A Novel *In Vitro* Human Model of Hemangioma

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Hemangioma, the most common tumor of infancy, is characterized by a proliferation of capillary endothelial cells with multilamination of the basement membrane and accumulation of cellular elements, including mast cells. The initial rapid growth is followed by an inevitable but slow involution. The currently available therapies are empirical and unsatisfactory because what is known of the cellular and molecular basis of hemangioma development is rudimentary. Advances in the understanding of its programmed biologic behavior has been hampered by the lack of a valid human model.

We report here a novel *in vitro* culture system that is a useful human model of hemangioma. A small fragment of hemangioma biopsy is embedded in fibrin gel in a well of culture plates and incubated in a serum-free, buffered-salt, minimal medium. A complex network of microvessels grows out from the tissue fragments. Biopsies taken from all three phases of hemangioma development were cultured successfully; proliferative phase samples developed microvessels in 1 to 4 days, involuting phase in 5 to 7 days, and involuted phase in 7 to 12 days. The relative growth rates of the microvessels in the culture of biopsies taken from different stages of hemangioma development reflect the growth patterns seen clinically.

This model has been validated using histochemistry, immunohistochemistry, and reverse transcriptase-polymerase chain reaction. Comparison of the number, localization, and phenotype of endothelial and mast cells and the distribution of basement membrane constituents (type IV collagen, perlecan, and laminins) and growth factors (*basic* fibroblast growth factor, vascular endothelial growth factor, transforming growth factor-βs) in the biopsy and the tissue after culture shows that many of the characteristics of the original tissues were retained in culture.

This *in vitro* human model of hemangioma overcomes some of the deficiencies associated with earlier models. It offers an opportunity for studying the precise cellular, biochemical, and molecular basis of hemangioma. It may also help to elucidate the mechanisms of action of existing therapies and may lead to the identification of novel treatments for hemangioma.

KEY WORDS: Angiogenesis, Culture, Hemangioma, Immunohistochemistry, Reverse transcriptase-polymerase chain reaction.

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Hemangioma, a primary tumor of microvasculature, is characterized by a proliferation of capillary endothelial cells with multilamination of the basement membrane and accumulation of cellular elements, including mast cells, macrophages, plasma cells, and pericytes (1–4). Thirty to 40% of hemangiomas are first noticed at birth, usually as a pink spot, blanched area, or telangiectasia (4), and exhibit a rapid postnatal growth for 8 to 12 months (proliferative phase), followed by a slow regression for the next 1 to 5 years (involuting phase), with continued improvement in the remaining children until 10 to 12 years (involuted phase) (5). Normal skin is restored in 50% of patients when involution is complete (4, 6). The trigger that initiates hemangioma seems to be a local event, not a hereditary predisposition (7).

Currently available experimental models include the induction of hemangioma in mice by transgenic endothelial cells (8), mouse endothelial cells that have been transformed by polyoma middle T oncogene (9, 10), and endothelial cells obtained from murine and human hemangioma (11). Each of these models has deficiencies; they use animal rather than human cells and tissue, or they involve viral transformation of endothelial cells, which is not known to occur in human hemangioma, or they use a cell culture system that is not influenced by the metabolism of neighboring cells as occurs *in*

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vivo. Mulliken *et al.* (12) cultured endothelial cells from human hemangioma on plasma clots. However, the absence of other cellular elements, as well as the necessity of plasma, makes this system less than ideal, especially for investigating the role of modulating molecules. Therefore, none of these systems simulates the cellular and extracellular matrix characteristics of human hemangioma.

We describe here an *in vitro* tissue culture system that uses human hemangioma specimens that are angiogenically active in a serum-free medium. Using histochemistry, immunohistochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR), we have established that a number of characteristics of hemangioma were retained in the cultured samples.

MATERIALS AND METHODS

Tissue Samples

Ten hemangioma biopsies were obtained from nine patients (Table 1) according to a protocol approved by the Wellington Ethics Committee. The specimens were kept moist with normal saline and cleansed of all blood clots with phosphate-buffered saline. Samples from them were snap-frozen in liquid nitrogen for RNA isolation, fixed in formalin for histochemistry and immunohistochemistry, and cultured as described next.

Tissue Culture

Pieces of fresh operative hemangioma tissue were submerged in serum-free MCDB131 medium (GIBCO BRL, Gaithersburg, MD). Under an operating microscope, the tissue was cut at room temperature into 1-mm explants and embedded in fibrin gel. For the gel, sheep fibrinogen fraction I (Sigma, St. Louis, MO) (3 mg/mL) was dissolved in MCDB131 medium with streptomycin (0.1 mg/mL) and penicillin (100 U/mL), supplemented with ϵ-amino-caproic acid (0.3 mg/mL) (Sigma) to prevent fibrinolysis. Human plasma thrombin (final concentration 0.5 U/mL) (Serva Feinbiochemica, Heidelberg, Germany) was added to the fibrinogen, and 0.7 mL of the mixture was immediately placed in each well of a culture plate (Nunclon; Nunc Inter Media, Roskilde, Denmark). After gel formation, the tissue explants were placed on top of it. A second aliquot of fibrinogen-thrombin-medium mixture (0.8 mL) was added so that the tissue fragment was sandwiched between two layers of fibrin gel. After gel formation, 1.5 mL of the same supplemented MCDB131 medium was added to each well. For each biopsy, 24 well culture plates were used so that multiple analyses with replicates could be carried out. The plates were incubated at 37° C in 3% CO_2/97% air in a humidified environment.

The medium was changed every 5 days. After the first passage, some of the cultured tissue samples were placed in Trizol (GIBCO, Life-Technologies) for RNA isolation, and the remainder were fixed in formalin and embedded in paraffin for histochemical and immunohistochemical analyses.

Quantitative Assay of Angiogenesis

Images of the cultures were captured using a Pixera digital image camera professional system (Digitech, Miami, FL) attached to an inverted microscope (Olympus CK2; Olympus, Tokyo, Japan). The images were taken approximately every 3 days to assess the rate of growth of the neovessels. The neovascular area in each image was outlined and calculated using the program Image 1.62b7 software (National Institutes of Health, Bethesda, MD) as described earlier (13). This area is expressed relative to the area of the explant.

Histochemistry

Three-μm-thick sections from the paraffin blocks were attached to poly-L-lysine–coated glass slides (Sigma). Deparaffinized sections were stained with hematoxylin and eosin. Mast cells were detected using Csaba stain as described (14).

TABLE 1. Details of Primary Cultures of Hemangiomas Including Age of Patient at Biopsy, Sex, Site of Lesion, Developmental Phase of Hemangioma, and Time in Culture Before New Vessels Were Detected

Patient	Age	Sex	Site	Phase	First sprouts (days)
1	18 days	F	Perianal	Proliferative	2–4
2	3 mo	F	Axilla	Proliferative[a]	1–4
3	5 mo	F	Axilla	Proliferative[b]	2–4
4	5 mo	M	Forehead	Proliferative	3–4
5	8 mo	F	Chest	Proliferative	3–4
6	13 mo	M	Scalp	Involuting	5–7
7	13 mo	F	Elbow	Involuting	6–7
8	5 yr	F	Upper lip	Involuting	5–7
9	6 yr	M	Glabella	Involuted	7–10
10	8 yr	F	Submentum	Involuted	9–12

[a] Tissue obtained before intralesional triamcinolone injection.
[b] Tissue obtained 5 weeks after intralesional triamcinolone injection.

Immunohistochemistry

Immunohistochemistry was performed as described previously (15–18) with several modifications. Three-μm-thick sections were cut, dewaxed, and predigested with protease XXIV (Sigma) (0.5 mg/mL in 50 mM/l Tris HCl pH 7.5, for 10 min at 37° C), pronase E (Serva Feinbiochemica) (0.05 mg/mL in Tris-buffered saline [TBS], for 10 min at 37° C), or pepsin (Sigma) (0.01 mg/mL in 0.1 M HCl at 37° C for 10 min). Endogenous peroxidase was blocked during incubation with secondary (bridging) antibodies (Abs) in 0.5% casein/TBS by the addition of 1% D-glucose (BDH, Poole, UK) and 0.85 units/mL glucose oxidase type VIII (Sigma). Dilutions of primary Abs used in the incubations and details of unmasking procedures are given in Table 2. All primary Abs were diluted with TBS/1% bovine serum albumin, and the sections were incubated overnight at 4° C. Controls included the omission of primary Ab and substitution with an irrelevant monoclonal Ab of the same isotype, or with normal rabbit or goat serum for polyclonal Abs. Mouse monoclonal and rabbit polyclonal Ab binding were detected with a sensitive three-step biotin-streptavidin/immunoperoxidase technique, using biotinylated anti-mouse immunoglobulins (Igs) (BioGenex, San Ramon, CA; 1:50) or biotinylated anti-rabbit Igs (BioGenex; 1:50) for 30 min followed by incubation with peroxidase-conjugated streptavidin (BioGenex; 1:50) for 30 min at room temperature. The reactivity of goat polyclonal Abs was revealed using rabbit anti-goat Igs (DAKO; 1:100) as bridging reagents and goat peroxidase-antiperoxidase (DAKO; 1:50), both incubated for 30 mins at room temperature. The sections were exposed to 0.05% diaminobenzidine in 50 mM/l Tris HCl, pH 7.5, counterstained with hematoxylin, dehydrated, and mounted in DPX (BDH).

Cell Counting

Mast cells that stained positively for tryptase and chymase were counted at 20× magnification using a 25-point Zeiss integrating grid. For each section, a minimum of three adjacent fields were counted by each of the two observers (STT and MV).

Reverse Transcriptase-Polymerase Chain Reaction Analysis

RNA was isolated from the hemangioma biopsy tissues before and after culturing using Trizol reagent (GIBCO). RNA was reverse transcribed to cDNA and amplified using the Perkin Elmer (Norwalk, CT) rTth RT-PCR kit as described (17–21). Briefly, a 10-min RT step was followed by 40 cycles of three-stage PCR using temperatures of 94° C for denaturing, 72° C for extension, and specific annealing temperatures for each primer set (Table 3). The amplified products were electrophoresed on 2% agarose gel in Tris-acetic acid- EDTA buffer. The quality of the amplifiable RNA was established using hypoxanthine phosphoribosyl transferase as the control gene. The other genes studied were for the cytokines basic fibroblast growth factor (*b*FGF) and transforming growth factor-βs (TGF-β1, -β2, and -β3). All of the primers used (Table 3) were designed using the Oligo Program based on published human sequences and were synthesised by Operon Technologies (Alameda, CA).

RESULTS

In Vitro Hemangioma Culture

For the *in vitro* culture, biopsy tissues from five hemangiomas in the proliferative (Fig. 1A), three in the involuting (Fig. 1B), and two in the involuted (Fig. 1C) phases were used. Histology of each of these phases is shown in Figures 1D–F. Each fragment of the hemangioma tissues in culture gave rise to a complex array of microvessels emanating from its perimeter (Fig. 2). The outgrowths stained positively with Von Willebrand factor (Fig. 3), confirming the presence of endothelial cells.

TABLE 2. Antibodies and Unmasking Procedures for Immunohistochemistry

Marker	Primary antibody	Staining dilutions	Unmasking procedure	Source of primary antibody
Perlecan	Mouse mAb clone MAB458	1:25	Protease	Chemicon, Temecula, CA
Collagen IV	Goat pAb	1:400	Pronase E	Southern Biotechology, Birmingham, AL
Laminin α1 chain	Mouse mAb clone 4C7	1:100	Protease	Gibco BRL, Gaithersburg, MD
Laminin α2 chain	Mouse mAb clone 5H2	1:200	Protease	Gibco BRL, Gaithersburg, MD
Laminin β1 chain	Mouse mAb clone 4E10	1:200	Protease	Developmental Studies, Hybridoma Bank, Iowa City, IA
Laminin β2 chain	Mouse mAb clone C4	1:3000	Protease	Gibco BRL, Gaithersburg, MD
Laminin γ1 chain	Mouse mAb clone 2E8	1:200	Protease	Gibco BRL, Gaithersburg, MD
vWf	Rabbit Ab	1:8000	Pepsin	Dako Corp., Carpinteria, CA
CD31	Mouse mAb clone JC/70A	1:25	Pronase	Dako Corp., Carpinteria, CA
Mast cell tryptase	Mouse mAb clone AA1	1:1000	Pronase E	Dako Corp., Carpinteria, CA
Mast cell chymase	Mouse mAb clone MAB1254	1:200	Pronase E	Chemicon, Temecula, CA
VEGF	Goat pAb clone	1:30	–	R&D Systems, Minneapolis, MN
*b*FGF	Rabbit pAb	1:40	–	Santa Cruz Biotech, Santa Cruz, CA

TABLE 3. The Genes Analysed and the Primer Sequences Used for Reverse Transcriptase-Polymerase Chain Reaction

Gene	Primers (sense and antisense)	Annealing temperature	Reference no.
HPRT	5′CCTTTGGGCGGATTGTTGT3′ 5′TTTTTTTTTTTTTTAAATTTTTGGGAAT3′	52°C	17, 20
TGF-β1	5′GGAAACCCACAACGAAATCTATGAC3′ 5′TTCCCCTCCACGGCTCAAC3′	56°C	21
TGF-β2	5′GCGAGAGGAGCGACGAAGAG3′ 5′AGCCTGGGTTGGAGATGTTA3′	56°C	21
TGF-β3	5′CTGGCGGAGCACAACGAACT3′ 5′GGACTCTCTTCTCAACAGCCACTCAC3′	56°C	21
*b*FGF	5′AGCGACCCTCACATCAAGC3′ 5′AAAAGAAACACTCATCCGTAACACA3′	60°C	19

HPRT, hypoxanthine phosphoribosyl transferase; TGF, transforming growth factor; *b*FGF, basic fibroblast growth factor.

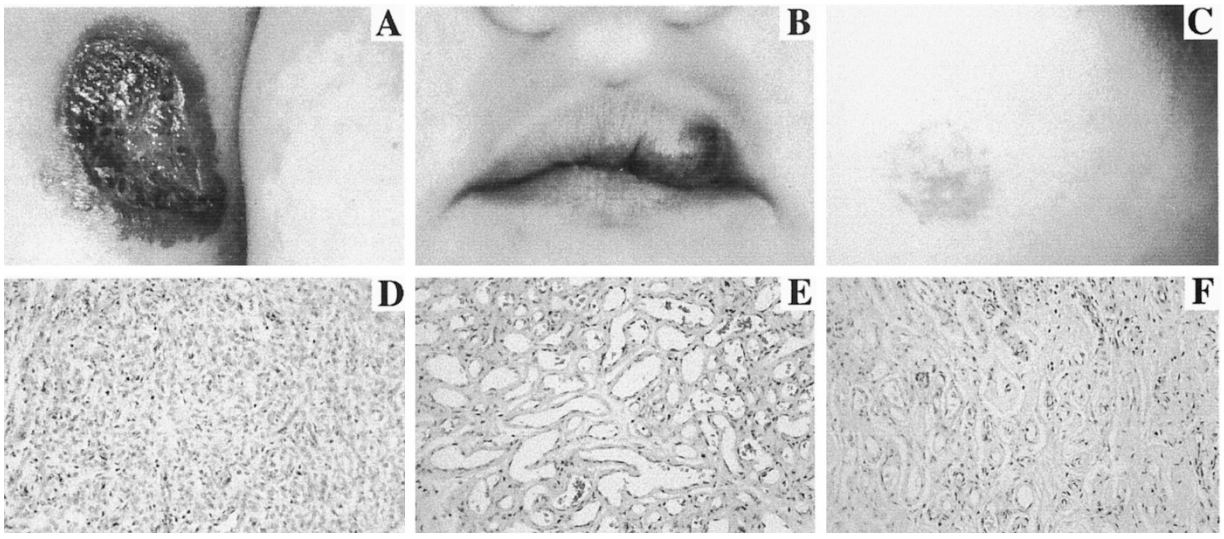

FIGURE 1. Hemangioma at different phases of development. **A,** an ulcerated proliferative hemangioma in the perianal region of an 18-day-old infant. **B,** an involuting hemangioma on the upper lip of a 5-year-old girl. **C,** an involuted hemangioma in the submental region of an 8-year-old girl. **D–F,** hematoxylin and eosin staining of sections from biopsies from **A, B,** and **C,** respectively (original magnification, 357×).

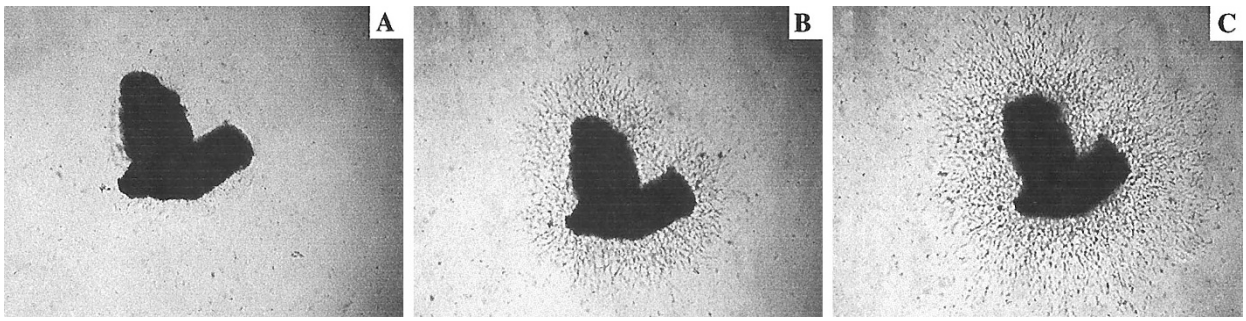

FIGURE 2. Human hemangioma in culture. Tissue taken from a proliferative hemangioma embedded in fibrin gel and cultured, as described in Materials and Methods, at 2 days (**A**), at 4 days (**B**), and at 6 days in culture (**C**) (original magnification, 22.5×).

Quantitation of the *In Vitro* Hemangioma Angiogenesis

The extent of the vessel outgrowths was quantified by digital image analysis. Tissue samples cultured from proliferative phase lesions were found to give rise to microvessels within 1 to 4 days in culture. The first sprouts of microvessels were seen between 5 and 7 days when tissue was taken during involuting phase, whereas involuted biopsy tissues took 7 to 12 days to grow new microvessels. Relative growth rates are shown in Figure 4.

Phenotypic Features of Hemangioma Tissue after Culture

Examination by histochemistry and immunohistochemistry of the expression of a number of cellular components, matrix proteins, and growth factors previously described for hemangioma (5, 22) showed that these phenotypic characteristics were retained in culture. RT-PCR also demonstrated that the transcripts for the growth factors studied were present in both the biopsies and the tissues after culture.

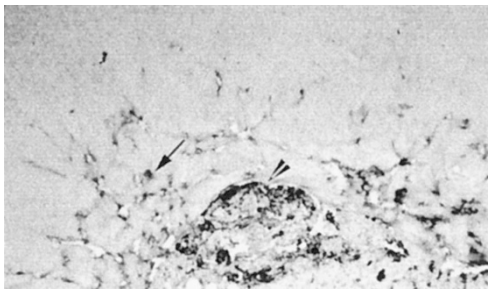

FIGURE 3. Section of a representative hemangioma culture. Positive staining for Von Willebrand factor is localized to the neovessels of the biopsy (***arrow***) and tissue of the culture (***arrow head***). (Original magnification, 43×).

Immunohistochemical staining of the biopsy and the tissue after culture with anti-CD31 (Figs. 5A, B) and anti–Von Willebrand factor (data not shown) showed a similar distribution that was localized to endothelial cells. Csaba stain showed that in both the biopsies and the explants, mast cells were present and expressed the biogenic amine phenotype (data not shown) in all three phases. The distribution of tryptase-positive mast cells was also similar (Figs. 5C, D). There was no significant quantitative difference in the number of tryptase-positive mast cells in the original biopsy and tissue after culture. (Proliferative: tissue: mean = 33.60 ± 2.10 [SEM] [n = 10 fields]; culture: 34.00 ± 2.01 [SEM] [n = 6 fields]; N.S. Involuting: tissue: mean = 50.83 ± 2.55 [SEM] [n = 12 fields]; culture: 46.17 ± 4.47 [n = 6 fields], N.S.). In both the original and the tissue after culture, the α-1 chain of type IV collagen (Figs. 5E, F), β2 (Figs. 5G, H), α1 and γ1 chains (data not shown) of laminin, and perlecan (Figs. 5I, J) were detected in the basement membrane. The α2, β1, and γ2 chains of laminin were not present (data not shown) in either biopsy or tissue after culture. *b*FGF (Figs. 5K, L) and vascular endothelial growth factor (data not shown) localized predominantly to the endothelial and mast cells in both the biopsy and the tissue after culture.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA isolated from the biopsy and the sample after culturing was of good quality and was amplifiable. Figure 6 shows the constitutively expressed hypoxanthine phosphoribosyl transferase gene transcripts from the hemangioma samples before and after culture. Transcripts of cytokines TGF-β1, -β2, and -β3 and *b*FGF identified in biopsies were also present in the tissue after culture (Fig. 6).

DISCUSSION

Hemangioma offers a unique model to study angiogenesis as a primary tumor of microvasculature in which angiogenesis is initially excessive, followed by inhibition and regression of the newly formed blood vessels (23). The cellular and biochemical events that initiate and regulate its proliferation and natural regression are unknown. Rudimentary knowledge of the pathogenesis of hemangioma has led to empirical and unsatisfactory therapies (22–24). Identification of the molecular trigger for hemangioma is of great interest both clinically and biologically. Presumably, the triggering event involves a potent stimulator of angiogenesis (7). Identification of the molecules involved may provide information that could be used for controlling common infantile hemangioma and angiogenesis. In the present study, a model that mimics as closely as possible the features of the hemangioma was developed.

Each of the models currently available to study hemangioma has deficiencies. For example, in the transgenic mouse models, a "hemangioma" develops after introduction of virally transformed endothelial cells (8–10). The natural history of these tumors, which often undergo malignant transformation and grow throughout life, is very different from human hemangiomas (7). Furthermore, the relevance of this oncogene incorporation to the development of human hemangioma has not been established, because there is no evidence that human lesions contain transformed endothelial cells. In another model, mice expressing Fps/Fes protein-tyrosine kinase transgene with an activating mutation have multiple hemangioma-like tumors at specific sites (25). However, these tumors never regress, and the mice die of internal hemorrhage. Hence, the value and validity of such models are questionable. Similarly, endothelial cells derived from murine (11) and human (11, 12) hemangioma and grown as pure cultures are devoid of the other cells and associated extracellular matrix that normally are present *in vivo*. Therefore, these cellular elements' and matrix constituents' influence on and interaction with the endothelial cells cannot be determined.

In this article, we describe a human *in vitro* model of hemangioma that overcomes some of the deficiencies associated with other previously described systems. Unlike many of the models, our culture system uses human hemangioma biopsies. Thus, any disadvantages arising from the use of animal models is eliminated. Consequently, the cellular and molecular mechanisms involved in this culture system would more likely mimic the *in vivo* situation. Another advantage is the ability of the neovessels to grow from the tissue sections in the absence of exogenous serum. Neovascularization in a minimal medium allows agents such as growth factors and antibodies to be added so that their

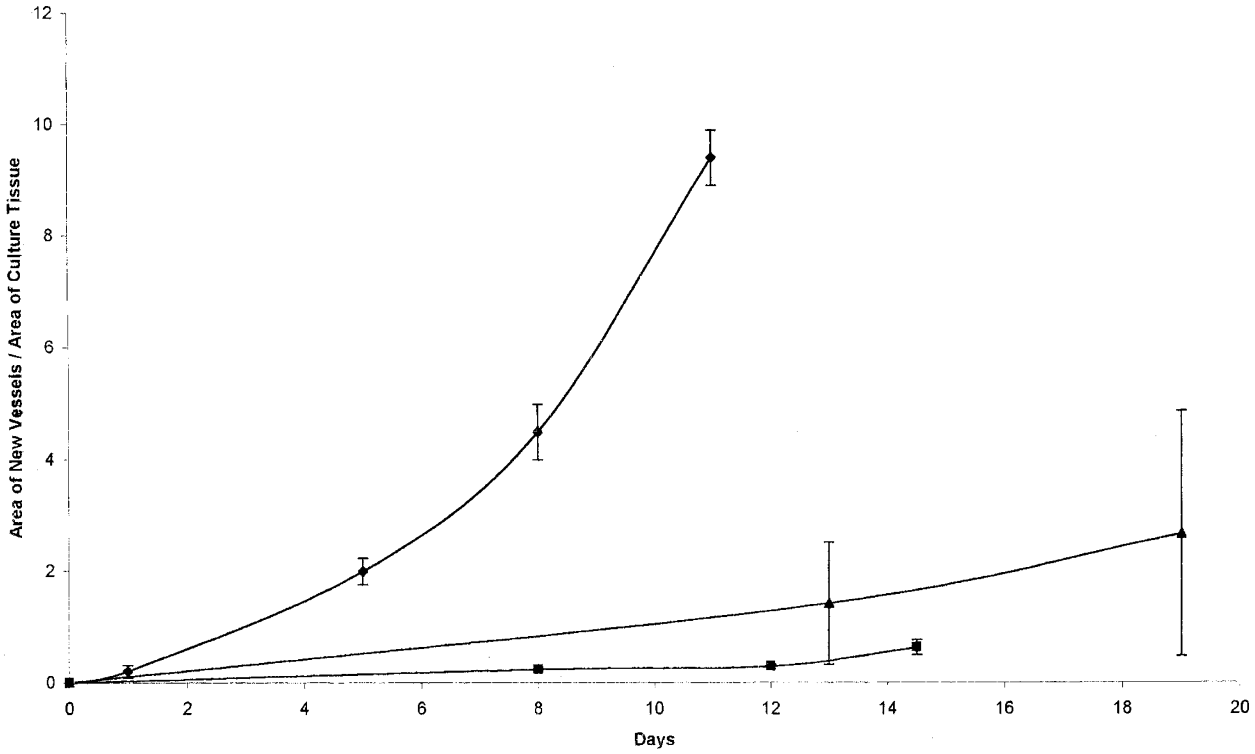

FIGURE 4. The relative growth rates of microvessels from hemangioma tissue in culture. The ratio of the area occupied by new vessels to the area of hemangioma tissue section from which they emanate. ♦–♦, proliferative; ▲–▲, involuting; ■–■, involuted. These areas were measured at several time points as described in Materials and Methods. Each value is the mean of 3 to 5 wells. Error bars represent standard deviation.

effect can be studied without the interaction or influence of serum constituents.

The more *in vivo* features of a lesion that can be maintained in a model system, the more valid the imitation is. In our model, both endothelial and mast cells that are characteristic of hemangioma (1–5) maintain similar phenotype and number in the tissue after culture. The basement membrane constituents of the cultured section show a similar distribution to that of the biopsy specimens. Of particular note is the retention of the $\beta 2$ laminin chain that is characteristic of vascular basal lamina. Expression of growth factors (*e.g.*, vascular endothelial growth factor, *b*FGF, TGF-βs) known to be relevant to angiogenesis is retained in culture and is demonstrated both transcriptionally and translationally. These growth factors have been demonstrated to be major promoters of angiogenesis in general and of hemangiomas in particular (5, 22, 26–29). Their presence in this model attests to the validity of this culture system for investigating the mechanism involved in hemangioma proliferation and involution. Further support for the utility of the system is provided by the observation that an angiogenic response occurs with biopsies taken at any phase of hemangioma development. The slower rates of neovascularization in biopsies obtained at involuting and involuted phases compared with those from the proliferative phase provide further evidence that the *in vivo* phenotype is maintained in culture. Furthermore, cultures can be maintained through several passages, allowing the growth of microvessels to be observed for several weeks. However, a section of the original tissue is necessary for the maintenance of repeated cultures, which suggests that factors from within the hemangioma tissues are essential for the maintenance of angiogenesis (Tan ST, Velickovic M, Davis PF, unpublished observations).

Although there are features of this model to be confirmed, it does demonstrate several properties that have been observed in similar models based on aortic rings (30) and human placental vessels (31) cultured in fibrin gels. Thus, it is likely that the role of modulators that have been shown to be effective in these other systems can be investigated in this pathologic lesion using the model described. This model offers opportunities to elucidate mechanisms regulating the programmed biologic behavior of hemangioma. It also provides a means to study the mechanisms of various existing and experimental pharmacologic therapies such as steroids and interferon-α. As well it may assist in identifying novel chemotypes for the treatment of hemangioma.

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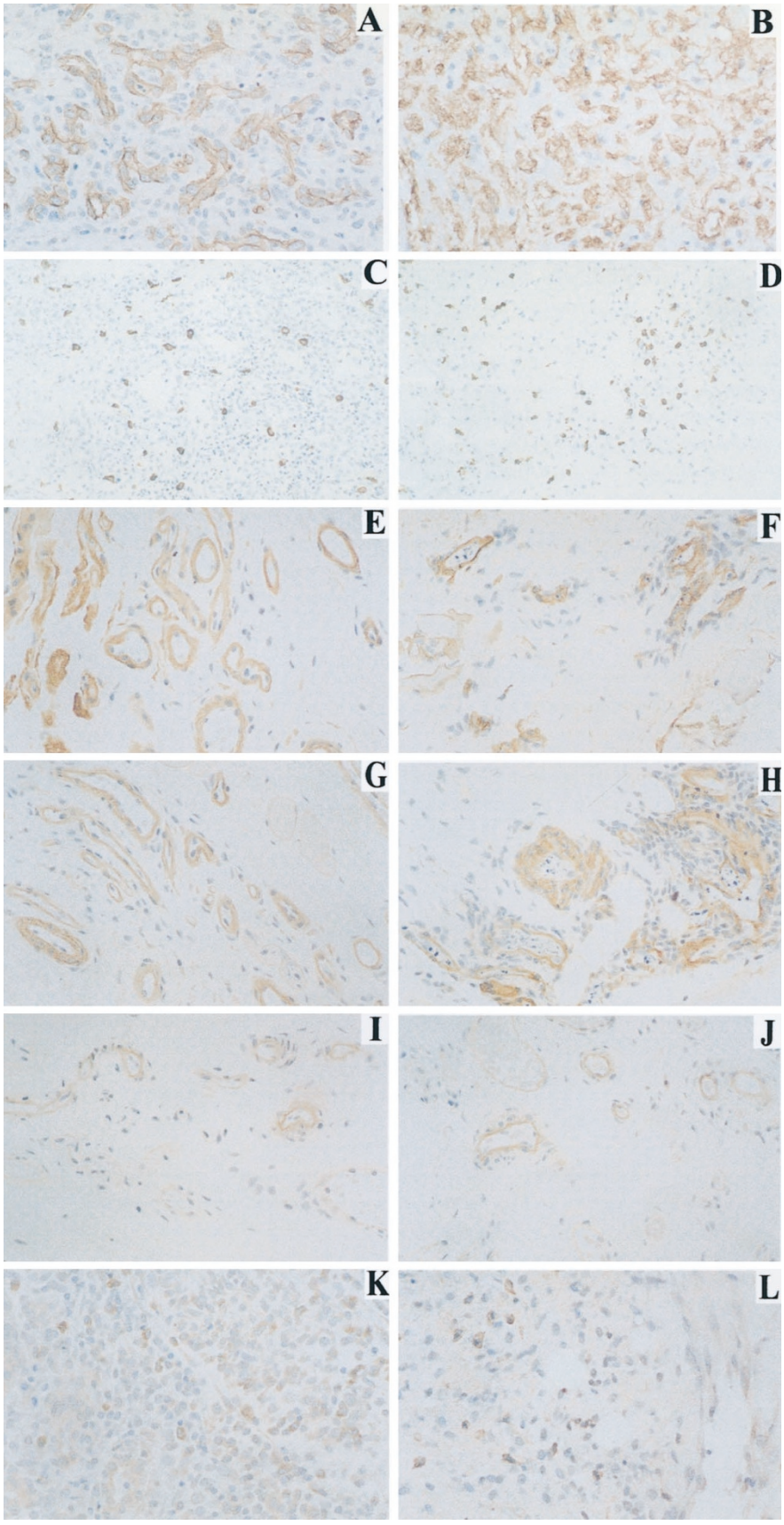

FIGURE 5. Immunohistochemical localization of constituents in hemangioma biopsy tissues (**A, C, E, G, I, K**) compared with the tissues after culture (**B, D, F, H, J, L**). Hemangioma tissues from proliferative phase: **A–D, K,** and **L**; involuting phase: **E–J. A** and **B**: anti-CD31; **C** and **D**: mast cell tryptase; **E** and **F**: anti α1-chain type IV collagen; **G** and **H**: laminin β2 chain; **I** and **J**: perlecan; **K** and **L**: basic fibroblast growth factor. (Original magnification, 185×).

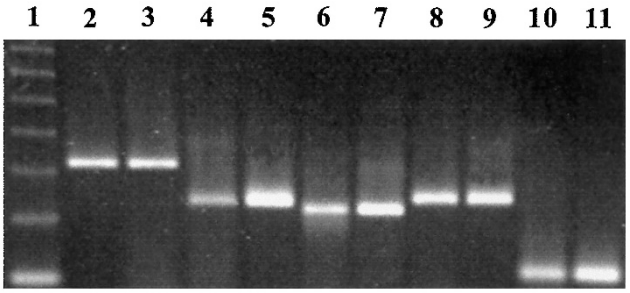

FIGURE 6. Ethidium bromide stained 2% agarose gel showing reverse transcriptase-polymerase chain reaction products in biopsy (lanes 2, 4, 6, 8, and 10) and tissue after culture (lanes 3, 5, 7, 9, and 11), taken from a proliferative hemangioma. Lane 1 is the 123*bp* DNA ladder; lanes 2 and 3: hypoxanthine phosphoribosyl transferase (~395*bp*); lanes 4 and 5: transforming growth factor (TGF)-β3 (~312*bp*); lanes 6 and 7: TGF-β1(~301*bp*); lanes 8 and 9: TGF-β2 (~363*bp*); lanes 10 and 11: basic fibroblast growth factor (~145*bp*).

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