Embryonic Stem Cell Model Systems for Vascular Morphogenesis and Cardiac Disorders

Tom Doetschman, Marcia Shull, Ann Kier, J. Douglas Coffin

To better understand the formation of the cardiovascular system and its disease states, models amenable to manipulation must be developed. In this article we present two models. One is a small animal model for an inflammatory disorder that can lead to heart failure. Production of this model is based on the ability of blastocyst-derived embryonic stem cells, which can be genetically altered in vitro by a technique called gene targeting, to reconstitute an entire animal when reintroduced into a blastocyst and allowed to colonize the germ line of the resulting chimeric embryo. The other model is based on the capacity of embryonic stem cells to differentiate in culture into embryo-like structures called embryoid bodies. Embryoid bodies contain angioblasts, or prevascular endothelial cells, which can be induced to undergo aspects of vascular development by manipulation of culture conditions. (Hypertension. 1993;22:618-629.)

Key Words • embryonic stem cells • transfection • transforming growth factor beta • myocarditis • fibroblast growth factor • angiogenesis • vasculogenesis

Recent advances in genetic engineering provide unique opportunities to develop model systems for poorly understood developmental processes and human diseases. The embryonic stem (ES) cell is one of the vehicles by which these models are generated. ES cells are established as a cell line from the inner cell mass cells of a mouse blastocyst. These cells are naturally immortal and can be cultured and repeatedly frozen and thawed without losing their stem cell characteristics. Genetic manipulation of ES cells can occur either by addition of a new gene or by gene targeting, in which a specific endogenous gene is modified. The former is accomplished by transfection of a gene into the cells followed by nonhomologous or random integration of the introduced gene. The latter is accomplished by a molecular event in which homologous recombination occurs between a prechosen endogenous gene (target gene) and transfected DNA (targeting DNA), which contains a selectable marker gene flanked by sequences homologous to that of the endogenous gene. Homologous recombination results in the alteration of the target gene.

ES cells are cultured in their undifferentiated stem cell state on a feeder layer of primary embryonic fibroblasts that inhibit differentiation of ES cells. When undifferentiated ES cells are introduced back into a blastocyst by microinjection, a technique called blastocyst injection, they will associate with the inner cell mass cells of the embryo and chimerize the resulting animal. A germ line chimera will be produced when ES cell derivatives colonize the germ line and generate viable germ cells. Genetic alterations made in ES cells can be stably transmitted through several generations with the use of these techniques. Thus, blastocyst injection of targeted ES cells and subsequent breeding of germ line chimeric mice allow specific, preplanned genetic modifications to be transmitted into mice. We have used this procedure to generate an animal model for inflammatory heart disease. Mice were produced in which the gene for the growth and differentiation factor transforming growth factor-β1 (TGF-β1) has been ablated. With this animal model we can now study the etiology and pathogenesis of the disease state and test therapeutic procedures to intervene in the disease process.

When undifferentiated ES cells are injected subcutaneously or intraperitoneally into syngeneic mice, they will form solid and cystic tumors called teratocarcinomas (ESTCs). These tumors contain many differentiated cell types, including muscle, cartilage, bone, connective tissue, secretory epithelium, keratinized epithelium, and melanocytes. Not only are these tumors angiogenic, in that they will attract and induce growth of host endothelial cells, but they will also differentiate into vascular endothelium, demonstrating that ES cell derivatives have the potential for two modes of vascular development—angiogenesis and vasculogenesis.

ES cells can also be cultured in suspension in a differentiation state. In the absence of a feeder layer, ES cells spontaneously differentiate into embryo-like structures called embryoid bodies (EB) that recapitulate several aspects of embryogenesis. These include formation of postimplantation embryonic tissues such as embryonic endoderm, ectoderm, and mesoderm, as well as more advanced embryonic processes such as hematopoiesis, cardiogenesis, and angiogenesis. The in vitro vasculogenesis system presented here models the very first formation of the vascular network that occurs in the embryo. The advantages of this culture system are (1)
ES Cell Approaches to Cardiovascular Model Systems

FIG 1. Diagrammatic summary shows embryonic stem (ES) cell experimental system. Cross-hatching designates agouti coat color, which is dominant over black. Diagram reviews establishment of ES cell lines from blastocysts, genetic modification of ES cells, and differentiation of ES cells into three different pathways: embryoid bodies in vitro after removal of feeder layer cells, teratocarcinomas after subcutaneous or intraperitoneal injection into syngeneic hosts, or whole animals via blastocyst injection and the formation of germ line chimeras.

large amounts of EB with the potential to undergo vasculogenesis can be cultured, (2) culture conditions can be manipulated to alter the course of vasculogenesis, and (3) EB can be genetically manipulated for the study of the role of these genes in vasculogenic processes.

A diagrammatic summary of the ES cell experimental system is shown in Fig 1. Included are the establishment of ES cell lines, genetic modification of ES cells, the formation of EB and tumors from ES cells, and the potential of ES cells to reconstitute mice by means of blastocyst injection and germ line chimera production.

Transforming Growth Factor-β1–Deficient Mouse Has Acute Inflammatory Disorder

Gene targeting in ES cells provides a unique opportunity for the investigation of the in vivo function of genes thought to be important in cardiogenesis, vasculogenesis, and cardiac function, because animals deficient in the
particular gene of interest can be produced and the resulting phenotype examined in detail. The generation of a new mouse strain deficient in TGF-β1 is an example of such an investigation. TGF-β1 is a homodimeric polypeptide member of the TGF-β superfamily of multifunctional growth, differentiation, and morphogenesis factors. It exhibits multiple biologic activities, including modulation of cellular differentiation, regulation of cell proliferation,

**FIG 2.** Schematic shows targeted disruption of murine transforming growth factor-β1 (TGF-β1) gene in embryonic stem cells. a: Targeting construct consisting of TGF-β1 4.0-kb SmaI genomic fragment containing exon (E) 6 and part of exon 7. A neomycin resistance gene (neo) lacking the polyadenylation signal (XhoI–SalI fragment from pMC1neo+) was inserted into the BamHI site in exon 6 of TGF-β1. 102 nucleotides (34 amino acids) from the amino terminus of the mature peptide. b: Restriction map of the wild-type TGF-β1 genomic locus surrounding the targeting site. c: Predicted structure of the disrupted TGF-β1 allele. Arrowheads represent positions of primers used for polymerase chain reaction (PCR) analysis. The 5′ primer is located 68 nucleotides upstream of the stop codon in neo. The 3′ primer is located 74 nucleotides downstream of the SmaI site in exon 7 of TGF-β1 and is not contained in the targeting vector. Locations of probes used in Southern analysis are shown. Probe A is from the neomycin resistance gene (XhoI–SalI fragment from pMC1neo). Probe B is a 0.6-kb TGF-β1 genomic fragment (BamHI–SmaI). Probe C is a 0.7-kb TGF-β1 genomic fragment (EcoRI) not contained in the targeting vector. Restriction enzymes are indicated by B, BamHI; Sm, SmaI; and S, SstI.

**FIG 3.** a: Southern blot shows parental D3 embryonic stem (ES) cells and three polymerase chain reaction–positive clones. Genomic DNAs (15 mg) were digested with SstI, electrophoresed through 0.8% agarose, transferred to a nylon membrane, and hybridized with probe B. The observed pattern corresponds to that expected from homologous recombination of the targeting vector into the transforming growth factor-β1 (TGF-β1) locus. Difference in intensity of the bands in the digest of clone 1-1 is due to the fact that this clone contained a mixed population of targeted and untransfected cells. b: Genotype of offspring from interbreeding mice heterozygous for the targeted TGF-β1 allele. Tail DNAs from parents (116 and 119) and offspring (159 through 167) were digested with SstI, fractionated by electrophoresis through 0.8% agarose, transferred to nylon membranes, and hybridized with a TGF-β1 probe (Probe B, Fig 1). The upper band (6.7 kb) represents the wild-type allele; the lower band (2.5 kb) represents the targeted allele. +/+ Indicates homozygous wild-type; +/-, heterozygote; and −/−, homozygous targeted.
Inflammation in Transforming Growth Factor-β1–Deficient Mice

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<tr>
<th>Tissue</th>
<th>Mutants Affected/Mutants Examined</th>
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<td>Heart</td>
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<td>Lung</td>
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<td>Lymphocytic</td>
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<td>Striated muscle</td>
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and control of extracellular matrix protein production and degradation, and can exert both stimulatory or inhibitory effects on the same cell, depending on cell type and culture conditions.18-20

The three mammalian TGF-βs are expressed in numerous embryonic and adult tissues.21-29 Expression of all three isoforms in the developing heart has suggested that these growth factors may be involved in cardiogenesis.30-32 In the 7-day postcoitum mouse embryo, TGF-β1 mRNA is detected in the progenitor cells of the heart, the cardiac mesoderm cells; at 8 days, expression is detected in all endocardial cells; and by 9.5 days, expression in heart is limited to the endothelial cells overlying the cushion tissue of the atrioventricular canal and outflow tract.30 Expression of TGF-β1 mRNA persists in endothelia of the heart valves up to 1 week postpartum.30 During embryonic development, TGF-β1 mRNA is also found in endothelial cells lining major blood vessels and possibly in capillary endothelial cells undergoing angiogenesis.23,27,30

TGF-β1 may also modulate postnatal cardiac growth and development. Neonatal growth of the rodent heart involves three phases.33,34 During fetal and early neonatal periods (birth to 4 days postpartum), the heart enlarges as a result of cardiomyocyte hyperplasia. Approximately 6 to 14 days postpartum, a transition from hyperplastic to hypertrophic growth occurs, resulting from karyokinesis without cytokinesis. At approximately 14 to 21 days postpartum, an increase in heart mass results from hypertrophic growth. TGF-β1, although present in the fetal endocardium, is low to undetectable in the fetal myocardium.26,30 However, TGF-β1 mRNA and immunoreactivity are expressed in the neonatal and adult myocardium,26,34 with transcript levels becoming near maximal at approximately 7 to 14 days postpartum, the period of transition from hyperplastic to hypertrophic growth.26 Furthermore, in primary fetal and neonatal cardiomyocyte cultures, TGF-β1 inhibits mito-

gen-stimulated DNA synthesis.34 Thus, TGF-β1 may function to regulate neonatal heart growth and development by modulating cardiomyocyte proliferation and differentiation.

In the adult mouse, TGF-β1 immunoreactivity has been detected in cardiac myocytes of atria and ventricles, connective tissue of the heart valves, and the fibrous cardiac skeleton, suggesting a role in adult cardiac function. TGF-β1 may be particularly important in cardiac physiology during disease states. TGF-β1 mRNA levels are elevated in infarcted rat heart35-37 and ischemic pig heart,38 with the appearance of an additional TGF-β1 transcript in the infarcted heart.37 TGF-β1 appears to protect against injury to the heart resulting from myocardial ischemia and infarction.39 In isolated perfused rat hearts subjected to ischemia and reperfusion, TGF-β1 administration before isolation of the heart prevents injury to the coronary vasculature, preventing the decrease in endothelium-derived relaxation response to acetylcholine and eliminating superoxide ion production that normally accompany ischemia.39 In rats subjected to experimental myocardial infarction induced by occlusion of the left coronary artery, TGF-β1 administration reduces loss of myocardial creatine kinase and blocks the increase in circulating tumor necrosis factor-α activity normally observed after ischemia and reperfusion.39 TGF-β1 mRNA expression is enhanced by hemodynamic overload induced experimentally in rats by constriction of the abdominal aorta.40 In primary cultures of neonatal rat cardiac myocytes, TGF-β1 induces the fetal pattern of contractile protein expression characteristic of pressure-overload hypertrophy, inhibiting expression of α-myosin heavy chain mRNA, while upregulating expression of the three fetal contractile protein genes—β-myosin heavy chain, α-skeletal actin, and α-vascular smooth muscle actin.41,42 To investigate the role of TGF-β1 in vivo, particularly its possible role in cardiogenesis and cardiac function, we disrupted the TGF-β1 gene in mouse ES cells and derived mice lacking functional TGF-β1 gene activity.10

The targeting vector, consisting of 4.0 kb of TGF-β1 genomic sequence containing a neomycin resistance gene inserted into exon 6 (Fig 2), was introduced into D3 ES cells by electroporation. Cells were grown in the presence of G418 for 12 to 14 days to allow identification of neomycin-resistant colonies. ES cells containing a disrupted TGF-β1 gene were identified by polymerase chain reaction analysis and confirmed by genomic Southern blot analysis using a TGF-β1 genomic fragment probe (Probe B, Figs 2 and 3). Three clones contained the desired targeting event, yielding a targeting efficiency of 1 in 2×10⁶ electroporated cells.

Chimeric mice were generated by injecting targeted ES cells into C57BL/6J blastocysts and implanting the blastocysts into the uterus of pseudopregnant F1 (C3H×C57BL/6J) recipients. Mating of coat color chimeric males to CF1 females allowed the identification of animals that transmitted the ES cell–derived agouti coat color and the modified TGF-β1 allele to their offspring. Heterozygous offspring carrying one wild-type and one disrupted TGF-β1 allele are phenotypically normal and fertile. Heterozygous animals were intercrossed, and offspring homozygous for the disrupted TGF-β1 allele
were identified by Southern blot analysis of tail DNA biopsies (Fig 3).

Animals homozygous for the mutant allele were approximately 85% of the weight of their normal littersmates until approximately 20 days after birth. At this time, they exhibited a severe wasting syndrome accompanied by a multifocal mixed inflammatory cell response and tissue necrosis that led to organ failure and death. No other obvious developmental abnormalities were detected. Histological analysis of 15 homozygous animals and six normal wild-type or heterozygous littermates revealed in the mutant animals a marked degree of mixed inflammatory cell infiltration and tissue necrosis in several organs, including heart, stomach, liver, lung, pancreas, salivary gland, and striated muscle. Histological results are summarized in the Table.

The heart was affected in all mutant animals examined, although the degree of inflammation and necrosis varied among animals. In several cases, a generalized and extensive inflammatory cell infiltration involving the pericardium, myocardium, and endocardium of atria and ventricles was observed (Fig 4a and 4b). The heart infiltrates appeared to consist primarily of lymphocytes and plasmacytic cells. In addition to cardiac muscle, striated muscles of some animals also exhibited inflammation. In two animals, inflammation and necrosis of the diaphragm were severe enough to contribute to breathing difficulty and possibly death (Fig 4c). Perivascularitis was observed in the lungs of 10 of the 15 mutant animals (Fig 4d).

Inflammation and tissue damage were observed in many organs in addition to those of the cardiovascular system, including inflammation and ulceration of the stomach submucosa, inflammatory cell infiltration in the portal triad area of the liver, inflammation of the serosa of internal organs (stomach, intestine, kidney, ovary, and testis), inflammation of conjunctiva and ocular striated muscle, and multifocal inflammation of the pancreas and salivary glands. Relative to normal linterm controls, homozygous mutant animals frequently exhibited slightly enlarged lymph nodes, smaller spleens containing less white pulp, and Peyers patches that were fewer in number and had less distinct germinal centers.

Peripheral blood samples from homozygous mutant animals and an equal number of age-, sex-, and parent-matched homozygous wild-type or heterozygous controls were analyzed for total numbers and differential distributions of leukocytes. In mutant animals, the average number of white blood cells was usually elevated compared with controls because of greater absolute numbers of neutrophils and monocytes.

The presence of cytokines important in immune and inflammatory responses was determined by polymerase chain reaction analysis of mRNAs from spleen, liver, and lung from one control and two homozygous mutant animals. As expected, no wild-type TGF-β1 transcript was detected in the mutant animals. Interferon-γ, tumor necrosis factor-α, and macrophage inflammatory protein-1α, major mediators of inflammation, were elevated in liver and lung of the mutant animals relative to controls, consistent with the inflammation observed in these tissues.

Intercrosses of heterozygous animals carrying one wild-type and one disrupted TGF-β1 allele have yielded a total of 806 live offspring. Of these, 280 were homozygous wild-type, 413 were heterozygous, and 113 were homozygous for the disrupted TGF-β1 allele, revealing a significant deviation from the expected Mendelian ratios. The statistically significant decrease in the number of homozygous mutant animals suggests the occurrence of some type of embryolethality in the TGF-β1-deficient animals. In addition, the frequency of heterozygotes, relative to wild-type animals, is reduced. This may reflect a loss of heterozygous animals or a reduction in conceptions involving mutant germ cells due to the involvement of TGF-β1 in normal haploid germ cell function.

The survival of homozygous mutant animals to birth and the lack of obvious developmental abnormalities (other than reduced growth rate) may reflect both maternal rescue and compensation by other TGF-β isoforms or by additional genes. With regard to cardiogenesis, TGF-β2 is expressed at high levels in the developing heart, and TGF-β3 may function in induction of cardiac cushion tissue. Thus, it is conceivable that TGF-β1 may play a role in cardiogenesis, but in its absence the functioning of TGF-β2, TGF-β3, or both is sufficient for normal or nearly normal cardiac development. It is also possible that developmental abnormalities resulting from TGF-β1 deficiency are subtle ones that were not detected in the initial survey of pathology in mutant animals. In the heart in particular, although gross dissection and examination revealed no structural abnormalities, more detailed analyses may reveal alterations in expression of cardiac proteins and/or subtle structural defects. TGF-β1 has been reported to influence expression of certain cardiac genes; therefore, examination of cardiac-specific gene expression in the TGF-β1-deficient animals may help define the role of TGF-β1 in regulating the developmental program of cardiac gene expression. Expression of members of the...
actin and myosin gene families in TGF-β1-deficient animals is currently being examined because treatment of primary cultures of cardiac myocytes with TGF-β1 has been reported to elicite expression of fetal contractile protein isoforms. In addition, the potential role of TGF-β1 in modulating postnatal transition from hyperplastic to hypertrophic heart growth suggests that a detailed examination of heart development in homozygous mutant animals may reveal cardiac abnormalities resulting from perturbations in the transition from hyperplastic to hypertrophic growth. Quantitative analyses of cell densities of cardiomyocytes and nonmuscle cells in hearts from wild-type and TGF-β1-deficient mice will be determined to address this question. However, to eliminate possible interference from the effects of inflammatory cytokines, which are elevated in the mutant animals, preinflammatory hearts will be examined, requiring determination of the developmental stage at which the inflammatory response is initiated in the heart.

In summary, animals homozygous for the disrupted TGF-β1 allele exhibit a multifocal, mixed inflammatory cell infiltration and tissue necrosis in numerous organs, including heart and lung. The inflammatory cell infiltration into the heart and the perivasculitis observed in TGF-β1-deficient mice suggest that these animals may be useful for investigating inflammatory disorders affecting the cardiovascular system, including myocarditis, transplant rejection, and graft-versus-host reactions. Further investigation into the developmental and/or immunologic causes of the inflammatory response and determination of the multisytem interdependency of these responses should clarify the potential of this mouse as a model for these cardiac disorders. TGF-β1-deficient cells derived from homozygous mutant animals can be used for examining processes that TGF-β1 is reported to affect, including angiogenesis and hematoipoiesis. Embryonic and fetal tissue explants can be used to study in vitro organ development in the absence of TGF-β1. Isolated perfused hearts from TGF-β1-deficient animals can be used to investigate physiological properties and responses of hearts in the absence of TGF-β1 activity. Finally, rescue of homozygous mutant animals by exogenous administration of TGF-β1 can be performed. The latter two experiments involving properties of isolated perfused hearts and rescue of mutant animals are being conducted, and the results will be published elsewhere. Studies such as these should help define the role of TGF-β1 in cardiogenesis and cardiac function in normal and disease states.

**In Vitro Model for Vasculogenesis**

Descriptions of blood vessel development in the quail and mouse embryo suggest that the embryonic vascular pattern arises via two fundamental modes of vessel formation. In the first mode, termed vasculogenesis, blood vessels in the blastodisk arise de novo by aggregation and alignment of angioblasts after they differentiate from mesoderm. Vasculogenesis can also be thought of as the differentiation of angioblasts to vascular endothelial cells. In the second mode, termed angiogenesis, invasion of vascular areas in the embryo occurs by means of vascular sprouting from endothelial cells in existing vessels. Tumor angiogenesis and neovascularization during wound healing are forms of angiogenesis. The term vascular morphogenesis subsumes the two modes of vessel formation, vasculogenesis and angiogenesis, and also angioblast differentiation from mesoderm.

The experiments reported here focus on the development of an in vitro system to study the role of growth factors in vascular morphogenesis. First, we demonstrate that angioblasts are present among the various mesodermal derivatives in EB. Second, we describe conditions in which EB-derived angioblasts undergo vasculogenesis to form vascular cords. Third, we demonstrate the utility of this system for functional studies on factors thought to regulate vasculogenesis.

Previous studies have shown that differentiating ES cells have the potential to form vascular cords, but it was not clear whether they could do so in EB culture. When ES cells are injected intraperitoneally into syngeneic mice, the resulting ESTCs can develop vascular cords de novo, demonstrating the "potential" of differentiating ES cells to undergo vasculogenesis if put in a favorable environment. When EB are grafted onto a chorioallantoic membrane, they induce vascular sprouting of host endothelial cells but do not appear to undergo de novo vessel formation, demonstrating the presence of angiogenic factors or factors but no vasculogenesis. Other studies have shown that EB contain blood islets and that cells associated with them are capable of taking up an endothelial cell marker, Dil-acetylated low-density lipoprotein (DAL), by endocytosis. However, because other cell types can also take up DAL, it was not clear whether the EB actually contained endothelial cells.

To demonstrate the presence of angioblasts and endothelial cells in EB, we have used three labels: Bandeiraea simplicifolia lectin (BSL), anti-human von Willebrand factor antiseraum, and DAL. BSL and DAL label angioblasts and vascular endothelial cells; von Willebrand factor stains endothelial cells in larger vessels but not in capillaries. DAL is phagocyted by macrophages, angioblasts, and endothelial cells, and BSL labels several epithelia, including murine angioblasts and endothelial cells. However, only cells in the endothelial lineage can be labeled by both BSL and DAL. Double fluorescence tests with BSL and DAL have shown doubly labeled cells in EB in suspension culture. None of the doubly labeled cells in this study stained with antisera against von Willebrand factor, which is localized to a subset of the endothelium and appears later in vascular development. Having shown that EB can support angioblast differentiation from mesodermal cells, our second objective was to find conditions in which EB-derived angioblasts will form vascular cords. Because cultured endothelial cells will form vascular cords on Matrigel substrate, we have conducted bioassays to demonstrate that EB cultured on Matrigel will sprout vascular cords. To determine the utility of this system for functional studies on regulatory molecules, we added known angiogenic factors such as fibroblast growth factor (FGF)-1 and FGF-2; this also demonstrated that FGFs stimulate growth and differentiation of angioblasts into vascular endothelium in EB. FGFs are among the best studied growth factors for tumor angiogenesis; they facilitate endothelial regrowth in vascular lesions and are angiogenic in cornal and chorioallantoic membrane assays. In the embryo, expression data have shown that FGF-1, FGF-2, and FGF-5 are associated with blood vessel formation. Here,
FIG 5. Photomicrographs show embryoid bodies and teratocarcinoma blood islets. a: Bandeiraea simplicifolia lectin labels endothelium of a blood islet in an oblique frozen section of a 10-day embryoid body (bar=25 μm). b: Transmission electron micrograph of a teratocarcinoma. Note the classic structure of a blood islet, with an outer layer of endothelial cells (e) surrounding hematopoietic cells in the center (bar=10 μm).

we demonstrate that ES cell in vitro differentiation provides an excellent model system for functional analysis of morphogens in vascular development.

ES cells cultured in suspension in the absence of feeder layers results in their differentiation into EB. After 8 to 10 days of suspension culture, EB vary from 1 to 5 mm in size, and approximately 10% of these contain pigmented blood islets in cystic EB (Fig 4e). The configuration of the blood islets in EB are different from the blood islets seen in ESTCs. Blood islet EB are globular in nature, and few cordlike structures are associated with them (Fig 4e); ESTC blood islets have a more reticular structure, reminiscent of a capillary network (Fig 4f). Morphologically, the EB and ESTC blood islets are similar (Fig 5a and 5b, respectively) because they both contain hemangioblasts surrounded by a shell of endothelium-like cells. However, these cells in EB fail to develop the vascular cords seen in ESTCs. Moreover, after 14 days of EB suspension culture, the blood islets begin to lose their pigmentation and disappear, with a simultaneous loss of endothelial cells.

Considerable differences are noted for EB when cultured in versus on Matrigel (Fig 6). When 3- to 10-day EB are cultured on Matrigel, they maintain their integrity as cystic EB but sprout cellular processes. After 1 to 2 days of culture on Matrigel, an area of altered matrix appears around each EB, and long, thin cellular processes grow in a directed manner between the EB (Fig 6a). These processes are similar to those described for several cell types grown on Matrigel, and the directional growth is believed to be caused by alteration of the matrix by the tissues resulting in "matrix lines" or "tracks." These processes occasionally contain one to two cells along their distance and fail to label with the DAL and BSL markers, suggesting that they are not endothelial in their composition.

Culture of 3- to 10-day EB in Matrigel causes an immediate loss of the cystic structure of the EB as it flattens and spreads (Fig 6b). After 3 to 4 days of culture in Matrigel, endothelial cells migrate a considerable distance from the EB, far beyond the other cell types shown in Fig 6b. The small stellate cells found throughout the culture shown in Fig 6b can be labeled with both DAL (Fig 7a) and BSL (Fig 7b), indicating that they are angioblasts. They often migrate to the borders of the Matrigel, then grow as a monolayer. Occasionally, these cells begin to align as rudimentary vascular cords, but this is limited.

Previous experiments had indicated that several exogenously added growth factors did not enhance blood islet formation (data not shown). We believe that they could not penetrate the outer endoderm layer of the EB in suspension culture, thereby preventing the factors from reaching the target cells. Therefore, we added FGF-1 and FGF-2 to EB/Matrigel cultures, allowing access to tissues inside the EB. Addition of FGF-1 or FGF-2 to the Matrigel culture greatly enhances the outgrowth of angioblasts and vascular cord formation (Fig 8). This suggests that FGF is acting as a potent morphogen during vasculogenesis and perhaps during angioblast differentiation from mesoderm. No difference is seen between FGF-1 and FGF-2 in this effect,
Matrigel cultures show embryoid bodies (EB). a: EB cultured on Matrigel form modified areas around the EB and send cellular processes between the EB within the first 48 hours of culture. Occasionally, a cell body can be seen along the process. These types of cords fail to label with Dil-acetylated low-density lipoprotein or Bandeiraea simplicifolia lectin (bar=55 μm). b: EB in Matrigel immediately flatten and lose their cystic structure. Unlike EB cultured on Matrigel, endothelial cells show a propensity to migrate away from the EB cultured in Matrigel, beyond the visible cells to the periphery of the Matrigel (bar=290 μm).

Matrigel culture of ESTCs in the presence of FGF (Fig 9) results in the outgrowth of more DAL/BSL-positive angioblasts than was the case with EB Matrigel culture. Moreover, development of endothelial cell cords from the ESTC is also increased. Curiously, unlike EB Matrigel cultures, no difference is evident in the number and size of beating cardiac muscle foci is apparent when FGF is added to EB in Matrigel.

Paracrine culture of ESTCs in the presence of FGF (Fig 9) results in the outgrowth of more DAL/BSL-positive angioblasts than was the case with EB Matrigel culture. Moreover, development of endothelial cell cords from the ESTC is also increased. Curiously, unlike EB Matrigel cultures, no difference is evident in the number and size of beating cardiac muscle foci is apparent when FGF is added to EB in Matrigel. In contrast, ESTCs spontaneously undergo vasculogenesis. They contain blood islets that differentiate into a capillaryplexus, thus attaining a more advanced stage of vascular development than the EB in which blood islets are globular and eventually degenerate. These differences may be due to the lack of morphogenetic factors in the EB cultures that are supplied by the animal in ESTCs. Thus, the different properties between ESTCs and EB are exploited by allowing systematic addition or deletion of morphogens to differentiating EB to gain the efficacy of the ESTC and ultimately the in vivo environment. We believe this model system can be used to delineate the cellular and molecular mechanisms for the actions of regulatory molecules in blood vessel growth and development.

Summary

The unique property of ES cells to differentiate into a wide variety of embryonic tissues in vitro and to reconstitute a mouse when reintroduced into the proper embryonic environment provides us with numerous opportunities to develop model systems for various
aspects of embryogenesis in culture and for various genetic disorders in whole animals. We have described here a model for studying early events in mammalian vascular morphogenesis that have been difficult to approach in the past because of the lack of a manipulable system in which these events occur. We have also described a mouse generated through targeted ablation of the TGF-β1 gene in ES cells. The TGF-β1-deficient

FIG 7. Photomicrographs show outgrowth of migrating angioblasts from embryoid bodies cultured in Matrigel. a: Cells labeled with Dil-acetylated low-density lipoprotein (bar=31 μm); b: cells labeled with Bandeiraea simplicifolia lectin (bar=28 μm).

FIG 8. Photomicrographs show cord formation from an embryoid body in Matrigel culture in the presence of added fibroblast growth factor (10 ng/mL). a: Phase-contrast photomicrograph; b: positive Dil-acetylated low-density lipoprotein staining of endothelial cords. Similar cords were also stained with Bandeiraea simplicifolia lectin (not shown). Bars=100 μm.
FIG 9. Photomicrographs show cord formation from a teratocarcinoma in Matrigel culture in the presence of added fibroblast growth factor, a: Phase-contrast photomicrograph; b: positive Dil-acetylated low-density lipoprotein staining of endothelial cords. Similar cords were also stained with Bandeiraea simplicifolia lectin (not shown). Bars=100 μm.

mouse develops an acute, multifocal inflammatory reaction that leads to organ failure and death. Nearly all TGF-β1-deficient animals develop myocarditis and a few succumb to heart failure, indicating that this animal may be a potentially useful model for investigating aspects of myocarditis and its role in heart failure.

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