesis (Poole and Coffin, 1991). Subsequent sprouting of these vessels through the process of angiogenesis gives rise to most of the embryonic vasculature, including the cardinal veins and vessels of the limb buds (Feinberg, 1991), embryonic brain (Risau, 1991a), kidney and the CAM. However, there are other organs of endodermal origin that become vascularized by the process of vasculogenesis; these include the lung, liver, pancreas and intestine (Sherer, 1991). In these
organs, angioblasts differentiate from mesenchyme in the developing organ then join up with the major vessels of the embryo. This process is not unlike that seen in the yolk sac which is an endodermal organ whose vasculature develops in the adjacent mesoderm and links up with the dorsal aorta via the vitelline artery.

In summary, vessel formation during both angiogenesis and vasculogenesis follows a similar series of events. In these processes angioblasts or angioblast-like cells migrate through a Fn-rich matrix (Risau and Lemmon, 1988) toward the area requiring vascularization. The migrating cells become non-migratory and assemble into cords during angiogenesis and clusters during vasculogenesis. This is followed by synthesis of the basement membrane protein, Ln, and formation of a vascular lumen (Risau and Lemmon, 1988); deposition of collagen IV occurs subsequently. Both processes are believed to be initiated by the stimulatory effects of growth factors.

Role of vascular endothelial growth factor and its receptors in endothelial cell differentiation

in vitro and in vivo

Vascular Endothelial Growth Factor (VEGF) is believed to be a specific mitogen for endothelial cells that is structurally related to Platelet Derived Growth Factor (PDGF) (for reviews see Ferrara et al., 1992; Dvorak et al., 1995). It has also been termed vasculotropin and vascular permeability factor (VPF) due to its ability to induce vascular leakage in vivo (Senger et al., 1983). VEGF was first characterized in media conditioned by bovine pituitary folliculo-stellate cells (Ferrara et al., 1989; Leung et al., 1989) and later in media conditioned by various transformed cell lines (Levy et al., 1989; Conn et al., 1990). VEGF has been shown to induce
angiogenesis *in vivo* in models such as the chicken CAM (Plouet et al., 1989; Leung et al., 1989; Connolly et al., 1989), and the rabbit corneal assay (Levy et al., 1989). Four molecular isoforms of VEGF are known and are products of alternative splicing of the same gene. Two members are known to be secreted while the other two are cell-associated (Dvorak et al., 1995). Endogenous VEGF can induce angiogenesis *in vivo* and its expression can be induced experimentally under hypoxic (Pierce et al., 1995; Shweiki et al., 1995) and hypoglycaemic (Shweiki et al., 1995) conditions and by tumour cells of low and high grade glioma (Plate et al., 1994).

Cellular responses to VEGF are also elicited via cell membrane-associated receptors specific for this growth factor (reviewed by Neufeld et al., 1994). Three cellular receptors for VEGF have been identified: VEGFR-1 which is encoded by the *flt-1* gene, VEGFR-2, encoded by the KDR/flk-1 gene and VEGFR-3. All are described as receptor tyrosine kinases which have been localized to ECs in developing mouse embryos (Yamaguchi et al., 1993) and bind VEGF with high affinity (DeVries et al., 1992; Millauer et al., 1993). The receptors belong to the PDGF receptor subfamily and have a structure consisting of: i) an extracellular domain with seven immunoglobulin-like domains, ii) a transmembrane domain, and iii) a cytoplasmic tyrosine kinase sequence interrupted by a kinase insert (Neufeld et al., 1994). The *flt-1* gene product has not been shown to undergo autophosphorylation upon binding while the *flk-1* gene product has been demonstrated to do so. VEGFR-1 is expressed in endothelial cells of low grade glioma and VEGFR-1 and 2 are expressed in ECs of high grade glioma (Plate et al., 1994). VEGF can directly induce an angiogenic response *in vivo*. However, VEGF receptors are often not expressed in adult tissues suggesting that an additional stimulus may be required for this process to take place.
VEGF and its receptors are expressed during embryogenesis and are associated with the vascularization of various tissues. Breier et al. (1992) detected VEGF mRNA expression in ventricular neuroectoderm of the 17 d post-coital (pc) mouse brain; adult levels were much lower. Expression was also high in embryonic and adult choroid plexus and glomeruli. Quail VEGF-C mRNA is strongly expressed in areas rich in lymphatics (Eichmann et al., 1998). Location of VEGF receptors has also been carried out in developing embryos. *In situ* analysis of *flk-1* mRNA showed expression in ECs during mouse development including 7.5 d yolk sac mesoderm (Quinn et al., 1993), blood islands (Millauer et al., 1993), ECs of the developing brain vasculature (Millauer et al., 1993; Quinn et al., 1993), ECs of aorta, cardinal vein, umbilical artery and vein, small vessels in limb of 12.5 d pc mouse embryo and adult mouse glomerulus (Quinn et al., 1993). The co-localization of VEGF and *flk-1* expression was demonstrated in several areas of vessel formation including the developing renal corpuscle, choroid plexus and yolk sac of the mouse embryo (Dumont et al., 1995). In these tissues, the renal or choroidal epithelium and 7.5d yolk sac endoderm expressed VEGF while the adjacent capillary plexus or yolk sac mesoderm expressed the receptor. Similarly, *flt-1* (VEGFR-1) mRNA was detected in the mesoderm of embryonic day (E) 7.5 embryos with VEGF detected in the adjacent endoderm (Brier et al., 1995). In later stages, *flt-1* was expressed in peripheral cells of blood islands, dorsal aorta ECs, blood vessels of developing organs and the choroid plexus. *Flk-1* expression has also been detected in the undifferentiated mesoderm of the quail embryo which was localized to ECs by E 2 (Flamme et al., 1995). Expression was also co-localized with VEGF in several organs in murine embryos. Intact mouse E 7.5 embryos, yolk sacs and yolk sac mesoderm alone all expressed *flk-1* and all but the yolk sac mesoderm produced endothelial tubes when cultured (Palis
et al., 1995). VEGF did not induce capillary formation in the mesoderm cultures which suggests that cell-cell or cell-ECM interaction is required for this process. These results indicate that VEGF is involved with endothelial differentiation in endodermal structures and possibly in phenotype regulation in specialized vascular structures such as the glomerulus and choroid plexus.

The importance of VEGF in vasculogenesis and angiogenesis in the embryo has been demonstrated by a number of studies. Exogenous VEGF administered to 5-6 somite quail embryos alters the pattern of vasculogenesis by inducing blood vessel formation in normally avascular regions of the embryo and causing excessive vessel fusion to produce sinusoidal vessels (Drake and Little, 1995). Individual vessels including the dorsal aorta were indistinct due to fusion with the inflow tract of the vitelline vessels. Avian wing bud with VEGF overexpression through chicken fibroblast transfection with VEGF DNA demonstrated an increase in vascularization and vascular permeability but no change in the vascular pattern indicating that a permissive environment is required for vessel formation (Flamme et al., 1995). VEGF may therefore influence the degree of vascularization of tissues rather than dictate vascular patterning.

Receptor mutation experiments have also demonstrated separate roles of the two VEGF receptors during vasculogenesis. A homozygous flt-1 mutation in mouse embryos was lethal by E 8.5 and resulted in disruption of vasculature but not cell differentiation (Fong et al., 1995). Angioblasts were seen within the lumens of blood islands, dorsal aorta and heart. Other vessels were abnormally large with ECs in lumens. The authors suggest that flt-1 signalling may regulate cell-cell or cell-matrix interactions. A homozygous flk-1 mutation in mouse embryos resulted in absence of blood islands at E 7.5 and was lethal at E 8.5 - 9.5 (Shalaby et al., 1995). Yolk sac mesoderm appeared undifferentiated and single-layered which may suggest that flk-1 marks the
proposed hemangioblast progenitor and that signal transduction through this receptor promotes
differentiation of this cell type. These results indicate that VEGF stimulation of ECs will be
manifested differently depending on the receptor which binds the ligand. Flk-1 signalling may
promote EC differentiation while flt-1 signalling may be involved in phenotype modulation.

VEGF stimulates ECs to proliferate, migrate, organize into tubes and increase their
production of other bioactive proteins. Early studies of VEGF demonstrated its ability to promote
EC proliferation (Gospodarowicz et al., 1989; Connolly et al., 1989) and migration (Favard et
al., 1991). Proliferation and elongation of ECs was observed in 3D collagen gels using
glioblastoma-conditioned medium containing VEGF (Goto et al., 1993). FGF-2 acted
synergistically with VEGF to increase EC proliferation and cord formation. VEGF also
stimulated vessel formation from rat aortic explants cultured in 3D collagen I gels (Nicosia et al.,
1994b). VEGF has been shown to increase plasminogen activator inhibitor-1 (PAI-1) production
(Pepper and Montesano, 1990), urokinase-type plasminogen activator (u-PA) activity and u-PA
receptor protein and mRNA in bovine aortic ECs (BAEC) and bovine microvascular ECs (BMEC)
(Mandriota et al., 1995) but FGF-2/VEGF synergism in u-PA activity induction could not be
demonstrated. Urokinase-type PA receptor expression was shown to be associated with migration
(Pepper et al., 1993). In vivo, VEGF may stimulate ECs to increase proteolytic activity, migrate
out from the parent vessel wall, and to proliferate and assemble into tubes within the interstitial
matrix.
Role of extracellular matrix and adhesion molecules in endothelial cell differentiation *in vitro* and *in vivo*

The extracellular matrix (ECM) is composed of insoluble protein molecules which form structural scaffolding upon which cells are located and can migrate, differentiate or proliferate (see review by Yurchenco and O'Rear, 1994). The ECM may also store soluble factors which can often be bound to the insoluble proteins in an inactive form. Most cell types will produce some ECM material. Epithelial tissues produce a specialized ECM called a basement membrane with which they interact. Cells can bind to the ECM through the integrin receptors and signalling through these ECM receptors can influence cell phenotype. ECs can also interact with one another through integrins and other cell adhesion molecules including vascular endothelial cadherin (VE-cadherin) and platelet-endothelial cell adhesion molecule (PECAM-1).

**Extracellular Matrix**

Separating the vascular ECs from the surrounding connective tissue is the endothelial basement membrane. It is composed primarily of proteins produced by the ECs and under normal conditions is the only ECM with which ECs are able to interact (reviewed by Yurchenco and Schittny, 1990; Paulsson, 1992; Yurchenco and O'Rear, 1994). The basement membrane is comprised of collagen IV, Ln, heparan sulphate proteoglycans, SPARC (secreted protein, acidic and rich in cysteine), entactin/nidogen and fibulin (Yurchenco and O'Rear, 1994). All these components will associate with one another to produce a supramolecular structure for cell attachment which also serves as a filtration barrier and a growth factor reservoir. Under abnormal conditions ECs may come in contact with the interstitial matrix. This is composed of collagen
types I and III, Fn, vitronectin (Vn) and others. Exposure to this matrix will elicit different responses of ECs in order to re-establish vascular integrity.

Much of the information about basement membrane components has been obtained from Engelbreth-Holm-Swarm (EHS) tumour. This cell line produces abundant amounts of basement membrane components including collagen IV, Ln, Fn, SPARC, and entactin and is available commercially as Matrigel. Culturing cells on Matrigel is often used in an attempt to mimic *in vitro* the conditions of an epithelium on its basement membrane. These basement membrane components may not reflect the exact composition of all basement membranes as some components are tissue-specific. Variations in molecular isoforms and ratios of the constituents also exists between different tissues. Matrigel is known to contain protease activators and growth factors which may influence cellular activities and therefore observations of cells cultured on this matrix may be misleading.

Changes in the extracellular matrix composition surrounding developing blood vessels have been observed and it is thought that these changes may influence cellular activities. Risau and Lemmon (1988) demonstrated in stage 11 chicken embryos that Fn is deposited in the periphery of blood islands during yolk sac vasculogenesis. Laminin was later detected in the endothelial basement membrane after blood island and capillary plexus formation is complete. Similarly, during brain angiogenesis, Fn was initially detected surrounding the sprouting endothelial cells. Concomitant with lumen formation, Ln was deposited in the forming basement membrane after the capillary sprout had been established. The authors suggest that the deposition of Fn provides a matrix for angiogenesis and is indicative of vessel immaturity while the appearance of Ln in the basement membrane is indicative of vessel maturity. Homozygous Fn null mice embryos have
various mesodermal abnormalities including malformed vasculature (George et al., 1993). In these embryos there was an apparent decrease in mesenchymal cell migration, lack of somites, notochord and yolk sac vessels, and variable heart and dorsal aorta formation. Blood cells could also be seen in the coelom and dorsal aortae were distended if present. Given these results, it is reasonable to assume that EC interactions with the ECM components may play a role in modulating cellular activities during these processes.

Changes in the extracellular matrix composition surrounding angiogenic blood vessels during wound healing and inflammation have been observed and it is thought that these changes may influence cellular activities. In a murine corneal assay, Ln was shown to appear in the migrating and proliferating tips of new vessels during inflammation (Form et al., 1986). The appearance of collagen IV in new vessels correlated with lumen formation. Studies of biopsied guinea pig skin wounds showed that increased Fn production in blood vessels near the wound; this was in conjunction with EC proliferation and capillary ingrowth (Clark et al., 1982). The intensity of the signal decreased as capillary ingrowth and EC proliferation ceased. Similar changes may also be seen with in vitro angiogenesis models. Rat aortic explants cultured in plasma clots produce microvessel sprouts which initially deposit Fn with collagen V but later accumulate Ln with collagen IV in the subendothelial matrix (Nicosia and Madri, 1987). ECs which had formed sprouts were shown to stain for collagen IV when the sprouts organized into tubule structures. Using rat aortic explants in collagen gels, microvessel sprouting into interstitial collagen from the cut edge of a parent vessel was shown to be enhanced by Ln as well as Ln complexed to entactin (Nicosia et al., 1994a). However angiogenesis was inhibited by high concentrations of this complex. An increase in microvessel length without an increase in
proliferation was observed following addition of soluble Fn (Nicosia et al., 1993). RGD peptides, which compete for the Fn receptor on the ECs, abolished this effect. The inhibitory effect of high Ln concentrations may be an EC response to an environment more closely resembling basement membrane. The production of specific ECM components in areas of vessel formation indicates that these cells may be responding to the ECM or that these components are produced in response to vessel formation. ECs can also influence their own activities by producing stimulatory or inhibitory ECM components.

ECM molecules are important for vessel production in vivo and EC tube formation in vitro. The basement membrane components, Ln and collagen IV have been shown to be required for these processes. The basement membrane collagen inhibitor GPA1734 decreased total collagen synthesis (Maragoudakis et al., 1988a) and angiogenesis in 9 to 12 d chicken embryo CAM (Maragoudakis et al., 1988b). The anti-tumour compound D609 was also shown to have this effect (Maragoudakis et al., 1990). In addition, GPA1734 and D609 were shown to inhibit EC invasion into subcutaneous Matrigel plugs in response to peptides containing the Ln binding site SIKVAV and capillary invasion into Matrigel by cultured ECs (Haralabopoulos et al., 1994). Endothelial cells in culture will assemble into tubes under certain conditions. Cells cultured in 3D collagen gels organize into anastomosing networks of tubes which are ultrastructurally similar to capillaries (Montesano et al., 1983). On Matrigel, ECs readily form tubes (Kubota et al., 1988; Form et al., 1986; Grant et al., 1989; Haralabopoulos et al., 1994) but this was not observed when ECs were cultured on plastic, plastic coated with one or both of Ln and collagen IV, agarose, or collagen I gels. There is evidence suggesting that attachment to Ln is necessary for this process. Laminin is required for tube formation on collagen I gels (Kubota et al., 1988).
Tube formation was also shown to be inhibited by antibodies to Ln or to synthetic B1 fragment of Ln (Form et al., 1986), synthetic peptides containing the Ln binding sites RGD, YIGSR (Grant et al., 1989) and SIKVAV (Grant et al., 1992). Cells attached to Ln via RGD site of A chain while YIGSR on the B1 chain induced cell-cell interactions and tube formation (Grant et al., 1989). Peptides containing SIKVAV were also shown to induce vessel formation on the chicken CAM and yolk sac and in a murine implanted disc assay (Grant et al., 1992). Collagen has also been demonstrated to be a requirement for in vitro tube formation. ECs grown in conditioned medium show an increased ability to form tubes when cultured on basement membrane collagen substratum than when cultured on interstitial collagen substratum (Madri and Williams, 1983). These results suggest that EC interaction with basement membrane components promotes angiogenesis and tube formation and may be analogous to the process of lumen formation during vasculogenesis and angiogenesis. Indeed, lumen formation has been shown to occur concomitant with the appearance of Ln during these processes (Risau and Lemmon, 1988).

In culture, endothelial cells have been shown to adhere, spread and migrate on various ECM components including Ln, Fn and collagen IV. EC adhesion is believed to occur through the helical domain of collagen IV (Herbst et al., 1988), the DGEA sequence of collagen I (Staatz et al., 1991), the RGD sequence of Fn (Cheng and Kramer, 1989), and the RGD, YIGSR (Grant et al., 1989), and SIKVAV (Grant et al., 1992) sequences and E8 fragment of Ln (Aumailley et al., 1991). Addition of soluble Fn to fibrin gels increased EC migration and was concentration dependent (Fournier and Doillon, 1994). Culture studies have also shown that EC proliferation may be modulated by ECM components. Laminin was shown to stimulate EC proliferation and this effect could be modulated by collagen IV (Form et al., 1986). ECs grown on Fn-coated
surfaces showed increased spreading and proliferation with increases in Fn coating density (Ingber, 1990). Soluble Fn or Fn-coated beads with ECs in suspension did not affect growth suggesting that tension dependent alterations in cell shape are required for Fn-mediated effects. The influence of ECM on proliferation is significant because during angiogenesis, proliferation takes place outside of the parent vessel where sprouting cells are within a different environment. This illustrates the different responses to ECM by ECs and indicates that changes in environment can influence cellular activities.

Integrins and other cell adhesion molecules

Integrins are a family of receptors whose ligands are the molecules comprising the ECM (see Hynes, 1992; Bosman, 1993 and Luscinskas and Lawler, 1994 for reviews). They are transmembrane heterodimeric glycoproteins composed of one each of fifteen α and eight β subunits. The pairing of the subunits determines the ligand specificity. The cytoplasmic domain of the β subunit anchors the integrin receptor to the cytoskeleton via α-actinin and talin and also co-localizes with vinculin and other focal contact proteins. Through this arrangement cells can bind to insoluble proteins of the ECM and transmit mechanical forces to the cytoskeleton for cell spreading and migration. On endothelial cells, integrins bind to elements of the basement membrane during quiescence or to interstitial molecules during migration, proliferation and differentiation associated with vasculogenesis and angiogenesis (Luscinskas and Lawler, 1994). Integrins may also form inter-endothelial junctions (Lampugnani et al., 1991) and contribute to vascular integrity along with VE-cadherin and PECAM which form homotypic bonds between ECs (Dejana et al., 1995).
Endothelial cell integrins tend to be of two types: the $\beta_1$ integrins and $\beta_3$ integrins. Beta 1 integrins are generally associated with ECM binding while $\beta_3$ integrins tend to bind plasma-derived components (Humphries, 1990). Ligands for the $\beta_1$ integrins include collagen (receptor is $\beta_1$ with $\alpha_1, \alpha_2, \alpha_3$ or $\alpha_4$), Ln ($\beta_1$ with $\alpha_1, \alpha_2, \alpha_3, \alpha_6$ or $\alpha_7$), Fn ($\beta_1$ with $\alpha_2, \alpha_3, \alpha_4, \alpha_5$ and $\alpha_6$), fibrinogen and vitronectin ($\beta_1$ with $\alpha_5$) (Humphries, 1990). These integrins recognize specific short peptide sequences which are found on the ECM proteins, including RGD, YIGSR, DGEA, and SIKVAV. The integrin $\alpha_6\beta_3$ has affinity to the RGD sequence of fibrinogen, Fn, Ln, thrombospondin, and vitronectin. ECs also express $\alpha_6\beta_4$ which is generally referred to as a basement membrane receptor. Immunohistochemical studies of large vessel and capillary ECs in situ have demonstrated the presence of $\alpha_1, \alpha_2, \alpha_3$ and $\alpha_6$ subunits of collagen/Ln receptors but $\alpha_5, \alpha_\gamma$, and $\beta_3$ subunits of Fn/fibrinogen receptors were detected only on large vessel ECs (Albelda et al., 1992). Similar results were obtained for cultured large vessel and capillary ECs with the exception of $\alpha_1$. This data demonstrates the ability of ECs to alter their integrin profiles when exposed to changes in their environment such as during inflammation and angiogenesis. It also indicates a difference between large vessels and microvessels which may reflect their different origins, roles in normal body functions or their responses to injury or inflammation.

In non-embryonic normal human tissue, integrin profiles have been shown to differ between normal capillary ECs and also in angiogenic vessels. Immunohistochemical studies of capillary ECs in situ have demonstrated the presence of $\alpha_1, \alpha_2, \alpha_3$ and $\alpha_6$ subunits of collagen/Ln receptors (Albelda et al., 1992). The integrin, $\alpha_4\beta_3$, was localized to angiogenic vessels in granulation tissues but was absent from normal vessels of skin (Brooks et al., 1994a). During FGF-2- or TNF-α-induced angiogenesis on chicken embryo CAM there was an increase in this
Integrin. Integrin expression clearly seems to be altered in reactive ECs. These differences may therefore reflect different functions of these cells in their respective environments.

Interruption of integrin binding \textit{in vivo} has demonstrated the role of these receptors during processes such as angiogenesis. Beta 1-null teratomas have impaired growth and vessel formation compared to teratomas produced by normal embryonic stem cells (Bloch et al., 1997). In this study, endothelial cells present within the teratoma were of mouse host origin and not derived from \(\beta_1\)-null cells. Similarly, \(\beta_1\)-null chimeric mice do not produce \(\beta_1\)-null endothelial cells (Fassler and Meyer, 1995). This indicates a requirement of \(\beta_1\) integrins for vessel formation.

Antibodies to \(\alpha_v\beta_3\) inhibited angiogenesis on CAM and, in tumours cultured on CAM, caused tumour regression and vessel disruption (Brooks et al., 1994b). Vessels undergoing normal angiogenesis in CAM did not express \(\alpha_v\beta_3\) while those treated with FGF-2 or TNF-\(\alpha\) showed an increase in this integrin (Brooks et al., 1994a). These authors suggest that induction of angiogenesis causes ECs to express \(\alpha_v\beta_3\) and enter cell cycle while anti-\(\alpha_v\beta_3\) induces apoptosis in activated cells and does not affect quiescent vessels. Anti-\(\alpha_v\beta_3\) decreased neovascularization of cornea and CAM by FGF-2 and TNF-\(\alpha\) while anti-\(\alpha_v\beta_5\) decreased neovascularization of cornea and CAM by VEGF, TGF-\(\alpha\) or phorbol ester (Friedlander et al., 1995). RGD cyclic peptide blocked neovascularization by both groups of growth factors. This indicates that neovascularization in response to different growth factors may be mediated by different integrins.

Integrin binding and signalling are important events during embryonic and adult angiogenesis and may follow a common mechanism (Drake et al. 1995).

Integrins are believed to be involved in vasculogenesis. Wu and Santoro (1994) detected \(\alpha_2\) subunits on mature and immature ECs in E 13.5 mouse embryos, most prominently on ECs
that had aligned into tubes. In this study it was assumed that the integrin detected was $\alpha_2\beta_1$. Integrin-ECM interactions may also influence EC differentiation and vessel development. Anti-$\beta_1$ injected into quail embryos at 4-10 somites inhibits dorsal aorta vasculogenesis. Angioblasts assemble into tubules which results in failure of aortic primordia to form patent vessels (Drake et al., 1992). Anti-$\alpha_3\beta_3$ injected into this region at the 3 to 4 somite stage results in inhibition of lumen formation, fragmentation of the dorsal aortae and impaired anastomosis of lateral vascular plexus vessels with each other and the dorsal aorta (Drake et al., 1995). Studies of gene mutations have also demonstrated the importance of integrins in differentiation of ECs. A loss-of-function mutant of the murine $\alpha_5$ gene had mesodermal abnormalities including vascular leakages (Yang et al., 1993). Dorsal aortae and yolk sac vessels released blood cells into the extracoelomic space. These findings indicate the importance of integrin signalling in the development of vessels during vasculogenesis. VE-cadherin may also play a key role during vasculogenesis. It is co-expressed with $flk-1$ in yolk sac mesodermal cell aggregates of E 7.5 mouse embryos and in peripheral EC precursors of blood islands and ECs of embryonic vessels in E 9.5 mouse embryos (Breier et al., 1996).

Endothelial cells in culture are known to express several different integrins, including $\alpha_1\beta_1$ (Albelda et al., 1989; Defilippi et al., 1991; Klein et al., 1993), $\alpha_2\beta_1$ (Albelda et al., 1989; Languino et al., 1989; Lampugnani et al., 1991), $\alpha_3\beta_1$ (Albelda et al., 1989; Klein et al., 1993), $\alpha_5\beta_1$ (Albelda et al., 1989; Lampugnani et al., 1991; Klein et al., 1993), $\alpha_5\beta_1$ (Albelda et al., 1989; Klein et al., 1993), $\alpha_6\beta_1$ (Klein et al., 1993), $\alpha_6\beta_4$ (Klein et al., 1993) and $\alpha_4\beta_3$ (Albelda et al., 1989). Immunohistochemical studies of cultured large vessel and capillary ECs have demonstrated the presence of $\alpha_2$, $\alpha_3$ and $\alpha_6$ subunits of collagen/Ln receptors but $\alpha_5$, $\alpha_6$ and $\beta_3$ subunits of
Fn/fibrinogen receptors were detected only on large vessel ECs (Albelda et al., 1992).

There is evidence that integrins mediate EC adhesion, spreading and migration while also contributing to cell-cell adhesion and monolayer permeability. Antibodies to the \( \alpha_5\beta_1, \alpha_2\beta_1, \alpha_3\beta_1 \) and \( \alpha_4\beta_3 \) receptors inhibited EC adhesion to their ligands as did soluble purified receptors (Albelda et al., 1989). Endothelial adhesion to Ln has been shown to be prevented by antibodies to \( \alpha_6\beta_1, \alpha_4\beta_3 \) and the \( \alpha_2 \) subunit (presumably blocks \( \alpha_5\beta_1 \)) (Languino et al., 1989). Isolated EC receptors that bind to Ln include \( \alpha_1\beta_1, \alpha_2\beta_1, \alpha_6\beta_1, \) and \( \alpha_4\beta_3 \) although \( \alpha_4\beta_3 \) is not RGD-sensitive (Albelda et al., 1989). Cultured ECs have \( \beta_1 \) receptors aligned with stress fibers while \( \beta_3 \) receptors are located at focal adhesions (Albelda et al., 1989). Beta 1 organization is influenced by Ln and Fn while \( \beta_3 \) is influenced by Fn (Basson et al., 1990). EC migration on Ln was shown to be RGD-sensitive and \( \beta_1 \) organization dissipated. In addition, both \( \beta_1 \) and \( \beta_3 \) integrins have been demonstrated at vinculin-positive focal adhesions (Albelda et al., 1989). Cultured ECs organize \( \alpha_2\beta_1 \) and \( \alpha_4\beta_1 \) along cell-cell contacts and maintain monolayer continuity (Lampugnani et al., 1991). Antibodies to these receptors and RGD peptides have been shown to disrupt cell-cell contacts. In addition, anti-\( \alpha_4\beta_1 \) increased the permeability of the monolayer. VE-cadherin and PECAM are also located at cell-cell contacts in vitro although VE-cadherin is associated with adherens junctions and is located more apically than PECAM-1 (Ayalon et al., 1994). Endothelial cells migrating into an in vitro wound showed a decrease in VE-cadherin (Lampugnani et al., 1995) in mechanically wounded EC monolayers. These results indicate both a specific role for integrins and also the ability of integrins to bind multiple ligands.

Integrins play a role during in vitro tube formation in 2D and 3D cultures. EC monolayers form tubes in response to soluble collagen I (Jackson et al., 1994). This was shown to be blocked
by anti-$\alpha_2\beta_1$ while antibodies against the individual subunits were less effective and RGD peptides were ineffective. In 3D cultures containing collagen I in the medium, EC tube formation was also blocked by anti-$\alpha_2\beta_1$, but not by RGD peptides (Jackson et al., 1994). EC tube formation on Matrigel was blocked by antibodies to the subunits of $\alpha_6\beta_1$ (Bauer et al., 1992; Davis and Camarillo, 1995) and the concentration required was lower than that required to block adhesion (Bauer et al., 1992). ECs contracted the gel and organized matrix guidance pathways through which cell processes of adjacent cells were able to form contacts (Davis and Camarillo, 1995). This indicates that $\beta_1$ integrins with $\alpha_2$ or $\alpha_6$ may mediate tube formation.

Growth substances have been shown to influence integrin expression. Alpha 2 and $\beta_1$ subunits and $\alpha_2\beta_1$ integrin were increased by FGF-2 in 2D culture (Enenstein et al., 1992). FGF-2-treated ECs showed an increase $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_\gamma\beta_3$ and endothelial cell adhesion molecule and the appearance of $\alpha_6\beta_1$ (Klein et al., 1993). A decrease in $\alpha_1\beta_1$, $\alpha_\gamma\beta_1$ and $\alpha_6\beta_3$ was observed. ECs were shown to have an increase in both $\alpha_\gamma$ and $\beta_3$ in response to FGF-2 (Sepp et al., 1994). TGF-$\beta$ and IFN-$\gamma$ decreased $\alpha_6\beta_3$ and inhibited FGF-2-induced increases in expression and morphological changes. Certain growth factors can increase or decrease integrin expression in ECs although culture conditions may influence this effect. Growth factor stimulation can enhance integrin-mediated processes due to the change in integrin profile. This change in profile may mediate EC activities observed during angiogenesis and vasculogenesis.

Rationale

Extracellular matrix components such as Fn and Ln have been shown by previous investigators to influence endothelial cell behaviour in culture, but a comprehensive description
of the changes in ECM deposition during dorsal aorta formation has not been reported. Previous studies have not attempted to correlate the expression patterns with morphological changes. Fibronectin and Ln have been suggested to be involved in blood vessel development in the embryonic chicken (Risau and Lemmon, 1988) but no study has examined the distribution of these molecules prior to stage 11 when key morphological events take place. Furthermore, there have been no reported studies investigating these molecules during vasculogenesis as it pertains to the dorsal aorta. These ECM components could potentially act as ligands for the integrins $\alpha_2\beta_1$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$. These three integrins have been suggested to play a role in blood vessel formation. Although several integrin subunits including $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_6$, $\alpha_v$, $\beta_1$, $\beta_4$, and $\beta_3$ have been identified on endothelial cells in culture, there has not been any comprehensive description of the changes in integrin subunit expression during dorsal aorta formation in the chicken embryo. Previous developmental studies of integrin expression have not examined the early stages at which dorsal aorta development occurs. Furthermore, the stages selected tend to provide a discontinuous time line and have not attempted to correlate the expression patterns with morphological changes.

My research project employs a variety of complementary techniques to define roles for the molecules that are believed to be important in vivo vessel formation using the chicken embryo as a model. The chicken embryo model offers several advantages. In contrast to commonly used mammalian models, such as the mouse, in which the staging system is based on time post-coitum, staging of the chicken embryo is event- and feature-dependent and therefore more precise. In addition, this model is inexpensive, fast and simple and allows for rapid analysis of many specimens. Unlike the mouse embryo, the chicken embryo is easily accessible and amenable to surgical manipulation. I have examined vasculogenesis of the dorsal aorta because the
morphological events of this process have been well-characterized. Immunohistochemistry was used to localize ECM components, adhesion molecules and endothelial markers which have not previously been investigated thoroughly during this process.

One approach to investigate the role of ECM and receptors has been to use gene knockouts, e.g. Flk-1, Fn, α5 and β1 integrin genes. The major limitation of this approach is that gene knockouts produce global effects. For example, in the case of the Fn gene knockout, the effects on vasculogenesis may be the consequence of a disturbance in cellular activities prior to the onset of the process. It may also result in embryonic lethality before the effects on a process can be completely evaluated. The approach employed in the present study is to block important receptor-ligand interactions at specific points in time by administration of antibodies and peptides. This method has not been fully utilized for the analysis of vasculogenesis.

Although the production of vessels is a normal process in the vascularization of tissue during development and wound healing, it can be an unregulated process in many diseases (Folkman and Shing, 1992). Vascularization of tumours allows increased growth and the potential for metastasis. In other diseases, excessive vessel growth impairs the function of the tissue as in arthritis and diabetic retinopathy, where vessels invade the synovial joint and the vitreous body of the eye, respectively. Specific molecular changes occur in endothelial cells which allow them to become angiogenic. Understanding the molecular events that regulate endothelial cell differentiation during normal vessel formation in the chicken embryo may reveal potential points of control and provide direction for therapeutic strategies.
Hypothesis

One or more of the $\beta_1$ integrins, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and/or $\alpha_6\beta_1$, is involved in lumen formation during dorsal aorta vasculogenesis in the chicken embryo and their subunits are expressed during dorsal aorta vasculogenesis by angioblasts prior to and during lumen formation. The ligand for the integrin(s) is(are) expressed in the same location as the angioblasts (in the case of $\alpha_2\beta_1$ and $\alpha_5\beta_1$, the ligand may be located on adjacent angioblasts). Peptides that prevent the binding of the integrin(s) to the ligand(s) will prevent lumen formation when injected into the embryo just prior to dorsal aorta vasculogenesis.

Objectives

The objectives of this study were to:

1. Confirm the pattern of morphological events that occur during dorsal aorta formation with previous studies.

2. Determine spatial and temporal distribution of the ECM components, Ln and Fn and the integrin subunits $\alpha_2$, $\alpha_5$, $\alpha_6$, and $\beta_1$ and compare the distributions of these molecules with morphological events.

3. Examine and interpret the effects induced by injection of integrin blocking peptides and antibodies.

4. Define roles for the integrins studied during dorsal aorta vasculogenesis based on information obtained for objectives 1, 2, and 3.
MATERIALS AND METHODS

Animal model

Fertilized White Leghorn chicken (Gallus domesticus) eggs (Coastline Chicks, Abbotsford, BC) incubated in a humidified atmosphere (56%) at 38°C were used in all studies. Experimental manipulation of embryos was carried out in ovo under sterile conditions. The location of the embryo within the egg was determined by placing the egg over a “candling box”, a sealed box containing a standard light bulb and single oval hole slightly smaller than the egg which allows light to exit. The shadow cast by the embryo on the shell was circled with a pencil. The shell in the pencilled area and at the blunt end of the egg containing the air space was then wiped with 70% ethanol. These areas were allowed to dry and then covered with clear adhesive tape. Watchmaker’s forceps were used to puncture the shell through the adhesive tape first at the blunt end of the egg, then at the pencilled area. Approximately 1.5 ml of albumin was withdrawn from the egg through the puncture in the blunt end using a sterile needle (21G) and syringe. This allows the embryo to fall away from the top of the shell and thereby facilitates handling and injection. A circular hole of about 1 cm in diameter was cut around the pencilled area using alcohol-cleaned scissors, beginning at the puncture site. A third hole was made in the shell approximately at the level of the embryo. Through this hole was inserted a 16 G needle which was then used to pierce the yolk sac. The end of the needle was placed under the embryo bevel side up. Sterile filtered India ink, (Pelikan, Hannover, Germany) which does not contain quick-drying agents, was diluted 1:40 with Tyrode’s saline (Appendix I; Stern, 1993) and was injected beneath the embryo to provide a contrasting background. (Other types of India ink contain
varnishes that are toxic to embryos, resulting in embryo death; Joy Richman, personal communication). The embryo was then staged according to the morphological criteria outlined by Hamburger and Hamilton (1951). If the embryo was not used immediately, the openings in the shell were covered with transparent tape and the egg was returned to the incubator.

**Processing of Embryos**

For studies of normal aorta development, embryos were removed at appropriate stages. Embryos that had been injected with blocking agents and control embryos were dissected 4 h post-injection. Watchmaker’s forceps were used to pierce and grasp the extraembryonic membranes close to the embryo. A 1 cm disk of extraembryonic membrane containing the embryo proper was dissected and removed using the forceps. The embryo was placed in a plastic petri dish (35 mm x 10 mm) containing phosphate-buffered saline (PBS). The vitelline membrane was removed and excess yolk was washed from the ventral side of the disk with PBS using a disposable pipette. The embryo was then spread flat in the dish with forceps and PBS was withdrawn using a pipette. The fixative appropriate for the processing method (see below) was added carefully to the dish by pipette so as not to disturb the disk. All subsequent steps were carried out in the petri dishes to ensure that the disks remained flat.

**Light microscopy**

Fixation was carried out in 2.5% (v/v) glutaraldehyde in 1 M phosphate buffer at pH 7.4 at room temperature for 15 min. Fixative was drawn off and embryos were rinsed in 3 changes of PBS for 5 min each. The embryos were subsequently dehydrated in 30%, 50%, 70%, 80%,
90%, and 95% (v/v) ethanol for 3 min each. Specimens were then infiltrated with a 1:1 solution of 95% ethanol: catalysed JB4 component A (Polysciences, Warrington, PA) for 1 h. This solution was replaced with 100% catalysed JB4 component A and specimens were left overnight at 4°C. Embryos were trimmed then placed in polyethylene moulding cup trays (6 x 12 x 5 mm; Polysciences, Inc., Warrington, PA) which were filled with a 1:25 solution of JB4 component B: catalysed JB4 component A. To facilitate polymerization, the moulds were placed in a sealed plastic container which was then flooded with nitrogen gas for 1 min and left for 1 h. The polymerized blocks were removed and trimmed with a razor blade and glued to EBH2 block holders (Polysciences, Inc., Warrington, PA). Step serial plastic sections were cut at 2 μm and every tenth section was stained with 0.25% (w/v) toluidine blue in 0.1 M sodium acetate buffer, pH 4.5.

Immunohistochemistry

For immunohistochemical experiments, embryos were fixed in Bouin's fixative at room temperature for 15 min followed by three rinses in PBS for 5 min. Embryos were embedded in gelatin using a modification of the method of Stern (1993). PBS was withdrawn from the dishes and replaced with 5% (w/v) sucrose in PBS for 2 h or until the tissue sunk to the bottom of the dish. Infiltration was then carried out with 20% (w/v) sucrose in PBS for a similar period of time. The petri dish and its contents were pre-warmed to 37°C and the solution was replaced with a 37°C solution of 7.5% (w/v) gelatin in 10% (w/v) sucrose/PBS for several hours with occasional swirling. The embryos were then oriented with forceps to lay flat in the dish and the gelatin was allowed to solidify at room temperature. A cube of the solid gelatin containing the
embryo was cut and removed from the petri dish, placed in a Tissue-Tek cryomold (Miles Inc., Elkhart, IN) and covered in Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen-cooled isopentane. Frozen serial sections were cut at 6 μm from similar axial levels and placed on Fisherbrand Superfrost/Plus slides (Fisher Scientific, Nepean, ON), allowed to air dry for 30 min and stored at -20°C.

**Immunohistochemical techniques**

Primary antibodies used in this study included anti-chicken α2 (clone MEP-17; gift of Kelly McNagny, European Molecular Biology Laboratory, Heidelberg, Germany), anti-chicken α5 (clone A21F7; Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA), anti-chicken α6 (clone P2C62C4; DSHB), anti-chicken β1 (clone W1B10; Sigma, St. Louis, MO and CSAT; gift of Clayton Buck, Wistar Institute, Philadelphia, PA), anti-chicken Fn (rabbit polyclonal; Chemicon, Temecula, CA), anti-human Ln (rabbit polyclonal; Monosan, Uden, the Netherlands), anti-CD 34 (clone MEP-21; gift of Kelly McNagny, European Molecular Biology Laboratory, Heidelberg, Germany). Antibodies were diluted with a diluent buffer (0.1 M PBS buffer pH 7.4 containing 1% (w/v) BSA, 1% (v/v) inactivated goat serum, 0.1 % (w/v) NaN3, 0.01% (v/v) Triton-X100) or, in the cases of A21F7 and P2C62C4, were applied neat. Secondary antibodies used in the studies included Oregon Green 514- or Texas Red X-labelled anti-rabbit (goat polyclonal, anti-IgG heavy and light chain) and Oregon Green 514- or Texas Red X-labelled anti-mouse (goat polyclonal, anti-IgG heavy and light chain) antibodies (Molecular Probes Inc., Eugene, OR).

Slides stored at -20°C were removed from the freezer and allowed to air dry for 15 min.
Rings of 1 cm in diameter were etched around the sections using a diamond pencil. Slides were then placed in PBS warmed to 37°C for 15 min to remove gelatin (Stern, 1993) and rinsed in PBS. The slides were then placed in a humidified chamber. Excess buffer was drawn off and 40 μl of antibody was applied within the etched ring. For primary antibodies, incubation was carried out for 16 h in a humidified chamber at room temperature while secondary antibodies were applied to sections for 2 h under the same conditions. Following incubation, the slides were washed in three changes of PBS. A negative procedural control, in which the primary antibody was omitted and replaced with diluent buffer only, was used with each series. After the final PBS rinse, the slides were mounted using Gelvatol (Appendix II; Stern, 1993) and coverslipped.

**Blocking antibodies and peptides**

Antibodies used in the injection experiments were anti-chicken β₁ integrin subunit (CSAT antibody; gift of Clayton Buck, Wistar Institute, Philadelphia, PA, and clone W1B10; Sigma Inc., St. Louis, MO), anti-chicken α₂ (clone MEP17 (McNagny et al., 1992); gift of Kelly McNagny, European Molecular Biology Laboratory, Heidelberg, Germany) and non-specific IgG (mouse monoclonal anti-rabbit, gamma chain specific; Sigma Inc., St. Louis, MO). Peptides used to block receptor-ligand interactions were RGD (Arg-Gly-Asp; Sigma, St. Louis, MO), YIGSR (Tyr-Ile-Gly-Ser-Arg; Sigma, St. Louis, MO), DGEA (Asp-Gly-Glu-Ala; Advanced Chemtech, Louisville, KY), and RGES (Arg-Gly-Glu-Ser; Sigma, St. Louis, MO). Lyophilized peptides were diluted with sterile Tyrode's saline to 10 mg/ml and 100 mg/ml. The commercially prepared antibody solutions for W1B10 were desalted to remove sodium azide (Appendix III). This was necessary due to the toxic effects of this substance (personal observations).
Administration of Antibodies and Peptides

Embryos that had reached the 5-6 somite stage (Stage 8+ to 9- according to Hamburger and Hamilton, 1951) were used for the experiments with blocking antibodies and peptides. The eggs were removed from the incubator and the tape covering the hole made earlier was cut away; the hole was enlarged slightly to allow access to the embryo. Two methods for delivery of antibodies and peptides were investigated: microsphere implants and micropipette injections.

Heparin-coated acrylic beads (Sigma Inc., St. Louis, MO) of 75-100 μm in diameter were soaked for 30 min in a solution containing an appropriate blocking agent. An incision was made in the ectoderm of stage 8+ to 9- embryos lateral to the last formed somite using a sharpened tungsten needle. Watchmaker's forceps were used to insert beads into the incision. The hole in the shell was re-sealed with transparent tape, and the eggs were returned to the incubator.

Micropipette injections were carried out as follows: a wedge-shaped notch was cut into the shell from the edge of the opening to provide a place to rest the shaft of the micropipette. Blocking antibodies and peptides were administered by injection with micropipettes pulled from borosilicate glass capillaries (TW 100F-4; World Precision Instruments, Sarasota, FL). Micropipettes were made using a Model 700a vertical pipette puller (DKI, Tujunga, CA) and bevelled with a K.T. Brown type micropipette beveller (Model BV-10, Sutter Instruments, Novato, CA). Micropipettes were sterilized by overnight exposure to ultraviolet light. The micropipettes were secured to the end of plastic tubing containing a cotton plug and reagents were drawn into the pipette by applying negative pressure to the other end of the tubing by mouth. Approximately 50 nl was drawn from a droplet (2 μl) of the appropriate reagent on a sterile weigh boat placed in a humidified chamber. The micropipette was stabilized by placing it in the notch
cut in the shell and the overlying vitelline membrane was pierced just caudal and lateral to each of the 3-4 most recently formed somites (Fig. 1). The volume of reagent was distributed among these injection sites. The hole in the shell was re-sealed with transparent tape.

Photomicrography

Photographs were taken using a Zeiss Axiophot photomicroscope. Kodak Ecktachrome EPH 1600 color reversal film for 35 mm slides (Kodak, Rochester, NY) was used for fluorescence photomicrography. Fujichrome 64T slide film was used for brightfield photomicrography (Fuji, Tokyo, Japan). Slides were then scanned using a Nikon LS-1000 slide scanner with Nikon Control Version 2.00 (Nikon, Tokyo, Japan). Scanned images were assembled into plates using Photoshop 4.0 (Adobe Systems Inc., San Jose, CA) and printed using a dye-sublimation printer.
Figure 1. Diagram showing Injection site for delivery of integrin blocking antibodies and peptides.
RESULTS

Normal development of the dorsal aorta in the chicken embryo

The normal course of dorsal aorta vasculogenesis was followed from stages 8 through 14 by examination of step serial cross sections of methacrylate-embedded chicken embryos. This was carried out to determine whether the present results were comparable to those reported previously (Gonzalez-Crussi, 1971; Hirakow and Hiruma, 1983) since breed, variation in incubation conditions or inconsistencies in staging may lead to differences.

Dorsal aortae were not present at stage 8 but individual rounded or spindle-shaped cells, presumably angioblasts, were occasionally seen and rarely, clusters of 2 or 3 cells (Fig. 2A), in the region between somites and the endoderm. Occasionally, these cells appeared to be emerging from the somite. At stage 9 they formed small aggregates of 2 to 4 cells. Cells within these clusters sometimes had a vacuolated appearance. Other groups of angioblasts had thin cellular processes extending out to form “C”-shaped arches while others occasionally appeared to completely enclose a lumen (Fig. 2B). By stage 10 the clusters contained 3-6 cells and were often completely enclosing a lumen. These cells tended to be attenuated except for their nuclei. The vessels generally appeared flattened with cell nuclei bulging into the lumen (Fig. 2C). Incomplete “C”-shaped vessels were occasionally seen. At stage 11 the dorsal aortae appeared inflated rather than flattened. Five to 10 cells could be seen in cross section at this stage and looked morphologically mature; the nuclei of these cells now displayed a flattened appearance (Fig. 2D). Individual blood cells were present within the lumens of the dorsal aortae at stage 12 (Fig. 2E). At stage 14, the left and right dorsal aortae began to converge along the midline at approximately
Figure 2. Normal dorsal aorta development in the chicken embryo.

(A) Stage 8 embryo. Note small group of angioblasts (arrow). (B) Stage 9 embryo. (C) Stage 10 embryo. Note cell nuclei protruding into the lumen. (D) Stage 11 embryo. (E) Stage 12 embryo. Blood cells are present in the lumen of the dorsal aorta. (F) Stage 14 embryo. Note convergence of the left and right dorsal aortae. (*) lumen of dorsal aorta. Toluidine blue-stained plastic sections. 275x.
the level of the fifth somite (Fig. 2F).

Immunohistochemistry of endothelial markers, ECM components and integrin subunits during normal development of the dorsal aorta

In the present study, immunohistochemistry was used to establish the temporal and spatial distribution of Ln, Fn, and the integrin subunits $\beta_1$, $\alpha_5$, $\alpha_6$ and the endothelial marker, thrombomucin during the period of dorsal aorta formation. Stage 8, 9, 10, 11, 12, and 14 ($n=3$ for each stage) embryos were fixed with Bouin's fixative, frozen in isopentane and cryostat sections were collected. The sections were stained using indirect immunohistochemistry as described in the preceding section. Positive tissue controls used included 9 d embryonic chicken brain (used in Fn and Ln immunohistochemistry), 5 d and 9 d embryonic chicken limb (used in integrin subunit immunohistochemistry), 4 d embryonic chicken eye (used in $\alpha_2$ subunit immunohistochemistry) and 9 d embryonic chicken heart (used in thrombomucin immunohistochemistry). Negative control procedures in which the primary antibody was replaced with either rabbit serum or a non-specific mouse antibody were included to assess the non-specific binding of the secondary antibodies. No non-specific binding of fluorochrome-labelled goat anti-rabbit IgG (used in Ln and Fn immunohistochemistry) (Fig. 3B) or fluorochrome-labelled goat anti-mouse IgG (used to detect antibodies to thrombomucin and all integrin subunits) (Fig. 3D) was observed.

Thrombomucin

In order to correlate the expression of endothelial markers with the developmental events
Figure 3. Assessment of non-specific binding of fluorochrome-labelled secondary antibodies. Fluorochrome-labelled goat anti-rabbit secondary antibody was applied following incubation with (A) anti-fibronectin and (B) rabbit serum. Fluorochrome-labelled goat anti-mouse secondary antibody was applied following incubation with (C) anti-\( \beta_1 \) subunit and (D) non-specific mouse antibody. Adjacent sections are shown. Immunofluorescence. 275x.
observed in this study and by previous investigators, the spatial and temporal distribution of the endothelial cell marker, thrombomucin, a sialomucin distantly related to CD 34, was examined using indirect immunohistochemistry with the monoclonal antibody MEP 21.

At all stages examined, MEP 21 immunoreactivity was strongest in the cells forming the interface between the splanchnic and somatic mesoderm. At stage 8 the staining pattern appeared punctate within the lateral plate mesoderm (Fig. 4B) and became more intense at stage 9 (Fig. 4D). Beginning at stage 10, the pattern appeared linear along the base of the cells lining the embryonic coelom and lining the apices of the cells of the neural tube (Fig. 4F) and staining in these regions subsequently increased (Fig. 5B, D, F). The ECs of the dorsal aorta occasionally expressed this marker at stage 10 and stage 11. By stage 12, the endothelium consistently expressed thrombomucin immunoreactivity albeit weakly (Fig. 5D). In addition, MEP staining was also present in a few cells of the somites and mesenchyme (Fig. 5D, F).

Fibronectin

Fibronectin was detected using an anti-chicken Fn polyclonal antibody. Fn was abundant at all stages (Table 1): strongest immunoreactivity for Fn was in the basement membranes of structures such as the neural tube, notochord, ectoderm, somites, and dorsal aortae. Fibronectin was also prominent in the extracellular spaces between mesodermal structures and also in spaces between mesoderm and ectoderm.

At stage 8, strong Fn immunoreactivity was in an incomplete line observed between the mesoderm and endoderm in the region of the developing dorsal aorta (Fig. 6A). By stage 9, when dorsal aorta lumen formation has begun to take place, the Fn immunoreactivity surrounded the
Figure 4. Immunolocalization of thrombomucin in the chicken embryo during dorsal aorta vasculogenesis. Stages 8-10.

Stage 8 embryo: (A) phase microscopy and (B) immunofluorescence. Note punctate staining in lateral plate mesoderm (L).

Stage 9 embryo: (C) phase microscopy and (D) immunofluorescence.

Stage 10 embryo: (E) phase microscopy and (F) immunofluorescence. Note staining lining the embryonic coelom (c) and neural tube (N). (*) lumen of dorsal aorta. 275x.
Figure 5. Immunolocalization of thrombomucin in the chicken embryo during dorsal aorta vasculogenesis. Stages 11-14.

Note the intense staining lining the embryonic coelom (c) and neural tube relative to the weak staining of the dorsal aorta endothelium.

Stage 11 embryo: (A) phase microscopy and (B) immunofluorescence.

Stage 12 embryo: (C) phase microscopy and (D) immunofluorescence. Dorsal aorta EC (arrowhead) is weakly stained.

Stage 14 embryo: (E) phase microscopy and (F) immunofluorescence. Note the punctate staining in the mesoderm of the somite (s). (*) lumen of dorsal aorta. 275x.
Table 1. Developmental expression of integrins and ECM during dorsal aorta formation

<table>
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</table>
Figure 6. Immunolocalization of fibronectin in the chicken embryo during dorsal aorta vasculogenesis.

(A) Stage 8 embryo. Note the discontinuous staining in the region of dorsal aorta vasculogenesis between the mesoderm (m) and the endoderm (e). (B) Stage 9 embryo. (C) Stage 10 embryo. (D) Stage 11 embryo. (E) Stage 12 embryo. (F) Stage 14 embryo. (*) lumen of dorsal aorta. Immunofluorescence. 275x.
developing dorsal aorta (Fig. 6B) and increased along the endothelial basement membrane during subsequent stages (Fig. 6C-F). In addition, immunoreactivity was also intense in all extracellular spaces within the embryo and this tended to increase as the embryo developed.

Laminin

Laminin was detected using an anti-Ln polyclonal antibody. At stage 8, staining was limited to basement membranes of the neural plate and ectoderm, where it appeared as a continuous line, and the basement membrane of the endoderm where it was punctate and discontinuous (Fig. 7A). At stage 9, the distribution was similar, but Ln immunoreactivity along the endodermal basement membrane became more continuous (Fig. 7B). By stage 10, there was occasionally a trace of immunoreactivity that was located in the developing basement membrane of the dorsal aorta (Fig. 7C; Table 1) and this became more prominent at stage 11 (Fig. 7D). The immunoreactivity was very intense in the basement membranes of the ectoderm, neural tube, notochord, and endoderm at stage 12 and began to appear surrounding mesodermal structures such as the somites (Fig. 7E). The dorsal aorta basement membrane now displayed a punctate, discontinuous distribution of Ln. By stage 14, the distribution remained similar but staining increased in intensity except in regions where the endothelium extended toward the midline of the embryo. Here, Ln immunoreactivity was often lacking (Fig. 7F). The staining around the mesodermal structures was more pronounced at stage 14.

Beta 1 integrin subunit

Expression of the $\beta_1$ integrin subunit was determined using the monoclonal antibody
Figure 7. Immunolocalization of laminin in the chicken embryo during dorsal aorta vasculogenesis.

(A) Stage 8 embryo. Note the linear staining in the basement membrane of the endoderm (arrow) and the punctate staining in the endoderm basement membrane (arrowhead). (B) Stage 9 embryo. (C) Stage 10 embryo. (D) Stage 11 embryo. (E) Stage 12 embryo. Note the intense staining in the basement membrane of the neural tube (N) and the appearance of laminin surrounding the somite (S). (F) Stage 14 embryo. Note the lack of staining around the endothelial sprout extending toward the notochord (arrowhead). (*) lumen of dorsal aorta. Immunofluorescence. 275x.
Figure 8. Immunolocalization of $\beta_1$ integrin subunit in the chicken embryo during dorsal aorta vasculogenesis by W1B10.

(A) Stage 8 embryo. (B) Stage 9 embryo. Note staining at cell-cell contacts in the forming dorsal aorta (arrowhead), the neural ectoderm (N) and notochord (n). (C) Stage 10 embryo. (D) Stage 11 embryo. (E) Stage 12 embryo. (F) Stage 14 embryo. (*) lumen of dorsal aorta. Immunofluorescence. 275x.
W1B10 which is specific for the chicken β₁ integrin subunit. In general, cells from all three germ layers showed staining with anti-β₁ primarily along cell-cell and cell-ECM contacts at all stages studied (Fig. 8A-F). The β₁ subunit was detected along cell-cell contacts of angioblasts assembling into tubes at stage 9 (Fig. 8B; Table 1) and along the base of the dorsal aorta endothelium from stage 10 on (Fig. 8C-F). The staining in the dorsal aorta endothelium appeared more intense relative to the adjacent mesoderm beginning at stage 11 (Fig. 7D-F). Strong immunoreactivity was also observed along the bases of cells of the neural ectoderm and the base and lateral sides of cells comprising the notochord beginning at stage 9 (Fig. 8B).

Alpha 2 integrin subunit

Using the monoclonal antibody MEP 17, immunoreactivity for the α₂ subunit could not be detected in control tissues including 9 d chicken embryo limb, 9 d chicken embryo heart and 4 d chicken embryo eyes. Therefore, it was not used to evaluate immunoreactivity in the dorsal aorta.

Alpha 5 integrin subunit

Using the monoclonal antibody A21F7, immunoreactivity for the α₅ subunit was not observed in stage 8 or stage 9 embryos (Fig. 9B,D). However, at stage 10, weak immunoreactivity was observed within occasional cells comprising the dorsal aorta and mesoderm (Fig. 9F; Table 1). Due to the attenuated shape of the cells, it was not clear if the expression pattern exhibited any polarity at this point. This subunit seemed to be almost exclusively expressed by cells of the dorsal aorta from stages 10 through 14 (Fig. 10B, D and F). At stages
Figure 9. Immunolocalization of α₅ integrin subunit in the chicken embryo during dorsal aorta vasculogenesis. Stages 8-10.

Stage 8 embryo: (A) phase microscopy and (B) immunofluorescence.
Stage 9 embryo: (C) phase microscopy and (D) immunofluorescence.
Stage 10 embryo: (E) phase microscopy and (F) immunofluorescence. Note the weak staining in the endothelial cell of the dorsal aorta (arrowhead) and in cells within the somite (s). (*) lumen of dorsal aorta. 275x.
Figure 10. Immunolocalization of $\alpha_5$ integrin subunit in the chicken embryo during dorsal aorta vasculogenesis. Stages 11-14

Stage 11 embryo: (A) phase microscopy and (B) immunofluorescence.

Stage 12 embryo: (C) phase microscopy and (D) immunofluorescence.

Stage 14 embryo: (E) phase microscopy and (F) immunofluorescence.

(*) lumen of dorsal aorta. 275x.
Figure 11. Immunolocalization of $\alpha_6$ integrin subunit in the chicken embryo during dorsal aorta vasculogenesis. Stages 8-10.

Stage 8 embryo: (A) phase microscopy and (B) immunofluorescence.

Stage 9 embryo: (C) phase microscopy and (D) immunofluorescence.

Stage 10 embryo: (E) phase microscopy and (F) immunofluorescence.

(*) lumen of dorsal aorta. 275x.
Figure 12. Immunolocalization of $\alpha_6$ integrin subunit in the chicken embryo during dorsal aorta vasculogenesis. Stages 11-14.

Staining is only observed in the mesoderm.

Stage 11 embryo: (A) phase microscopy and (B) immunofluorescence.

Stage 12 embryo: (C) phase microscopy and (D) immunofluorescence.

Stage 14 embryo: (E) phase microscopy and (F) immunofluorescence.

(*) lumen of dorsal aorta. 275x.
11 and 12, the staining pattern within the cells appeared diffuse (Fig. 10B, D) and by stage 14 was polarized to the cell endothelial cell bases (Fig. 10F). Five day embryonic chicken limb bud, which was used as a positive tissue control, exhibited staining in the endothelium of capillaries.

Alpha 6 integrin subunit

The distribution of $\alpha_6$ integrin subunit was determined using the monoclonal antibody P2C62C4. Immunoreactivity was not observed in the ECs of the dorsal aorta at any of the stages examined (Figs. 11B, D, F, 11B, D, and F; Table 1) and was limited to trace amounts in the bases of the cells comprising the splanchnic and somatic mesoderm from stages 11 to 14 (Figs. 12B, D, and F). In 5 day embryonic chicken limb bud, (positive control), immunoreactivity was observed in the basal aspect of the surface ectoderm.

Administration of blocking antibodies and peptides

Microsphere implants

Heparin coated acrylic beads soaked in a solution of CSAT antibody (2 mg/ml) were inserted lateral to the last formed somite ($n=10$). Although this technique did not adversely affect survival or morphology, these embryos did not exhibit somite displacement or dorsal aorta malformations similar to those described by Drake et al. (1992).

Injection by micropipette was chosen as the method of delivering the blocking antibodies and peptides rather than microsphere implantation due to the inability of the latter technique to deliver an adequate volume to achieve results similar to those of Drake et al. (1992).
Efficacy of injection technique

In order to assess the efficacy of the injection technique and to establish the pattern and time course of diffusion of the injected molecules, the following experiment was conducted.

Embryos of stages 8+ to 9- were injected lateral to the last 3-4 formed somites on the embryo’s right side with 50 nl of either mouse anti-chicken β1 integrin (CSAT) or non-specific mouse IgG as a control. At various times following injection (0, 1, 2, and 4 h), embryos were dissected and processed for cryostat sectioning. Frozen sections were stained with fluorochrome-labelled anti-mouse IgG. At least 3 specimens were examined at each time for both the anti-β1- and non-specific antibody-injected groups.

Embryos examined immediately following injection exhibited strong fluorescence in the extracellular spaces of the injected side surrounding structures such as the somites, notochord and the lateral plate mesoderm. (Fig. 13A, B). No difference in the pattern of diffusion for the anti-β1 (Fig. 13A, C, D, G) and non-specific (Fig. 13B, D, F, H) antibodies was observed except for what occasionally appeared to be some weak specific binding of the CSAT antibody to the β1 subunit. This resembled the staining pattern of anti-β1-stained embryos. By 1 h, the fluorescence on the injected side had decreased and extended to the extracellular spaces of the contralateral side (Fig. 13C, D). By 2 h, staining was only slightly greater on the injected side (Fig. 13E, F). By 4 h, the staining was weak and was the same on the contralateral and injected sides (Fig. 13G, H). 0/4

Of the 12 CSAT-injected specimens examined, 11 displayed immunoreactivity predominantly within the embryo. One embryo demonstrated staining only along the apical surface of the ectoderm.
Figure 13. Diffusion of injected antibodies in the chicken embryo.

Injected antibodies were detected with fluorochrome-labelled goat anti-mouse antibody: 0 h after injection of (A) CSAT and (B) non-specific IgG; 1 h after injection of (C) CSAT and (D) non-specific IgG; 2 h after injection of (E) CSAT and (F) non-specific IgG; 4 h after injection of (G) CSAT and (H) non-specific IgG. (*) approximate site of injection. Immunofluorescence. 275x.
Effects of anti-β₁ subunit injection

This experiment was carried out to evaluate the effects of injecting anti-β₁ subunit antibody on dorsal aorta development in ovo. A 50 nl volume of CSAT was injected into a group of embryos at stages 8+ to 9- (n=8) caudo-lateral to the last 3 to 4 formed somites into the region where the dorsal aorta is forming. The embryos were allowed to develop for 4 to 6 h and were then collected. The antibody W1B10 was injected into another group of embryos (n=8) to determine if any observed effects were due to some component of the CSAT antibody solution other than the antibody itself. A 1 in 10 dilution of the CSAT antibody was also used to assess the effect of concentration. As a control, a similar volume of non-specific IgG was injected into a group of embryos (n=6). In order to evaluate any changes due to the injection technique alone, micropipettes were inserted into embryos (n=4) at the appropriate site ("sham injection"). Gross morphology was assessed at the time of collection and specimens were further assessed by light microscopy following methacrylate processing, sectioning and staining with toluidine blue.

In 7/8 CSAT-injected embryos, an area extending 4-5 somites was observed in which the somites began to show lateral displacement, reached a maximum displacement in the mid-region of the area then receded back toward the midline. Beyond the injection site, subsequent development of structures was usually impaired: somites were decreased in size and had indistinct borders, and neural tube formation did not occur. Light microscopic examination of step serial sections revealed the absence of normally formed dorsal aortae. Instead, in 6/8 embryos dorsal aortae appeared as isolated, multicellular clumps in cross section (Fig. 14A, C; Table 2) which formed a cord located between the mesoderm and endoderm. In 7/8 embryos, somites were laterally displaced and did not appear typical: there was often no distinction between the somite
and other mesodermal structures (Fig. 14A, C; Table 2). Cells situated on the medial side of the somites often had cytoplasmic blebs protruding from the cells.

In the W1B10-injected embryos, displacement of somites and endothelial clumping were also present in 6/8 and 5/8 specimens, respectively (Table 2). These malformations were less frequent in embryos treated with a 1 in 10 dilution of CSAT (Table 2). Administration of non-specific IgG (Fig. 14B, D; Table 2) and MEP-17 anti-α2 subunit (Table 2) did not result in the aforementioned malformations. In these embryos, there were no morphological differences were observed in the dorsal aortae or other structures as compared to untreated embryos of the same stage.

Effects of integrin blocking peptides

Since many different integrins containing the β1 subunit may be present in the developing embryo, it is not clear which one(s) was (were) perturbed by the administration of anti-β1 antibodies. In order to determine which of these integrins is affected in CSAT-treated chicken embryos, the integrin recognition peptide sequences RGD, DGEA, and YIGSR were injected at the site of dorsal aorta vasculogenesis as in the anti-β1 antibody experiment. The RGD, DGEA and YIGSR peptides were used at two concentrations: 10 mg/ml and 100 mg/ml. As a negative control, RGES, a non-specific peptide, was used at the same concentrations. Use of the 10 mg/ml concentration was based on the result of Svennevick and Linser, (1993) in which RGD peptide at this concentration was shown to disrupt lens formation when injected the heads of chicken embryos in ovo. In order to evaluate any changes due to the injection technique alone, micropipettes were inserted into embryos (n=4) at the appropriate site ("sham injection").
Figure 14. Effects of injection of anti-β₁ subunit and non-specific antibody.

Embryos were collected 4 h following injection. (A) Anti-β₁ injected embryo. Note the lateral displacement of the somite (s) and malformed vessels (arrowheads). 135x. (B) Control embryo injected with non-specific antibody (135x). (C) Higher magnification of displaced somite and malformed dorsal aorta shown in (A). 275x. (D) Higher magnification of dorsal aorta shown in (B). (*) lumen of dorsal aorta. Toluidine blue stained plastic sections. 275x.
Table 2. Results of injection of integrin blocking antibodies

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<td>W1B10 (anti-β1)</td>
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<tr>
<td>sham injection</td>
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Of the 6 embryos injected with 10 mg/ml RGD, none exhibited any malformations that had been observed in CSAT- or W1B10-injected embryos, such as dorsal aorta malformations and somite displacement (Table 2). Furthermore, no malformations were seen in any of the embryos injected with 10 mg/ml DGEA or 10 mg/ml YIGSR (n=6 for each group; Table 2). Apoptotic cells were observed in the area of injection following administration of 100 mg/ml of RGD, DGEA, YIGSR and RGES in 3/13, 5/8, 5/6 and 2/7 embryos, respectively (Fig. 15A, B; Table 3). This effect was not observed with any of the peptides at a concentration of 10 mg/ml (Table 3). Sham injected embryos did not show any evidence of tissue necrosis; localized hyperplasia was occasionally observed (Fig. 15 C, D).
Figure 15. Effects of injection of integrin blocking peptides at high concentration (100 mg/ml).

Embryos were collected 4 h following injection of (A) RGD and (B) RGES (control peptide). Note the lighter staining cells, pyknotic nuclei and cell disaggregation in the area of injection. Sham injected embryos showed areas of hyperplasia (arrowhead) in the ectoderm (C) and in the mesoderm (D) in step serial sections. Toluidine blue stained plastic sections. 135x.
Table 3. Results of injection of integrin blocking peptides.

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<td>Sham</td>
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DISCUSSION

In this study, a comprehensive analysis of the spatial and temporal distribution of integrins, thought to be involved in dorsal aorta vasculogenesis, and their ligands was conducted using immunohistochemistry. Furthermore, a novel system for examining the effects of integrin blocking antibodies and peptides on dorsal aorta vasculogenesis in ovo is presented. The present system allows the introduction of reagents to the embryo in ovo and the ability to monitor for potentially longer periods than explanted embryos. It also ensures that there are not contributions to the effects seen by factors present within the culture medium or cellular interactions with matrix material of the gel in the culture medium. This is of some concern due to discrepancies seen between the various culture systems used to study vasculogenesis and angiogenesis in vitro. Of the β integrins, αβ₁, αβ₂, and αβ₃ have previously been suggested to be involved in vessel formation using various culture models. This study attempts to determine which of the integrins are involved in dorsal aorta vasculogenesis by introduction of peptides that will interfere with integrin-ligand binding.

Normal development of the dorsal aorta in the chicken embryo

A light microscopic study of toluidine blue-stained, step serial sections was performed to establish the pattern of morphological changes during dorsal aorta vasculogenesis in the chicken embryo. The results were compared with those of earlier publications to verify previous findings and confirm that there were no discrepancies due to variations in breed, incubation conditions or staging of embryos.
The temporal pattern of morphological changes during dorsal aorta vasculogenesis was similar to that reported previously by Hirakow and Hiruma (1983) using LM and TEM. A continuous cord of cells, presumably angioblasts, which will form the dorsal aorta was present by stage 9. These cells often appeared to form an incomplete tube which resembled a “C” in cross section. By stage 10 tube formation was complete. In cross section, endothelial cells formed a complete ring and their nuclei appeared to bulge into the lumen. By comparison, with SEM the dorsal aorta anlage appears as a cord of cells at stage 9- and as a tube at stage 9+ (Hirakow and Hiruma, 1981) and by LM and TEM as pairs of angioblasts incompletely surrounding a lumen (Hirakow and Hiruma, 1983). By stage 10 the tube was also complete but clefts were present between endothelial cells (Hirakow and Hiruma 1983). By stage 11, ECs assumed their typical squamous shape and the dorsal aorta had a definite rounded lumen. Beyond this stage no morphological changes were observed in the endothelium by LM.

No detectable differences in staging or in morphological events of dorsal aorta formation were observed between the present study and previous studies by Hirakow and Hiruma (1981, 1983). It was therefore assumed that relating the expression of integrins and ECM components to morphological events would be valid and that blocking agents were injected at the appropriate stages in order to repeat previous studies by Drake et al. (1992).

**Endothelial cell markers**

**Thrombomucin**

Indirect immunohistochemistry using the antibody MEP 21 was used to detect thrombomucin, a sialomucin distantly related to CD 34. This molecule, like CD 34, is
expressed by hematopoietic precursors, platelets and endothelia early in development and in adult tissue (McNagny et al., 1997). It has also been shown to be expressed in such tissues as the lining of the embryonic coelom (McNagny et al., 1997). Thrombomucin is believed to inhibit adhesion of platelets to the endothelium due to the highly negative charge of its carbohydrate residues.

In the present study, the endothelial marker, thrombomucin, was not detected in the region of developing dorsal aorta before the onset of lumen formation. Strong thrombomucin immunoreactivity was present along the splanchnic and somatic mesoderm outlining the embryonic coelom from as early as stage 9. Prior to this a punctate staining pattern was observed within the unseparated mesoderm at stage 8. The dorsal aorta endothelium began to exhibit trace immunoreactivity at stage 10 and this had increased slightly by stage 12 and remained weak at stage 14. In control tissues (9 d chicken brain), moderate staining was confined to the capillary endothelial cells. There was also immunoreactivity along the inner surface of the neural tube beginning at stage 10 and increasing in intensity at stage 12.

It is interesting that this endothelial marker was strongly expressed by cells lining the embryonic coelom and the neural tube. One possible explanation, as suggested by McNagny et al. (1997) is that this molecule may prevent adhesion between cells expressing it by virtue of its negative charge. Indeed, the embryonic coelom and inner surface of the neural tube remain as potential spaces from the time they are formed and persist in adulthood.

Although thrombomucin appears to be an early endothelial cell marker, it was not detected prior to dorsal aorta lumen formation and therefore could not be used to identify endothelial precursors.
Immunohistochemistry of ECM molecules and integrins

In the present study, immunohistochemistry was used to establish the temporal and spatial distribution of Ln, Fn, and the integrin subunits $\beta_1$, $\alpha_5$, and $\alpha_6$ during the period of dorsal aorta formation. The distribution of integrins was compared to the distribution of ECM and morphological changes. Embryos were Bouin's fixed, frozen in isopentane and cryostat sections were collected. The sections were stained using indirect immunohistochemistry. Negative control procedures in which the primary antibody was omitted and positive control tissues (9 d embryonic chicken brain for Fn, Ln and $\beta_1$ subunit, 5 d and 9 d embryonic chicken limb for $\alpha_5$ and $\alpha_6$ subunits and 4 d embryonic chicken eye), and were included with this study. The distributions of each molecule were assessed on adjacent sections of the same embryos to control for variations between individual specimens.

ECM components

Initially, Fn immunoreactivity was seen around mesenchymal cells present early on at the site of dorsal aorta formation. These cells are presumed to be angioblasts on the basis of their location. At the stage when these cells of the presumptive dorsal aorta organize into cords, Fn was detected along the bases of outermost cells of the cord. As tube formation progressed, Fn immunoreactivity became more intense in the developing basement membrane of ECs. This is the first report of the presence of this molecule during dorsal aorta vasculogenesis in chicken. Comparatively, Risau and Lemmon (1988) reported that Fn is distributed around blood islands during yolk sac vasculogenesis prior to lumen formation. The results of the present study provide further support for the hypothesis that Fn serves a scaffolding upon which angioblasts migrate,
aggregate and form tubes during vasculogenesis (Risau and Lemmon, 1988; Risau, 1995), interactions likely mediated through integrins.

In contrast to Fn, Ln was not associated with mesenchymal cells at the site of dorsal aorta formation at stages prior to lumen formation. Only after lumen formation did this ECM molecule begin to appear as part of the endothelial basement membrane. The appearance of Ln at the base of the endothelium in this study agrees with Risau and Lemmon (1988) in which Ln was immunolocalized in the basement membrane of blood islands in the chicken yolk sac and this correlated with lumen formation. The relatively late appearance of Ln during the initial morphogenesis of the dorsal aorta suggests that this molecule may not be directly influencing angioblasts in the formation of the dorsal aorta but may be deposited by ECs after endothelial cell differentiation has taken place (Bar and Wolff, 1972; Clark et al., 1982; Risau and Lemmon, 1988). It is interesting to note that at stage 14 in the region of convergence of the left and right dorsal aortae subjacent to the notochord, there is an absence of Ln immunoreactivity in the medial portion of the dorsal aorta endothelium. A possible explanation is that this represents loss of Ln in the endothelial basement membrane which may allow cells to reorganize to form a single large aorta. A loss of Ln in regions of endothelial sprout formation has been described during angiogenesis (Cliff, 1963).

Beta 1 integrin subunit

The β1 integrin subunit can form integrin heterodimers with several α subunits. Beta 1 integrins will bind to a number of ECM components including Fn and Ln. Therefore, one would expect concomitant appearance of β1 integrins and Fn and Ln as was observed in the present
study. Beta 1 immunoreactivity was also observed at sites of cell-cell contact, in particular between angioblasts organizing into tubes. The integrins $\alpha_2\beta_1$ and $\alpha_5\beta_1$ have been immunolocalized along endothelial cell borders in vitro (Lampugnani et al., 1991) but the ligands were not identified. It is possible that in the present study $\beta_1$ integrins are binding non-ECM proteins containing the appropriate recognition sequence.

An increase in $\beta_1$ integrin immunoreactivity was observed in the base of dorsal aorta endothelium concomitant with the appearance of Ln. This might represent the expression of a Ln-binding $\beta_1$ integrin. Based on the current literature, the potential candidates include $\alpha_2\beta_1$ (Languino et al., 1989) and $\alpha_5\beta_1$ (Kramer et al., 1990). Although Ln contains the RGD sequence which is recognized by $\alpha_5\beta_1$, this integrin has not been shown to bind Ln.

**Alpha 2 integrin subunit**

Antibodies recognizing the chicken $\alpha_2$ subunit were not commercially available. Immunohistochemical studies with MEP 17 failed to give positive results with control tissues. This molecule has been reported to be expressed in endothelium of E 13.5 mouse embryos (Wu and Santoro, 1994), human large vessel endothelium in situ and in human large vessel and microvessel endothelial cultures in vitro (Albelda, 1991), in embryonic chicken neroepithelium (Bradshaw et al., 1995) and in avian cartilage (Hirsch et al., 1994). The reason for the absence of immunoreactivity is not clear but the following are possible explanations. The antibody may have became inactivated during shipping or by contamination. It is also possible that the concentration of the antibody, which was obtained as a supernatant, was insufficient or that $\alpha_2$ subunit is expressed at levels undetectable with this method. Failure to obtain results prevented any
conclusions regarding the temporal and spatial pattern of expression of the \( \alpha_2 \) subunit.

**Alpha 5 integrin subunit**

Alpha 5 beta 1 is a Fn-binding integrin that recognizes the RGD peptide sequence and is the only known integrin containing the \( \alpha_5 \) subunit. (Bosman, 1993; Luscinskas and Lawler, 1994). The \( \alpha_5 \) integrin subunit has not been previously investigated during dorsal aorta vasculogenesis. Due to the extensive distribution of its primary ligand, Fn, and the \( \beta_1 \) subunit, with which it is assumed to be paired, throughout the embryo, it was expected that the \( \alpha_5 \) integrin subunit would be similarly ubiquitous. In particular, it was expected to be expressed in angioblasts that are migrating and aggregating within the Fn matrix and later along the bases of cells of the dorsal aorta endothelium.

The fact that the \( \alpha_5 \) subunit was not ubiquitously expressed was unexpected; it was largely restricted to endothelial cells and did not appear until after lumen formation had begun. Interestingly, there is a concomitant increase in \( \beta_1 \) and Fn expression in the dorsal aorta endothelium when these cells begin to express \( \alpha_5 \). Therefore, it would appear that \( \alpha_5 \) integrins are not involved in the initial aggregation of angioblasts, cord formation or lumen formation. One possibility is that \( \alpha_5 \beta_1 \) is involved in differentiation of the endothelium following lumen formation.

**Alpha 6 integrin subunit**

Alpha 6 beta 1 recognizes the YIGSR peptide sequence of Ln. The \( \alpha_6 \) subunit may also form heterodimers with the \( \beta_4 \) subunit, to form a basement membrane receptor. Considering the late appearance of Ln previously discussed, it was predicted that the \( \alpha_6 \) subunit would not be
detected until the formation of the dorsal aorta lumen.

The $\alpha_6$ subunit was not seen in the dorsal aorta endothelium during the stages studied. However, it was observed in the surface ectoderm of 5 d chicken embryo limb buds as reported previously by Bronner-Fraser et al. (1992). This lack of staining in the dorsal aorta was not expected but one possible explanation is that $\alpha_6$ integrins are present in such small amounts that they could not be detected by the method employed. Further, the antibody concentration in the supernatant may have been insufficient. It is also possible that this subunit does not appear in the dorsal aorta until vessel maturation occurs.

Based on these results, it is unlikely that $\alpha_6\beta_1$ is involved in dorsal aorta vasculogenesis. The $\alpha_6$ subunit was not detected and its ligand Ln was not present until after lumen formation.

**Injection of blocking antibodies**

The injection technique used in this study was shown to be an effective means by which reagents could be delivered in ovo to the site of dorsal aorta formation. The injected solutions were initially localized between the ectodermal and endodermal layers and were able to diffuse readily. The successful injection of integrin blocking antibodies and peptides during vasculogenic events in ovo has not previously been reported and was used to confirm that the effects of anti-$\beta_1$ injections reported by Drake et al. (1992, 1995) were not artifactual due to embryo explant culture conditions. The major differences between the present investigation and studies by Drake et al. (1992, 1995) are that Drake and coworkers explanted quail embryos, cultured them in an inverted orientation and injected them from the ventral side at the 3 to 4 somite stage. In the present study, embryos were injected in ovo from the dorsal side at the 5 to 6 somite stage; injection of embryos
at earlier stages resulted in poor survival rates. The same antibody, CSAT, was used at a similar volume and concentration and was obtained from the same source.

The effects of injecting anti-β₁ antibodies \textit{in ovo} were similar to those reported in the explant culture system by Drake et al. (1992). In 7/8 embryos, somites were displaced on the injected side and in 6/8 embryos, the dorsal aorta and some medially located vessels of the yolk sac were malformed, appearing as clumps of 3 to 6 cells in cross section. There are also regions having dorsal aorta malformations in which there is not a lateral displacement of somites. This observation was also described by Drake et al. (1992) and suggests that the vessel malformations were not due to the lateral displacement of somites. The contralateral side was also affected in 3/8 embryos and similar malformations were seen. Control embryos injected with a mouse monoclonal anti-rabbit IgG did not produce any malformations. The anti-β₁ antibody, W1B10, gave similar results which demonstrated that the effects elicited by CSAT were not due to other components of the antibody solution. A dose response effect was observed: at a lower concentration (1 in 10 dilution) of CSAT, 2/6 embryos showed a malformed dorsal aorta on the injected side.

The antibody MEP17, which is reported to block binding of α₂ to its ligand, was also injected but all 7 embryos appeared normal. As discussed in a preceding section, the activity of this antibody was in question therefore it is not possible to draw any conclusions from this experiment.

\textbf{Injection of peptides containing integrin recognition sequences}

It was expected that the RGD peptide would have elicited some response since this
sequence is recognized by several integrins, including $\alpha_5\beta_1$ and $\alpha_\pi \beta_3$. Previous work has shown that antibodies that block $\alpha_5\beta_1$, disrupt embryonic vessel formation (Drake et al. 1995) as well as angiogenesis (Brooks et al., 1994a,b). In a previous study of $\alpha_5$ null mouse embryos, yolk sac vessels were present but appeared to permit the leakage of RBCs into the embryonic coelom (Yang et al. 1993). The immunohistochemical results of the present study suggest that $\alpha_5\beta_1$ is involved in differentiation of the endothelium following lumen formation. However, these results also suggest that $\alpha_6\beta_1$ is not likely to be involved in Dorsal aorta vasculogenesis. The YIGSR peptide which is the recognition sequence for this integrin was therefore not expected to produce any malformations at the stages investigated. Although the distribution of $\alpha_2$ could not be determined, the possibility that the DGEA peptide, the recognition sequence for $\alpha_2\beta_1$, could cause vessel malformations since Davis and Camarillo (1996) demonstrated that $\alpha_2$ and $\beta_1$ blocking antibodies prevented lumen formation in 3D EC cultures. Injection of the RGES peptide, a sequence not involved in integrin-ligand interactions, was not expected to produce any malformations.

Injection of YIGSR and RGES has no effect for the reasons given above. However, the finding that injection of the peptides, RGD and DGEA, had no effect on vessel formation was unexpected. The positive results obtained in the anti-\(\beta_1\) injection experiments would indicate that this was not due to poor injection technique. Further, experiments were repeated with different batches of peptides and solutions were freshly prepared to eliminate possible loss of activity due degradation or contamination. Injection experiments were also carried out using high concentrations of peptides (100 mg/ml); this represents a ten-fold increase over that used by other investigators (Svennevick and Linser, 1993). In the case of RGD, it is possible that this peptide alone is insufficient to prevent binding of integrins to their ligands. One explanation is that the
RGD peptide may not have the same 3D configuration as the sequence would have in Fn and this may alter the binding affinity. This could be further investigated by using peptides which are held in a more rigid structure such as cyclic peptides or fragments containing the amino acids adjacent to this sequence in Fn, such as GRGDS (Nicosia et al., 1993) or GRGDSP (Lampugnani et al., 1991).

Another unexpected finding was that areas of tissue necrosis were observed at the injection site following administration of all peptides at high concentrations. At high concentrations, there may have been some precipitation of the peptides onto the surface of the cells. The surface of cells in the area of injection may be sufficiently coated with these charged peptides that binding of the cellular receptors with their ligands is prevented. The lack of certain receptor-ligand interactions may induce cells to undergo apoptosis (Brooks et al., 1994b).

General Discussion

Fn was localized at the site of dorsal aorta vasculogenesis prior to the onset of morphogenetic events and Ln appeared only after tube formation had commenced. This is similar to the expression pattern seen in yolk sac vasculogenesis (Risau and Lemmon, 1988). The $\beta_1$ subunit was expressed in cells undergoing tube formation while $\alpha_5$ appeared only after the onset of tube formation; $\alpha_6$ was not detected during the stages of dorsal aorta vasculogenesis investigated. The expression of the $\alpha_2$ subunit could not be determined. In light of the results of this study, it would seem that Fn and a $\beta_1$ integrin other than $\alpha_5\beta_1$ or $\alpha_6\beta_1$ could be involved in dorsal aorta tube formation. The possibility that $\alpha_2\beta_1$ plays a role in this process cannot be excluded. Laminin and an $\alpha_5$ integrin(s) appear after the onset of tube formation. These
molecules could be involved in the differentiation of endothelial cells. Alpha 6 integrin(s) may perform functions only associated with the differentiated ECs.

It is possible that tube formation may not involve interactions between β1 integrins and Fn. George et al., (1993, 1997) demonstrated with Fn null mice that vessels were present in the absence of Fn although they tended to be distended. In contrast, injection of anti-β1 in the chicken embryo prevents vessel formation as demonstrated in the present study and by Drake et al., (1992). Further, the present study showed that α5β1 integrin, whose primary ligand is Fn, was not present during the initial events of tube formation. If Fn is involved in the β1 integrin-mediated interactions, cells might interact with Fn through a β1 integrin other than α5β1 that recognizes the RGD sequence. An explanation for the findings of George et al. (1993) is that integrins that normally bind Fn can still interact with other ECM components containing the RGD sequence and thereby partially compensate for the absence of Fn.

Another possibility that must be considered is that the malformations induced by blocking β1 integrins may be mediated through disruption of cell-cell interactions rather than cell-ECM interactions. The present study has shown the presence of β1 immunoreactivity at points of cell-cell contact during dorsal aorta vasculogenesis, outlining mesodermal cells of the somites and along the medial and apical borders of cells comprising the neural tube and notochord. The effects observed following β1 injection, such as vessel malformation, somite displacement and failure of neural tube formation, may be due to disruption of cell-cell contacts. The process of somite condensation is believed to be dependent on cell-cell interactions (reviewed by Christ et al., 1998). Somites that formed after injection of CSAT were decreased in size and had indistinct borders suggesting cell-cell borders may be disrupted. Although the affected cells of the dorsal
aorta remain aggregated, the disruption of cell-cell interactions may alter the establishment of cell polarity or inhibit further cell differentiation. Indeed, cell junction formation in the dorsal aorta was shown to be inhibited in anti-\(\beta_1\) (Drake et al., 1992). The \(\beta_1\) integrins, \(\alpha_2\beta_1\) and \(\alpha_5\beta_1\) have been described at cell-cell contacts in endothelial monolayers and contacts have been shown to be disrupted by antibodies to these integrins as well as by RGD peptides (Lampugnani et al. 1991). Of these two integrins, the present study indicates that \(\alpha_5\beta_1\) is not likely to mediate cell-cell interactions during dorsal aorta vasculogenesis but \(\alpha_2\beta_1\) remains a possibility.

The vast array of effects produced by anti-\(\beta_1\) injection could not be accounted for by disruption of \(\alpha_5\beta_1\) or \(\alpha_6\beta_1\) interactions. The \(\beta_1\) integrin subunit and the ECM protein, Fn, and to a lesser degree, Ln, were expressed ubiquitously and very early. However, the \(\alpha\) subunits examined, \(\alpha_5\) or \(\alpha_6\), were not present at early stages and were not expressed by all structures that were affected by anti-\(\beta_1\). The observed effects of anti-\(\beta_1\) injections may be due to the blockade of other \(\beta_1\) heterodimer(s), possibly \(\alpha_2\beta_1\).

The process of vessel formation has been examined in many types of culture systems. What remains unclear is whether these culture conditions truly represent the \textit{in vivo} environment. Tube formation by endothelial cells cultured with soluble collagen I can be inhibited by anti-\(\alpha_2\beta_1\) (Jackson et al. 1994). In this system the ECs assembled the collagen along their apical surfaces and then wrapped around the fibers to produce lumens that contain ECM. However, this is not seen during \textit{in vivo} vasculogenesis (Hirakow and Hiruma, 1983) nor angiogenesis (Cliff, 1963). In contrast, anti-\(\alpha_2\beta_1\) increases lumen formation in invasive ECs from cultures grown on top of gels (Meyer et al. 1997). Endothelial cells cultured on Matrigel also have the ability to form tubes (Bauer et al. 1992; Davis and Camarillo, 1995). This process can be inhibited by either anti-\(\alpha_6\).
or anti-β₁. Grant et al (1989) found that tube formation on Matrigel is dependent on Ln and can be blocked by RGD and YIGSR suggesting that a Ln-binding integrin(s) is involved, possibly α₆β₁. From these studies, different conclusions are drawn regarding the roles of integrins during vessel forming processes and this is likely attributable to the differences in the culture systems employed. Due to the paucity of in vivo investigations with which culture studies may be compared, it is difficult to determine which of these can be viewed as a reliable model for vessel formation.

The present study may provide some insight. Studies with Matrigel lead to the conclusion that α₆β₁ is involved. However, the present study indicates the contrary; I did not detect the presence of α₆ in angioblasts during the early events of dorsal aorta formation. Matrigel contains components of the basement membrane. However, a completely formed intact basement membrane is not present around cells undergoing vasculogenesis and angiogenesis in vivo; it has been found to be deposited later as the vessel develops (Risau and Lemmon, 1988). It would appear then that a culture system involving Matrigel does not simulate the environment in which vasculogenesis or angiogenesis occur. It may simply provide an environment for the cells to attain a differentiated state without going through the process of vasculogenesis or angiogenesis. Studies with other culture media have shown observations not seen in normal vasculogenesis or angiogenesis including lumen formation around collagen fibrils (Jackson et al., 1994). In collagen I or fibrin gels, ECs initially begin forming vacuoles which may eventually form lumens (Davis and Camarillo, 1996). They also will form "C" shapes as seen during vasculogenesis of the dorsal aorta. This process was inhibited by antibodies to the subunits of α₂ and β₁ while antibodies to other subunits did not have this effect. They believe that this system may more closely resemble
vasculogenesis since the $\alpha_\text{v}\beta_3$ integrin, which has been shown to be important during angiogenesis (Brooks et al., 1994a,b) does not appear to be involved in this process. Indeed, the morphological changes during vessel formation described in the study were similar to those observed by Hirakow and Hiruma (1993) and in the present study. Furthermore, the involvement of $\alpha_2\beta_1$ in tube formation during normal vasculogenesis could not be excluded in the present study.
CONCLUSION

Integrin blocking experiments and immunohistochemical analysis of the subunits $\alpha_2$, $\alpha_5$, $\alpha_6$ and $\beta_1$ were conducted in order to determine the role of $\beta_1$ integrins during dorsal aorta vasculogenesis. A specific $\beta_1$ integrin that plays a role in dorsal aorta tube formation could not be determined. Of the integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$, only $\alpha_2\beta_1$ could not be excluded due to the absence of immunohistochemical data for the $\alpha_2$ subunit. Furthermore, of the in vitro investigations that suggest a role for these integrins in vascular tube formation, only the study by Davis and Camarillo (1996) which indicates a role for $\alpha_2\beta_1$ was in agreement with the present study which was based on immunohistochemical and morphological results. The ligand for the $\beta_1$ integrin(s) involved in dorsal aorta tube formation could not be Ln. Fibronectin could possibly act as a ligand but the lack of vessel disruption with the RGD peptide and the absence of $\alpha_2\beta_1$ during dorsal aorta tube formation makes this questionable. The ligand for the $\beta_1$ integrins may be a cell surface molecule since this subunit was observed along cell-cell contacts between angioblasts during dorsal aorta tube formation. This suggests that the dorsal aorta malformations induced by antibodies that block the $\beta_1$ subunit may be disrupting cell-cell interactions rather than cell-ECM interactions.

Further investigations could be conducted to determine the spatial and temporal expression of the $\alpha_2$ integrin subunit and develop strategies to prevent binding of integrins containing this subunit to their ligand(s). Possible approaches to this include the use of blocking antibodies, perhaps a purified sample of the MEP 17 antibody, or anti-sense oligonucleotides that would prevent translation of $\alpha_2$ subunit mRNA.


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Appendix I

Tyrode's saline Stock (10X; can be autoclaved)

Dissolve the following in 1 L of dH₂O:

- 80 g NaCl
- 2 g KCl
- 2.71 g CaCl₂·2H₂O
- 0.5 g NaHPO₄·2H₂O
- 2 g MgCl₂·6H₂O
- 10 g glucose

Before use, dilute 1:10 with dH₂O. The working solution may be buffered with bicarbonate.
Appendix II

De-salting procedure for removal of sodium azide.

Removal of sodium azide from commercially prepared antibody solutions was carried out in an Amicon Microcon (Millipore Corp., Bedford, MA) microcentrifuge filter.

1. Antibody solutions were serially diluted and concentrated 10 times with Tyrode's saline, then diluted to the appropriate concentration.

2. The concentrated, desalted solutions were sterile filtered in Amicon Microsep (Millipore Corp., Bedford, MA) 0.22μM filters prior to use.

3. All handling of the solutions from this point was carried out under aseptic conditions using sterilized pipette tips and sterile containers. Dilutions were made using sterile filtered Tyrode's saline.
Appendix III

“Gelvatol” mounting media

1. Add 2.4 g of Airvol 205 (Air Products, Utrecht, Netherlands) to 6.0 g glycerol. Stir with a stirring rod.

2. Add 6.0 mL of dH2O. Leave for about 4 hours at room temperature.

3. Add 12.0 mL of 0.2 M Tris buffer (pH 8.5).

4. Heat to 50°C for 10 minutes, mixing occasionally.

5. After Airvol dissolves, clarify by centrifugation (5000 g. for 15 minutes).

6. Add approximately 0.57 g of 1,4-diazobicyclo-(2,2,2)-octane (DABCO) (Sigma, St. Louis, MO) to make a 2.5% solution. Weigh in fume hood wearing eye goggles, respirator and gloves. Very corrosive, harmful upon exposure to mucous membranes.

7. Aliquot in air-tight containers and store at -20°C. Medium is stable at room temperature for several weeks after thawing.

8. Apply the mixture directly onto a washed/blotted slide above the tissue section. Place the coverslip carefully on top of the sections. The mixture is non-hardening, therefore coverslip edges must be completely sealed with nail polish if slides are not viewed immediately or if slides are to be stored.

Notes:

a. The solutes will not dissolve completely in steps 1 and 2. Stir the solutions so that the solute particles are evenly dispersed throughout.

b. Warm to room temperature before use. Store at 4°C after thawing.

DABCO is an organic compound which is claimed to decrease the amount of quenching of fluorescence in the stained specimen by scavenging free radicals produced by the excitation of the fluorochromes which may react with non-excited molecules of the fluorochrome.

Gelvatol is the trade name of a form of polyvinyl alcohol that sets solid after drying. An equivalent material is Airvol 205.