

Brain derived neurotrophic factor is an endothelial cell survival factor required for intramyocardial vessel stabilization

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SUMMARY

Brain derived neurotrophic factor, BDNF, is a neurotrophin best characterized for its survival and differentiative effects on neurons expressing the trk B receptor tyrosine kinase. Although many of these neurons are lost in the BDNF^{-/-} mouse, the early postnatal lethality of these animals suggests a wider function for this growth factor. Here, we demonstrate that deficient expression of BDNF impairs the survival of endothelial cells in intramyocardial arteries and capillaries in the early postnatal period, although the embryonic vasculature can remodel into arteries, capillaries and veins. BDNF deficiency results in a reduction in endothelial cell-cell contacts and in endothelial cell apoptosis, leading to intraventricular wall hemorrhage, depressed cardiac

contractility and early postnatal death. Vascular hemorrhage is restricted to cardiac vessels, reflecting the localized expression of BDNF and trk B by capillaries and arterioles in this vascular bed. Conversely, ectopic BDNF overexpression in midgestational mouse hearts results in an increase in capillary density. Moreover, BDNF activation of endogenous trk B receptors supports the survival of cardiac microvascular endothelial cells cultured from neonatal mice. These results establish an essential role for BDNF in maintaining vessel stability in the heart through direct angiogenic actions on endothelial cells.

Key words: Brain derived neurotrophic factor, Endothelial cell, Angiogenesis, Mouse

INTRODUCTION

Angiogenesis is a precisely regulated process that coordinates the assembly and differentiation of numerous cell types to form the arteries, capillaries and veins of the pre-existing vascular bed. The primitive vasculature, composed of an endothelial plexus, is patterned into arteries and veins by the recruitment of pericytes and vascular smooth muscle cells by soluble growth factors secreted by endothelial cells (Risau, 1997). In later steps of vessel formation, endothelial cells are stabilized by interactions with the extracellular matrix and ensheathment with pericytes. Several growth factors regulate vasculogenesis and angiogenic sprouting, including vascular endothelial growth factor (VEGF), fibroblast growth factors (bFGF and FGF-2), and the angiopoietins (reviewed in Darland and D'Amore, 1999; Carmeliet, 2000). In addition, platelet derived growth factor B, ephrin B2 and TGFβ have been shown to regulate later aspects of the angiogenic process, in the recruitment of mural cells and patterning of the vascular bed. Little is known about growth factors that regulate the stabilization and survival of the mature vasculature, although angiopoietin-1 has been proposed as a candidate molecule (Thurston et al., 2000).

Of these growth factors, only VEGF has been rigorously tested for its ability to initiate angiogenesis in the adult period in preclinical and clinical trials (Losordo et al., 1998; Mack et al., 1998; Hendel et al., 2000). Although delivery of VEGF by gene transfer can initiate an angiogenic response in ischemic tissues, exogenous VEGF also induces the formation of fragile and malformed vessels (Drake and Little, 1995; Springer et al., 1998; Thurston et al., 2000). In addition, endothelial cells of postnatal rodent vessels may become independent of VEGF for their continued survival within several weeks of birth (Gerber et al., 1999). Thus, defining the cellular steps and growth factors that direct and maintain microvascular assembly is necessary to identify therapeutic targets for repair and adaptive remodeling.

In recent studies, we have investigated the roles of the neurotrophins in regulating cardiovascular development and modulating the vascular response to injury (Donovan et al., 1995, 1996; Kraemer et al., 1999). The neurotrophins consist of a family of five related polypeptide growth factors: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophins 3, 4 and 6 (NT-3, NT-4 and NT-6, respectively) (reviewed in Lewin and Barde, 1996). These structurally related proteins mediate their actions on responsive

neurons by binding to two classes of cell surface receptor. The p75 neurotrophin receptor binds all neurotrophins and modulates signaling initiated by the second class of neurotrophin receptors, the *trk* family of receptor tyrosine kinases (Chao and Hempstead, 1995). *Trk A*, *trk B* and *trk C* selectively bind NGF, BDNF and NT-3, respectively, and *trk B* can also be activated by NT-4 (Snider, 1994). *Trk B* and BDNF are expressed at high levels by central and peripheral neurons, and gene ablation studies have demonstrated the critical roles for *trk B* and BDNF in neuronal differentiation and survival, with gene targeted animals exhibiting abnormalities in cerebellar function and peripheral sensory nerves (Jones et al., 1994; Ernfors et al., 1994; Schwartz et al., 1997).

However, BDNF and *trk B* mRNAs are expressed in several nonneuronal tissues, including muscle, heart and the vasculature, at levels comparable to those of the brain (Scarlsbrick et al., 1993; Timmusk et al., 1993; Donovan et al., 1995; Hiltunen et al., 1996). In pathological states, BDNF and *trk B* expression are induced in neointimal vascular smooth muscle cells of the adult rodent and human aorta following vascular injury (Donovan et al., 1995). To identify potential novel functions for BDNF during development, we undertook an analysis of mice lacking expression of BDNF, or overexpressing BDNF in the developing heart, to assess potential biological actions of this growth factor during cardiovascular development.

MATERIALS AND METHODS

BDNF mutant mice

Heterozygous (+/-) BDNF mice (Ernfors et al., 1994), (*Bdnf*^{tm1Jae} and C57BL/6J backcrossed *Bdnf*^{tm1Jae}, Jackson Laboratories) were intercrossed by brother/sister matings. For embryo analysis, the morning of the detection of a vaginal plug was considered day 0.5, and the gestational age was confirmed by morphological criteria including limb bud, eye development, length and weight at embryo harvest. The genotype of embryos or neonates was determined using head derived DNA and PCR amplification as described (Ernfors et al., 1994). Following sacrifice, bodies were fixed immediately in 3% paraformaldehyde in PBS for 18 hours. Dissected tissues were embedded in paraffin for histological analysis, or infiltrated with 30% sucrose in PBS prior to cryoprotection in 30% sucrose:OCT (1:1; Tissue Tek, Sakura, CA) for immunohistochemistry. For electron microscopic analysis, the hearts were immediately removed from newborn mice sacrificed by decapitation, and fixed in Karnovsky's fixative for 18 hours prior to embedding in Epon. 1 µm sections were stained with Toluidine Blue for initial evaluation, then ultrathin sections were counterstained with lead citrate and viewed with a JEOL electron microscope.

Immunohistochemical analysis

Monoclonal antiserum specific for smooth muscle specific α -actin (Dako, 1A4, 95 ng/ml) or biotinylated antiserum specific for CD 31 (Pharmingen, MEC 13.3, 5 µg/ml) was used to detect vascular smooth muscle cells or endothelial cells, respectively. In adult heart sections, Bandeira Simplicifolia Lectin I (Pierce, 33901, 5 µg/ml) was used to detect endothelial cells. Polyclonal antiserum specific for BDNF (R & D Systems, AF248, 5 µg/ml), β -catenin (Transduction Laboratories, C19220, 2 µg/ml) or *trk B* (Santa Cruz Immunochemicals, sc-8316 and sc-12-G, 2 µg/ml) were used on tissues which had been snap frozen in sucrose/OCT and cryostat sectioned. The specificity of BDNF antisera was confirmed by the absence of staining of neural tissues from BDNF^{-/-} mice. Additionally, preincubation of polyclonal

antisera with the immunizing peptide was used to confirm antibody specificity. Sections were treated with 0.1% hydrogen peroxide, followed by the primary and signal amplification utilizing the avidin:biotinylated horseradish peroxidase complex method (ABC Vectastain, Vector Labs). TUNEL procedure was performed as per the manufacturer's recommendation (Roche Molecular Biochemicals) using frozen sections. Proliferating cell nuclear antigen (PCNA) immunoreactivity was detected using paraffin embedded sections, pretreated with proteinase K, then Target Unmasking Fluid (Signet) as per the manufacturer's protocol, prior to incubation with monoclonal antibody PC10 (Signet, 1 µg/ml). Double immunofluorescence microscopy was performed using either FITC- or rhodamine-conjugated secondary antibodies and visualized using an Axioskop microscope or a Zeiss confocal microscope to generate 0.5 µm optical sections.

Generation of NesPIXpBDNF mice

The generation of transgenic mice has been described (Ringstedt et al., 1998). Briefly, the NesPIXpBDNF construct consisted of a region 5.8 kb upstream from the initiation codon of the mouse *nestin* gene, followed by a 1 kb fragment from the fifth exon of the mouse gene containing the complete BDNF protein coding sequence, a 300-bp-long SV40 polyadenylation signal and 5.4 kb of the *nestin* gene downstream sequence including introns 1, 2 and 3. The construct was injected into fertilized mouse oocytes, transplanted into pseudopregnant females, and embryos were harvested at E17.5-E18.5. The thoracic contents were either fixed in 4% paraformaldehyde for paraffin embedding or snap-frozen prior to frozen sectioning. Head tissue was used for genotyping using PCR as described (Ringstedt et al., 1998).

Capillary counts

Immunohistochemistry was performed on heart sections using the anti-CD 31 antiserum, which detects all vascular endothelial cells (Gerber et al., 1999) or anti-smooth muscle-specific α -actin, to detect vascular smooth muscle cells. Subepicardial regions were examined in a blinded manner, using NIH Image, or counted with a gridded eyepiece. Six fields were counted per section in each of four BDNF^{-/-} or BDNF^{+/+} littermates, or from four Nes-BDNF and four wild-type littermates.

Cell culture

Cardiac microvascular endothelial cells (CMEC) were isolated from the hearts of C57/Bl6 mice at postnatal day 3 according to established protocols (Lodge et al., 1992). In brief, minced hearts were digested with collagenase and DNase I, and endothelial cells were further purified by differential plating. CMECs were maintained on gelatin-coated plates in DMEM/F12 medium containing 5% fetal bovine serum, 0.1% mouse serum, insulin, transferrin and selenium (1:100, Gibco) and endothelial cell growth supplement (Intracell, 30 µg/ml) and used at passage 1 or 2. Approximately 0.5-1.0 × 10⁶ cells were isolated from 20 neonates, and cell purity was quantitated using acetylated LDL binding and CD 31 expression assessed by flow cytometry as described (Bergers et al., 1999). For TUNEL analysis or β -catenin immunolocalization, 4 × 10⁴ cells/cm² were plated on gelatin-coated Permanox slides, and cultured for 24 hours. Cells were washed and refed with X-Vivo20 (Biowhittaker, Walkersville, MD, USA) containing 0% serum ± the indicated growth factor, or in X-Vivo20 containing 10% serum for 24-48 hours, as indicated prior to fixation. For TUNEL assessment, 1500 cells/well were scored for TUNEL positivity. Immunofluorescence detection of β -catenin was evaluated blind in duplicate conditions in three independent experiments.

To assess annexin V binding, cells were cultured as above with the addition of 1 ng/ml bFGF to the medium. After 24 hours, cells were washed, and refed with X-Vivo20 containing 0% serum and growth factors as indicated, or X-Vivo20 containing 10% serum. After 48

hours, cell suspensions were generated using PBS/EDTA, washed in serum-free DMEM, and annexin V binding determined by incubating the cells with FITC-conjugated Annexin V (Immunotech, Miami, FL, USA) in DMEM containing 1.5 mM Ca²⁺ on ice for 10 minutes. After washing to remove unbound annexin V, cells were incubated with propidium iodide and samples analyzed by flow cytometry using a Coulter Elite system.

RT-PCR

Total RNA was extracted from CMEC (passage 1) and from adult murine brain and 1 µg was subjected to reverse transcription using murine leukemia virus transcriptase (Perkin-Elmer). Total RNA not incubated with reverse transcriptase was used as a negative control. RT-PCR was performed using primer sequences for BDNF as described, or modified for kinase active trk B to reflect codon usage in the murine sequence (Labouyrie et al., 1999). PCR products were resolved by electrophoresis in 7% acrylamide gels, and visualized with ethidium bromide.

Echocardiographic imaging

Within 48 hours of birth, all animals in a litter were subjected to sonographic imaging in a blind fashion by placement of a 40 MHz Scimed coronary probe in warmed gel on the anterior chest wall, using a Clear View Ultra Boston Scientific system (model I5006) with real-time image analysis. Images of the short axis and long axis of the heart were identified, and imaging proceeded for a minimum of 4 minutes per animal. The cardiac rate, chamber dimensions and wall motion were determined on each animal by analysis of recorded images, using the Diagnostic Off-line analysis system. Animals were sacrificed for genotyping and histological analysis within 4 hours of imaging.

RESULTS

BDNF and trk B are selectively expressed by vessels of the neonatal and adult mouse heart

Although expression patterns of BDNF and trk B in the peripheral and central nervous systems have been well described (Lewin and Barde, 1996), expression in the

developing and adult vasculature has not been comprehensively examined. In the adult mouse, vascular expression of BDNF was detectable in the heart and skeletal muscle, but absent in the liver, kidney, spleen (Fig. 1A,C-F). Within the heart, BDNF expression was restricted to arteries and capillaries, and not detectable in veins (Fig. 1A). Endothelial cells were identified using double immunofluorescence confocal microscopy to detect Bandeira lectin and BDNF, and colocalization was apparent in coronary arteries and cardiac capillaries (Fig. 1G,H). Vascular smooth muscle cells in large muscular arteries exhibited BDNF immunoreactivity (Fig. 1B and data not shown), consistent with prior reports (Donovan et al., 1995; Hiltunen et al., 1996; Scarisbrick et al., 1993). Thus, in the adult mouse BDNF is selectively expressed by endothelial cells within the heart and skeletal muscle, and by smooth muscle cells of large arteries.

In a developmental analysis of BDNF vascular expression, mouse tissues from embryonic day 13.5 (E13.5) to postnatal day 4 (P4) were examined. BDNF immunoreactivity was first detectable in a minority of vessels in the heart at E17.5, and is expressed by most cardiac arteries and capillaries by P2 (Fig. 1I). Using double immunofluorescence microscopy, colocalization of CD 31 and BDNF was apparent in arteries of the neonatal heart (Fig. 1J). As in the adult, BDNF expression is undetectable in the vascular beds of the neonatal kidney, liver and spleen (data not shown). Thus, the restricted pattern of vascular expression of BDNF is established at the time of birth, and persists into adulthood.

Immunohistochemical localization of trk B in the adult mouse reveals expression in vessels of the heart and skeletal muscle (Fig. 2A,C), but not within the vascular beds of many organs, such as the kidney and liver (Fig. 2D,F). In the heart, trk B is expressed by some capillaries, and by both endothelial and mural cells of coronary arteries, but not in coronary veins (Fig. 2A). Endothelial cells were identified by double immunofluorescence microscopy to detect Bandeira lectin and trk B, and colocalization was noted in some capillaries in the

Fig. 1. Expression of BDNF in adult and neonatal mouse tissues. Immunohistochemical detection of BDNF in sections from adult (A-H) or postnatal day 2 (P2) (I,J) wild-type mice. (A-F,I) VIP-based immunodetection yielded a red reaction product. (A) Left ventricle; (B) aorta; (C) diaphragm; (D) kidney; (E) spleen; (F) liver; (I) neonatal left ventricle. Immunohistochemistry using antisera preincubated with the immunizing peptide, or with control IgG to document antisera specificity, are shown in panel inserts. (G,H,J) Confocal double immunofluorescence detection of lectin (G,H) or CD 31 reactivity (J) (using a rhodamine-conjugated secondary antibody) and BDNF reactivity (using FITC-conjugated secondary antibody). (G, H) Adult left ventricle; arrowhead shows colocalization of actin and BDNF reactivity; (J): neonatal left ventricle. a, artery; v, vein; c, capillaries; ad, adventitia; l, lumen. Scale bars, 40 µm (A,D,E,F,I), 40 µm (B,C), 20 µm (G,H), 40 µm (J).

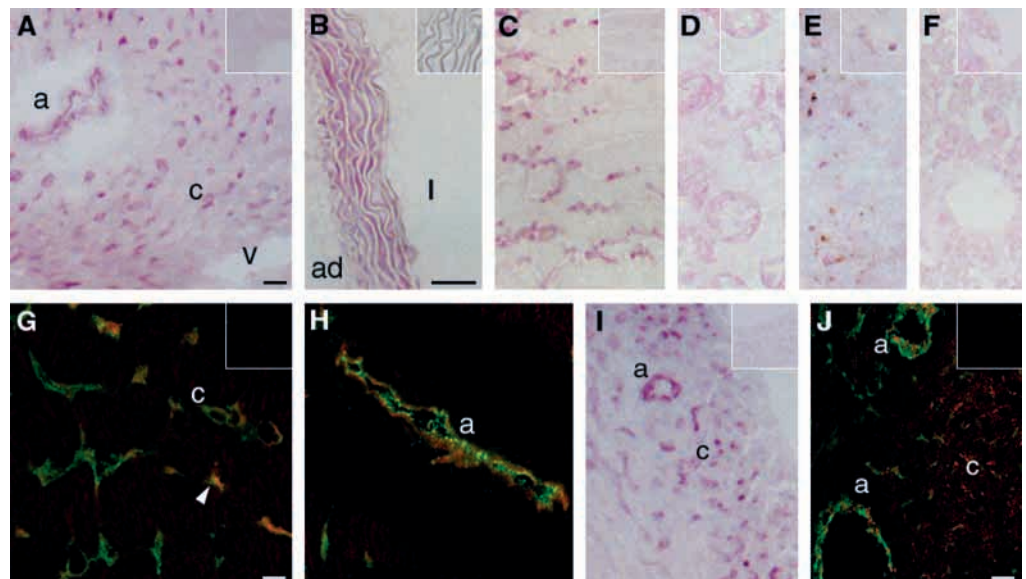


Fig. 2. Expression of trk B in adult and neonatal mouse tissues.

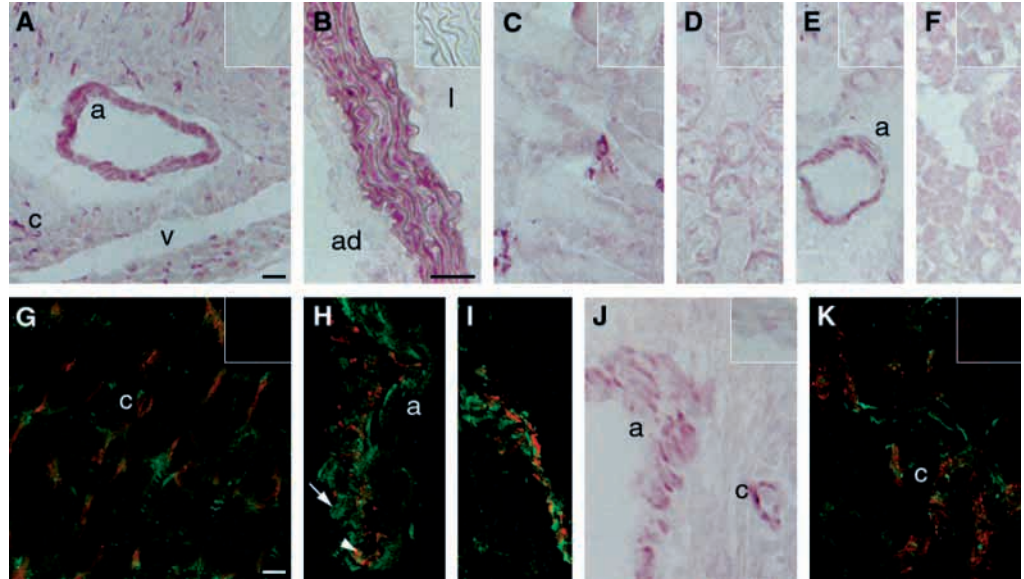
Immunohistochemical detection of trk B in sections of adult (A-I) and P2 (J,K) wild-type mice.

(A-F,J) VIP-based immunodetection yielded a red reaction product. (A) Left ventricle; (B) aorta; (C) diaphragm; (D) kidney; (E) lung; (F) liver; (J) neonatal left ventricle.

Immunohistochemistry using antisera preincubated with the immunizing peptide, or with control IgG to document antisera specificity, are shown in panel inserts. Antiserum specific for either extracellular or intracellular epitopes of trk B yielded comparable results.

(G-I,K) Confocal double immunofluorescence detection of

lectin (G,H) or CD 31 reactivity (I,K) (using a rhodamine-conjugated secondary antibody) and trk B reactivity (using FITC-conjugated secondary antibody). (G,H) Left ventricle; arrowhead shows colocalization of lectin and trk B reactivity; arrow, trk B expression alone. (I) Diaphragm; (K) neonatal left ventricle. a, artery; v, vein; c, capillaries; ad, adventitia; l, lumen. Scale bars, 40 μ m (A,D,E,F), 40 μ m (B,C,J), 20 μ m (G,H,I,K).



ventricular wall. However, analysis of the coronary arteries identifies not only cells that bind lectin and express trk B, but also cells in a mural distribution that express only trk B (Fig. 2G,H). In skeletal muscle, trk B was expressed by CD 31 positive cells in a capillary-like distribution (Fig. 2C,I). Vascular smooth muscle cells in large muscular arteries (Fig. 2B,E) expressed trk B, consistent with previous reports (Donovan et al., 1995; Scarisbrick et al., 1993). Thus, trk B is selectively expressed in the adult vasculature in a pattern overlapping with BDNF, by endothelial cells in the heart and skeletal muscle, and by smooth muscle cells of large arteries. However, in coronary arteries, BDNF expression appears to be restricted to endothelial cells, whereas trk B is expressed by both endothelial and mural cells.

To assess the onset of trk B vascular expression, sections of mice from ages E13.5 to P4 were examined. Trk B immunoreactivity was first detected at E18.5 in a minority of ventricular wall capillaries, but becomes prominent in coronary arteries and a subpopulation of capillaries by P2 (Fig. 2J). Coexpression of both CD 31 and trk B was detected in the ventricular wall using double immunofluorescence microscopy (Fig. 2K). Thus, BDNF and trk B are first expressed late in gestation selectively by the capillaries and arteries in the heart. This expression pattern persists into adulthood, by which time

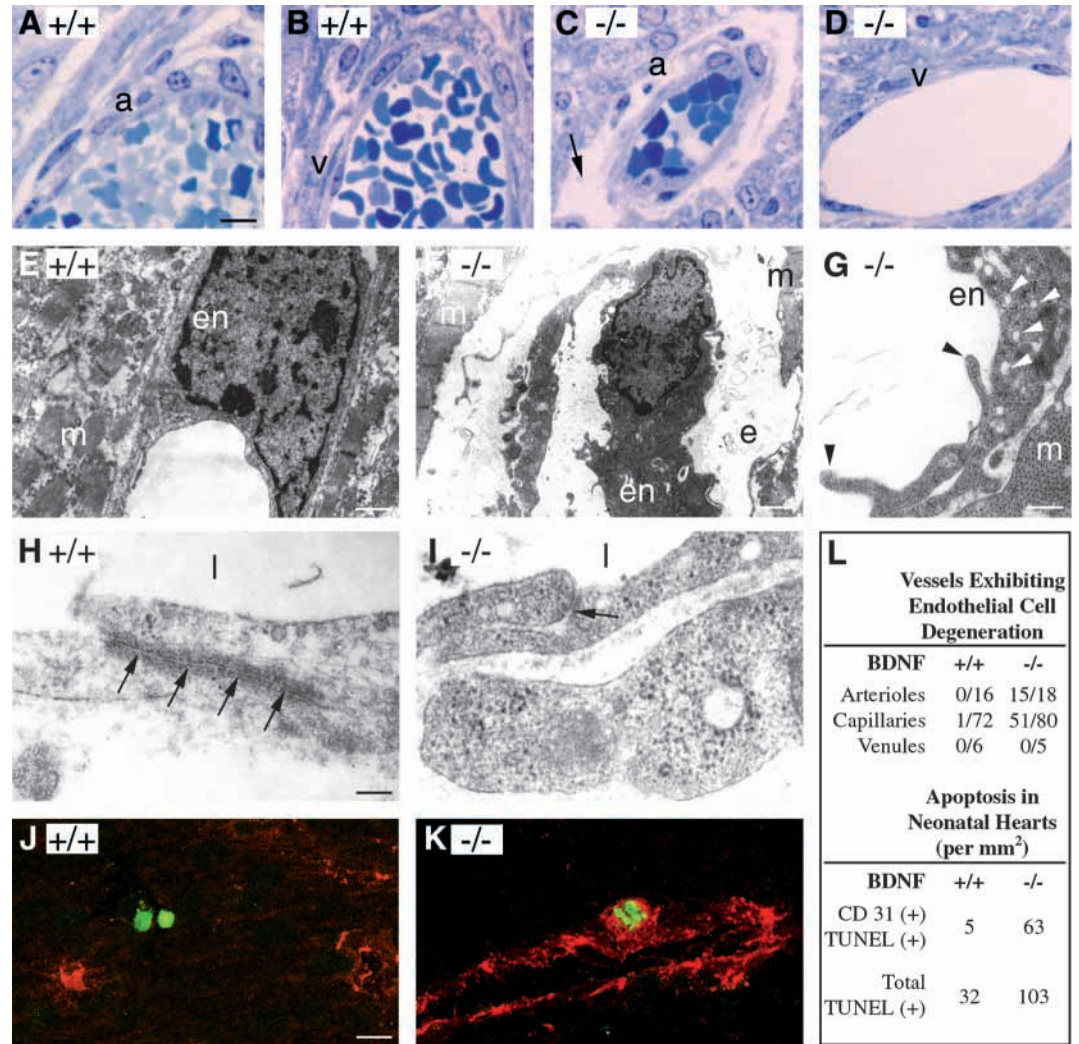
the vascular beds of other muscles also coexpress trk B and BDNF.

Vascular defects in *BDNF*^{-/-} mice

Given the expression of BDNF and trk B in selective vascular beds, we examined mice with a targeted deletion of the *BDNF* gene to identify potential actions of the gene in mammalian vascular development. *BDNF*^{-/-} mice demonstrate well-

Fig. 3. *BDNF*^{-/-} neonates exhibit ventricular wall hemorrhage. Histological analyses of *BDNF*^{+/+} (A,C,E) or *BDNF*^{-/-} (B,D,F-H) littermates sacrificed at P0. Hematoxylin and Eosin stained sections reveal hemorrhage in the epicardial third of both right and left ventricular walls of *BDNF*^{-/-} neonates (B,D, arrowheads) and an atrial septal defect (B). Pulmonary hemorrhage is detectable in *BDNF*^{-/-} (F) but not *BDNF*^{+/+} littermates (E). Hemorrhage was not detectable in other organs, such as kidneys (G), skin and spinal cord (H). ra and la, right and left atria; rv and lv, right and left ventricles; asd, atrial septal defect; s, skin; m, skeletal muscle; vb, vertebral body; sc, thoracic spinal cord. Scale bars, 150 μ m (A,B), 50 μ m (C,D), 100 μ m (E-H).

Fig. 4. Endothelial cell abnormalities in $BDNF^{-/-}$ animals. (A-D) Epon-embedded, Toluidine Blue stained thin sections of ventricles from P0 animals of the indicated genotype with abnormal arteriole (C), but normal venule (D) in $BDNF^{-/-}$ sections (arrow in C indicates perivascular edema). (E-I) Electron micrographs of ventricles from $BDNF^{+/+}$ or $BDNF^{-/-}$ P0 littermates. Vacuolated endothelial cells (G, white arrowheads) with a disorganized extracellular matrix, perivascular edema (F) and projections into the vessel lumen (G, black arrowheads) were consistently detected in capillaries and arterioles of $BDNF^{-/-}$ animals. $BDNF^{+/+}$ endothelial cells adhered to each other via well developed junctions over long distances (H, arrows) whereas junctional complexes between $BDNF^{-/-}$ endothelial cells occurred over shorter distances and were less frequent (I, arrow). m, cardiac myocyte; en, endothelial cell; e, perivascular edema. (J,K) Confocal immunofluorescence microscopy to detect CD 31 (rhodamine-conjugated secondary antibody) and TUNEL positivity (using FITC) in $BDNF^{+/+}$ and $BDNF^{-/-}$ E18.5 embryos. Most TUNEL positive cells in $BDNF^{+/+}$ embryos did not express CD 31, whereas the majority of TUNEL positive cells express CD 31 in $BDNF^{-/-}$ embryos. Note the apoptotic nuclear morphology in the $BDNF^{-/-}$ section (K). Scale bars, 10 μ m (A-D), 1 μ m (E), 2 μ m (F), 0.5 μ m (G), 0.2 μ m (H,I), 15 μ m (J,K). (L) Quantitative analysis of endothelial cell degeneration and apoptosis in $BDNF^{-/-}$ and $BDNF^{+/+}$ PO hearts. (Upper table) EM analysis of ventricular wall vessels from two $BDNF^{-/-}$ and two $BDNF^{+/+}$ PO littermates was performed blind. (Lower table) Double immunofluorescence analysis of CD 31 (+) and TUNEL (+) cells in six sections from two $BDNF^{-/-}$ or two $BDNF^{+/+}$ E18.5 embryos. The increase in endothelial cell degeneration and apoptosis in $BDNF^{-/-}$ animals is significant ($P < 0.002$, two-tailed t -test).



characterized losses of *trk B* expressing peripheral sensory and central neurons (Ernfors et al., 1994; Jones et al., 1994; Schwartz et al., 1997). However, most $BDNF^{-/-}$ pups die within 1-4 days of birth, with reduced body weight and cyanosis, suggesting the presence of potential defects in cardiovascular development. At postnatal day 0 (P0), heart size and the anatomical relationships of the heart and great vessels appeared normal. 14 of 15 $BDNF^{-/-}$ animals examined histologically, however, exhibited both atrial septal defects and intramyocardial hemorrhage, which ranged from focal areas within the left ventricular wall, to diffuse hemorrhage within the walls of both ventricles and the base of the atria (Fig. 3B,D). Intramyocardial hemorrhage was typically confined to the epicardial regions of the ventricular walls, and was rarely observed in the interventricular septum. Serial section analysis of three $BDNF^{-/-}$ neonates revealed hemorrhage only within the

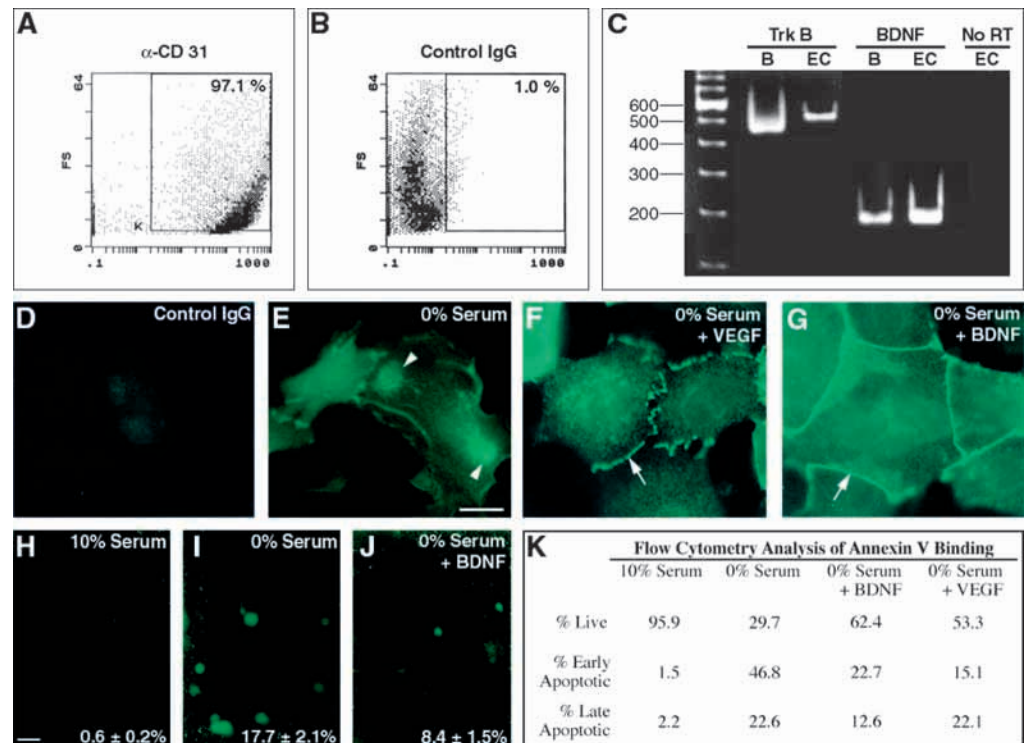
heart wall and lung, with no hemorrhage in the central nervous system, liver, kidney, skeletal muscle or skin (Fig. 3F-H).

To identify defects in vessel morphogenesis in $BDNF^{-/-}$ animals, ultrastructural analysis was performed on two $BDNF^{-/-}$ and two $BDNF^{+/+}$ animals at P0. Intramyocardial arterioles of $BDNF^{-/-}$ animals exhibited perivascular edema whereas the intramyocardial venules appeared normal (Fig. 4C,D). By electron microscopic analysis, endothelial cells within arterioles and capillaries appeared enlarged and focally degenerated, with a vacuolated cytoplasm and prominent plasma membrane blebbing (Fig. 4F,G). The majority of endothelial cells within arterioles and capillaries in sections of hearts from $BDNF^{-/-}$ animals exhibited cytoplasmic vacuolization, detachment from the underlying basement membrane, and perivascular edema, whereas no abnormalities in endothelial cells within veins were noted (Fig. 4L). Furthermore, in the capillaries of $BDNF^{-/-}$

Fig. 5. Effects of BDNF on cardiac microvascular endothelial cells (CMEC). (A,B) Flow cytometric analysis of CMEC incubated with anti-CD 31 antisera (A) or control IgG (B). 97% of cells exhibit CD 31 reactivity and 1.0% react with control IgG. X-axis is log fluorescence intensity. (C) RT-PCR analysis of transcripts for BDNF and kinase active trk B mRNA in CMEC (EC) and adult murine brain (B). Amplification of the BDNF sequence (181 bp) and trk B kinase domain sequence (471 bp) is detectable in CMEC and brain samples. Confirming the absence of DNA contamination, RNA samples amplified using primers without the reverse transcription step yielded no products (no RT).

(D-G) Immunolocalization of β -catenin in CMECs. Cells were cultured in the indicated conditions in the presence of BDNF (25 ng/ml) or VEGF (10 ng/ml) for 24 hours prior to

fixation and immunofluorescence detection using anti- β -catenin (E-G) or control IgG (D). With serum deprivation (E), β -catenin localizes to the nucleus (arrowheads, E); however, VEGF and BDNF maintain β -catenin clustering at regions of cell:cell contact (arrow, F,G). Results are representative of three experiments. (H-J) TUNEL analysis of CMEC. Cells were cultured in media containing 10% serum (H), 0% serum (I) or 0% serum plus BDNF (100 ng/ml) (J) for 48 hours. 1500 cells per sample were analyzed and means \pm s.d. of four samples are indicated ($P < 0.003$, two-tailed *t*-test). Results are representative of two experiments. (K) Flow cytometric analysis of annexin V binding of CMEC cultured as indicated in the presence of BDNF (25 ng/ml) or VEGF (10 ng/ml) for 48 hours prior to incubation with FITC-annexin V and propidium iodide for flow cytometry (1×10^4 cells per condition). Cells in early apoptosis bind annexin V but do not incorporate propidium iodide, while cells in late apoptosis bind annexin V and incorporate propidium iodide, due to a loss of membrane integrity. The results are representative of two independent experiments. Scale bars, 15 μ m (D-G), 30 μ m (H-J).



animals, the interendothelial cell contacts were less frequent, and occurred over shorter distances than observed in BDNF^{+/+} animals (Fig. 4H,I). This suggested that the remaining contacts in BDNF^{-/-} vessels were too weak to maintain an intact endothelial cell lining, resulting in numerous gaps between endothelial cells and protrusions of endothelial cells into the vessel lumen (Fig. 4G).

To determine whether these ultrastructural changes were accompanied by endothelial cell apoptosis, concomitant TUNEL analysis and CD 31 immunofluorescence detection was performed using sections from BDNF^{-/-} and BDNF^{+/+} E18.5 littermates (Fig. 4J-L). TUNEL-positive cells were more numerous in BDNF^{-/-} embryos than in BDNF^{+/+} littermates, and the majority of the TUNEL-positive cells in BDNF^{-/-} neonates exhibited CD 31 immunoreactivity, whereas in sections from BDNF^{+/+} embryos the majority of TUNEL-positive cells were CD 31 negative. These results suggest that BDNF deprivation results in apoptosis of endothelial cells in capillaries and arterioles of the late gestational heart.

BDNF promotes the survival of purified cardiac microvascular endothelial cells

To identify direct biological actions of BDNF on cardiac microvascular endothelial cells (CMEC), highly purified cultures were obtained from neonatal wild-type mice. Greater

than 95% of the cultured cells exhibited CD 31 expression and uptake of DiI-LDL (Fig. 5A and data not shown). RT-PCR analysis revealed that these cardiac microvascular endothelial cells express BDNF mRNA and kinase active trk B mRNA (Fig. 5C), confirming the immunohistochemical localization to endothelial cells within the neonatal heart (Figs 1I, 2J).

Although BDNF deficiency *in vivo* results in a loss of endothelial cell:cell contacts and apoptosis, we sought to determine whether BDNF supports the formation of endothelial cell contacts and survival using a model of serum deprivation. Nanomolar concentrations of BDNF maintained the clustering of β -catenin, an adherens junctional molecule (Dejana et al., 1999), to regions of cell-cell contact in CMEC exposed to serum deprivation (Fig. 5G). In contrast, cells exposed to serum deprivation exhibited nuclear β -catenin localization (Fig. 5E). VEGF treatment also maintained β -catenin clustering at most areas of cell-cell contact, consistent with reports using other populations of endothelial cells (Dejana et al., 1999) (Fig. 5F).

To determine whether BDNF inhibited endothelial cell apoptosis in the setting of serum deprivation, TUNEL analysis and annexin V binding assays were utilized. Nanomolar concentrations of BDNF were effective in inhibiting approximately 50% of the endothelial cell apoptosis induced by serum withdrawal, as quantitated by both TUNEL analysis

Fig. 6. Intramyocardial hemorrhage in BDNF^{-/-} embryos. Embryos of the indicated gestational ages were harvested from BDNF^{+/-} females. (A-D) Intramyocardial hemorrhage in Hematoxylin and Eosin stained sections from BDNF^{-/-} embryos can be noted (arrowhead, B,D). (E,F) Immunohistochemistry using anti-CD 31 to detect endothelial cells within the ventricular wall. Six fields per section from three BDNF^{+/+} or BDNF^{-/-} embryos were analyzed blind. There was no statistically significant difference in capillary density between them (inserts, E,F). Scale bar, 40 μ m.

and annexin V binding (Fig. 5H-K). BDNF treatment appeared as effective as VEGF in maintaining viability of this cell population. Thus, the ability of BDNF to promote the clustering of β -catenin at cell contacts and to maintain the survival of highly purified populations of CMEC suggests the direct action of BDNF on this trk B expressing cell population.

Onset of vascular defects

The ability of BDNF^{-/-} animals to survive until birth, and to form identifiable cardiac arteries, capillaries and veins (Fig. 4C,D), suggests that the early processes of vasculogenesis and sprouting angiogenesis are not greatly impaired. However, to establish the onset of intramyocardial vessel defects, 11 BDNF^{-/-} embryos and 6 BDNF^{+/+} littermates were examined from E11.5 to E19.5. Intramyocardial hemorrhage in BDNF^{-/-} embryos could be detected first at E16.5 (2 of 2 BDNF^{-/-} embryos), and was present in 3 of 3 BDNF^{-/-} embryos examined at E17.5, and absent in BDNF^{+/-} and BDNF^{+/+} littermates (Fig. 6A-D). The onset of hemorrhage in late gestation suggested that BDNF is not required for the initial stages of cardiac angiogenesis as gene-targeted embryos with defects in vasculogenesis and sprouting angiogenesis typically die in utero between embryonic days 9 and 13 (reviewed in Carmeliet, 2000). Indeed, the density of the intramyocardial capillary bed in the BDNF^{-/-} embryos at E18.5 was not different from that of BDNF^{+/+} littermates, as assessed by CD 31 immunoreactivity (Fig. 6E,F). These results, together with the morphometric analysis of neonates (Fig. 4L), suggest that the establishment of the cardiac vascular network, and its patterning to form arterioles, capillaries and veins, is not substantially perturbed in BDNF^{-/-} embryos. However, vascular hemorrhage in the heart is detectable from E16.5 onwards, during the developmental time-frame in which BDNF vascular expression normally commences.

Deficiency in BDNF results in defective atrial septation

In addition to vascular defects, BDNF^{-/-} animals exhibited abnormalities in atrial septation in 10 of 12 animals examined at P0 (Fig. 3B). In affected animals, the septum primum appeared to be largely vestigial, while the septum secundum exhibited varying degrees of hypoplasia. These defects resulted in incompetence of the foramen ovale with a prominent atrial septal defect involving both the septum primum and secundum. By morphometric analysis, atrial septal defects in excess of 100

μ m in the anterior-posterior plane were detected in four of the BDNF^{-/-} animals (Fig. 3B), with no atrial septal defects detected in 10 BDNF^{+/-} or BDNF^{+/+} littermates examined (Fig. 3A). Marked atrial enlargement and atrial wall thinning, with concomitant pulmonary congestion and intra-alveolar hemorrhage, were noted in the 10 BDNF^{-/-} animals with atrial septal defects (Fig. 3F). No defects in ventricular septal formation, valvulogenesis, ventricular trabeculation and septal muscle formation were detected.

To establish the onset of defective atrioseptal formation, BDNF^{-/-} and BDNF^{+/+} littermates were examined from E11.5 to E18.5 (2 BDNF^{-/-} and 2 BDNF^{+/+} embryos of each gestational day). The formation of the dorsal component of the septum primum is initiated at E10.5, and this process appears to be unaffected by deficient BDNF expression (Fig. 7A,D). However, by E14.5, when the septum primum has formed, and the dorsal and ventral ridges of the septum secundum are emerging in wild-type animals, BDNF^{-/-} littermates exhibit hypoplasia of the developing dorsal and ventral components of the septum secundum (Fig. 7B,E). The hypoplasia of the septum secundum in BDNF^{-/-} embryos is progressive at E16.5 and E18.5 (Fig. 7F and data not shown), and results in maldevelopment of the foramen ovale at P0 (Fig. 3B). To assess whether abnormalities in BDNF-mediated trk B signaling could result in septal hypoplasia, immunohistochemical localization of kinase active trk B and BDNF in the developing atria was undertaken. Expression of trk B and BDNF is detectable in the endocardium of the developing atria, in the region of the septum primum (Fig. 7I,J). These results suggest that BDNF-mediated trk B signaling is required for the persistence and continued growth of the atrial septum primum and septum secundum.

Echocardiographic imaging of BDNF^{-/-} mice

To assess the functional impairment of the BDNF deficient heart in vivo, we performed real-time echocardiography on all littermates within 48 hours of birth. 5 of 5 BDNF^{-/-} animals exhibited significant decreases in left ventricular ejection fraction (EF) as compared to 5 of 5 BDNF^{+/+} littermates (Table 1). Analysis of the end systolic and end diastolic left ventricular dimensions suggest that BDNF deficiency results in hypocontractility rather than chamber enlargement. The reduction in ejection fraction in the BDNF^{-/-} neonates is consistent with the histological and ultrastructural evidence of intramyocardial vessel fragility and hemorrhage which impacts on myocardial contractility.

Fig. 7. Atrial septal formation in BDNF^{+/+} and BDNF^{-/-} embryos. (A-F): Histological analyses of Hematoxylin and Eosin-stained sections to show atrial septal formation in BDNF^{+/+} and BDNF^{-/-} animals at the indicated ages. p, septum primum; s, septum secundum. (G-J) Immunohistochemistry using antiserum to CD 31 (H), BDNF (I) or kinase active trk B (J) to assess expression in the region of the atrial septum of E18.5 BDNF^{+/+} embryos. Preincubation of trk B antisera with the immunizing peptide confirms the specificity of the antisera (G). Scale bars, 80 μ m (A-F), 30 μ m (G-J).

BDNF overexpression in the gestational heart induces angiogenesis

To determine the developmental consequences of excessive local production of BDNF in the heart, we generated transgenic mice overexpressing BDNF under the control of the promoter and enhancer regions of the *nestin* gene, which direct expression in developing muscle and nervous system. As these mice die shortly before birth, transgenic embryos (E17.5-E18.5) arising from independent injections of the construct were harvested and found to focally overexpress BDNF in the cardiac ventricular walls (Fig. 8D,C). Histological analysis of the hearts from six BDNF overexpressing or six wild-type littermates revealed focal abnormalities in the ventricular wall of transgenic animals, characterized by an increase in the number of predominantly small diameter vessels (less than 10 μ m) (Fig. 8B). Immunohistochemistry using the endothelial cell marker CD 31 revealed a threefold increase in the density of endothelial lined vessels in these regions of the ventricles of transgenic, as compared to wild-type, littermates (Fig. 8E,F,I). No increased expression of smooth muscle cell α -actin was detected, suggesting that these small-diameter vessels lacked a smooth muscle cell ensheathment and were capillaries (Fig. 8 G-I). To determine whether the increase in vessel number reflected increased endothelial proliferation, immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was undertaken. In a quantitative, blind analysis of sections from three Nes-BDNF and three wild-type littermate hearts, no statistically significant difference in the number of PCNA-positive cells was detected (PCNA-positive cells =

Table 1. Echocardiographic imaging of BDNF^{+/+} and BDNF^{-/-} mice at P2

(A)	Ejection fraction (%)	
	BDNF ^{+/+}	BDNF ^{-/-}
	73%	42%
	77%	62%
	79%	31%
	72%	53%
	81%	56%
(B)	Left ventricular wall measurements	
	BDNF ^{+/+}	BDNF ^{-/-}
lvidd	1.10 mm	1.20 mm
lvlds	0.70 mm	1.00 mm
Diastolic volume	1.33 mm ³	1.73 mm ³
Systolic volume	0.34 mm ³	1.00 mm ³
Stroke volume	0.99 mm ³	0.73 mm ³
Ejection fraction	73%	42%

Neonates were imaged blind using a 40 Mhz Scimed coronary probe on the anterior chest wall. Diagnostic Off-line analysis of real-time images was performed to obtain the measurements of ventricular volumes.

(A) Ejection fractions for four BDNF^{+/+} and four BDNF^{-/-} animals from three litters.

(B) Representative measurements from BDNF^{+/+} and BDNF^{-/-} littermates. The ejection fractions of the BDNF^{-/-} and BDNF^{+/+} neonates were significantly different ($P < 0.004$, two-tailed t -test).

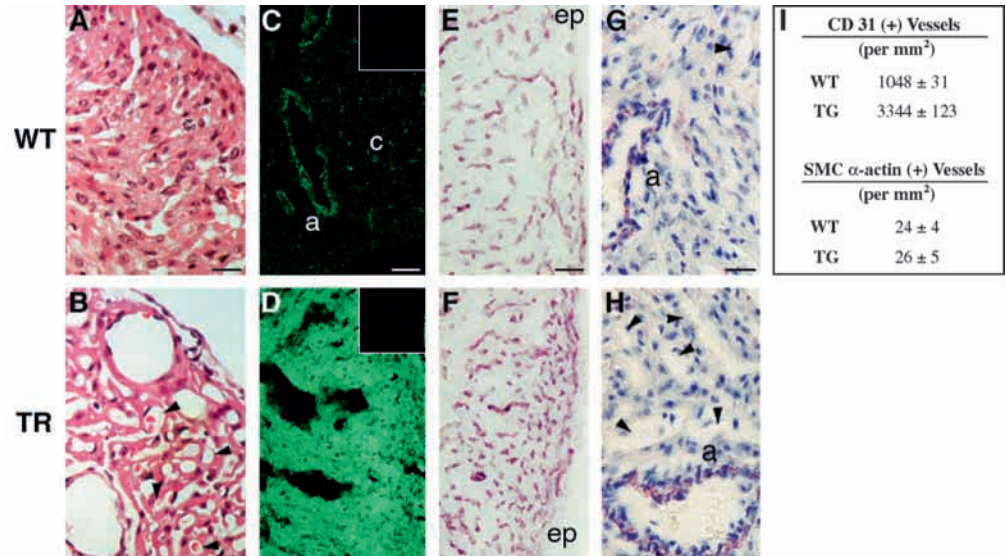
lvidd, Left ventricle internal diameter, diastolic; lvlds, left ventricle internal diameter, systolic.

2039 \pm 155/mm² (mean \pm s.d.) in Nes-BDNF as compared to 2018 \pm 176 in wild-type littermates, $P > 0.05$, two-tailed t -test), suggesting that the dominant action of BDNF is to promote survival, rather than to induce cell proliferation. Significantly, no evidence of intraventricular wall hemorrhage was noted in the BDNF overexpressing embryos, suggesting that excess BDNF does not alter vessel integrity.

DISCUSSION

While multiple growth factors and adhesion events promote angiogenesis during early development, little is known about the factors that maintain vessel integrity in the postnatal period. Our data demonstrate that BDNF is required to promote the stabilization of intramyocardial arteries and capillaries during late embryogenesis, through direct survival action on endothelial cells. The onset of expression of BDNF and trk B in the cardiac vasculature in late development is consistent with our observations of unimpaired cardiac capillary formation through mid-gestation, and the ability of BDNF-deficient embryos to form identifiable capillaries, arteries and veins. The ultrastructural defects in BDNF^{-/-} animals, with a loss of endothelial cell-cell contacts and endothelial cell apoptosis, is similar in part to the endothelial cell apoptosis observed in angiopoietin-1^{-/-} and VE-cadherin^{-/-} embryos (Suri et al., 1996; Carmeliet et al., 1999). However, the earlier embryonic lethality and the widespread vessel abnormalities distinguish the effects of deficient expression of angiopoietin-1 and VE-cadherin from BDNF. BDNF can appropriately maintain the clustering of β -catenin to endothelial cell-cell contacts which, together with recent reports that VEGF performs similar functions in a VE-

Fig. 8. Overexpression of BDNF in gestational hearts results in increased capillary density. Analysis of hearts from E18.5 wild-type (WT) littermate embryos (A,C,E,G) or Nes-BDNF transgenic (TR) embryos (B,D,F,H). (A,B) Histological analysis reveals abnormal vascularity of the Nes-BDNF ventricular wall, with abundant large and small diameter vessels (B, arrowheads). (C,D) Immunofluorescence detection of BDNF demonstrates increased BDNF expression in the ventricular wall of Nes-BDNF (D) as compared with wild-type littermates (C). Preincubation of the BDNF antisera with the immunizing peptide (panel inserts) confirms antisera specificity. Immunofluorescence of paired samples, using FITC-conjugated secondary antisera, was imaged by optical sectioning at identical settings. The results are representative of those observed with three transgenic and three wild-type embryos. (E,F) CD 31 immunoreactivity reveals increased vessel density in Nes-BDNF embryos as compared to wild-type littermates, detected using VIP substrate; ep, epicardial surface. (G,H) Smooth muscle α -actin immunoreactivity, to detect vascular smooth muscle cells, is similar in Nes-BDNF transgenic and wild-type hearts. Note the positivity of large vessels but absence of reactivity of small diameter vessels (arrowheads) in wild-type and Nes-BDNF embryos. In A,B,E-H the results are representative of those obtained in four transgenic and four wild-type littermates. Scale bars, 30 μ m (A,B), 100 μ m (C-F), 40 μ m (G,H). (I) Quantitation of vessel density, using CD 31 or α -actin immunodetection in wild-type or transgenic (TG) sections. Six fields from each of four wild-type or Nes-BDNF embryos were analyzed blind. The number of CD 31 (+) vessels is greater in the Nes-BDNF embryos; $P < 0.001$, two-tailed t -test.



cadherin-dependent process (Carmeliet et al., 1999), provide testable mechanisms by which BDNF may support the survival of cardiac endothelial cells in late gestation through adulthood.

The vascular phenotype observed upon BDNF overexpression in the gestational heart further supports the hypothesized role of BDNF in regulating endothelial cell survival and vessel stabilization. The increased density of small vessels, but no increase in vascular smooth muscle cell ensheathment, suggest that BDNF acts predominantly on cardiac endothelial cells, rather than supporting cells, during late gestation. Importantly no vessel hemorrhage was observed, distinguishing the effects of BDNF from those of VEGF, which can promote the formation of capillary networks with enhanced fragility (Drake and Little, 1995; Springer et al., 1998; Thurston et al., 2000).

Recent studies have identified several angiogenic factors that modulate reciprocal interactions between endothelial cells and pericytes or vascular smooth muscle cells, resulting in bidirectional signaling between endothelial cells and the support cells which ensheath them (Darland and D'Amore, 1999; Yancopoulos et al., 1998). The colocalization of BDNF and trk B to endothelial cells of intramyocardial arteries and capillaries, as well as the ability of BDNF to support cardiac microvascular endothelial cells survival in culture, provide evidence of autocrine, or local paracrine actions. Are there additional actions, however, of BDNF on vascular smooth muscle cells? In the adult, vascular smooth muscle cells of large vessels express neurotrophins, and expression of BDNF and trk B is increased by neointimal cells following vascular injury (Donovan et al., 1995). In addition, neurotrophins promote chemotaxis of trk expressing adult vascular smooth muscle cells, suggesting that neointimal neurotrophin

expression could influence medial smooth muscle cell migration following injury (Kraemer et al., 1999). However, overexpression of BDNF in the developing heart does not result in abnormal vessel ensheathment, consistent with the lack of trk B expression by vascular smooth muscle cells in late gestation. Further studies will be required to determine whether BDNF mediates direct chemotactic or survival effects on pericytes or vascular smooth muscle cells in adulthood.

The atrial septal defects in the BDNF^{-/-} animals are considerably larger than those described for secondary physiological septal defects. The developmental hypoplasia of these structures, and the local expression of trk B and the BDNF by the atrial endocardium, suggest that BDNF is required for septal development in addition to maintaining endothelial cell survival. The structural defects may reflect either primary survival deficiencies in the mesenchymal cells of the septae or endothelial cell dysfunction leading to increased apoptosis of these structures. Little is known about the factors regulating valve formation and septal development, although endocardial cell growth factors of the TGF β family and transcription factors of the Helix-Loop-Helix family play roles in these processes (Fishman and Chien, 1997; Schott et al., 1998). Interestingly, the coexistence of a competent valve system with markedly hypoplastic septal structures in BDNF^{-/-} animals supports an even more complicated profile of pathways.

Although endothelial cells line the vessels in all organs, local expression of growth factors confers specialization and functional heterogeneity of the endothelial cells lining great vessels and different vascular beds (Edelberg et al., 1998). Hemorrhage only within the heart, and probably secondarily in the lungs of BDNF^{-/-} mice, with normal development of other vascular beds, is most likely to reflect the regional vascular expression of BDNF

and trk B in the late gestational embryo. This restricted pattern of BDNF and trk B expression to endothelial cells of intramyocardial capillaries and arterioles suggests that endothelial cells in other vascular beds utilize different growth factors to ensure cell survival. Indeed, the heterogeneity of cardiac microvascular endothelial cell responsiveness to PDGF-AB suggests that local expression of growth factors can regulate microvascular endothelial cell function (Edelberg et al., 1998). In addition, the identification of ephrin B2 and its receptor Eph B4 as embryonic markers for endothelial cells within arterial or venous capillaries, respectively, suggests that endothelial cells are molecularly distinct prior to their ensheathment by vascular smooth muscle cells (Wang et al., 1998). One hypothesis to account for the restricted expression of the neurotrophins by vascular endothelial cells is that production of BDNF and trk B may be regulated by ephrin-B2:Eph-B4, or PDGF-AB mediated interendothelial signaling, questions that are most amenable to genetic dissection in the future.

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