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Mice with Hemangiomas Induced by Transgenic Endothelial Cells

A Model for the Kasabach–Merritt Syndrome

Nathalie Dubois-Stringfellow,*
Linda Kolpack-Martindale,† Victoria L. Bautch,*
and Richard G. Azizkhan†

From the Department of Biology, and Department of Surgery,† University of North Carolina at Chapel Hill, Chapel Hill, North Carolina*

Inoculation of an established endothelial cell line from transgenic mouse hemangiomas (Py-4-1) into histocompatible mice induced vascular tumor formation at the site of injection with 100% frequency. Histological and hematological studies revealed that the mice developed hemangiomas with hematological changes similar to those found in the Kasabach–Merritt syndrome in humans, including hemolytic anemia and thrombocytopenic purpura. Modifications in the red blood cell count, hemoglobin concentration, hematocrit, and platelet count were directly correlated with the size of the hemangioma. Thus transgenic endothelial cell injection into histocompatible mice provides an in vivo model system to study the pathobiology of hemangiomas as well as the investigation of angiogenesis inhibitors. (Am J Pathol 1994, 144:796–806)

Hemangiomas are angiomatous malformations/growths characterized by a proliferation of vascular endothelium. Hemangiomas occur in 10% of children and are the most common nonmalignant tumors of infancy.[1] Approximately 75% of these lesions are recognized in the neonatal period. The majority of capillary hemangiomas resolve spontaneously as the children grow older.[2] Developing or enlarging hemangiomas may obstruct, compress, or destroy vital structures and thereby cause disfigurement or serious disability. Occasionally, these lesions cause thrombocytopenia, consumptive coagulopathy, and microangiopathic hemolytic anemia.[3–6] Such complications are characteristic of the Kasabach–Merritt syndrome.[3] This syndrome is usually seen in early infancy but, it may occur in later childhood or adulthood.[5,7] The majority of vascular neoplasms in the Kasabach–Merritt syndrome occur at a single site on the limbs and the trunk.[7]

Transgenic mice expressing the entire polyoma virus early region gene develop multifocal tumors of the vascular endothelium.[8] These tumors, defined as hemangiomas, appear in adult mice, usually at different sites. In a previous report, we established a cell line, Py-4-1, from hemangiomas occurring in these mice.[9] Py-4-1 cells have retained vascular endothelial cell properties such as cobblestone appearance at confluence, contact inhibited growth, expression of von Willebrand factor (vWF), active uptake of acetylated low-density lipoprotein, and organization into capillary-like structures when cultured on a basement membrane-like matrix, Matrigel[9] (and unpublished data). Furthermore, Py-4-1 cells are tumorigenic in nude mice and histocompatible mice.[9]

Here the pathological and hematological features associated with the development of hemangioma after injection of Py-4-1 cells in mice are described. Two other murine models of induced vascular tumors have been reported.[10,11] In one model, syngeneic mice transplanted with hemangioendotheliomas developed hemangiomas.[10] However, the tumor was a mixed cell population, so nonendothelial cells present in the tumor may have contributed to tumor development. In a second model, inoculation of a cell line derived from ultraviolet-induced murine skin tumors was shown to induced hemangiomatous tumors in nude mice, but no hematological study was reported along with tumor progression.[11] In our model, we show that injection of a pure population of cultured

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Address reprint requests to Dr. Nathalie Dubois-Stringfellow, Biology Department, CB 3280, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

endothelial cells (Py-4-1) in histocompatible mice caused a single hemangioma to develop at the site of injection with 100% frequency. Furthermore, these mice developed many of the features of the Kasabach–Merritt syndrome,[3–6] including thrombocytopenia, anemia, and splenomegaly. This animal model may serve to study the features and complications of the Kasabach–Merritt syndrome and for therapeutic studies.

Materials and Methods

Mice and Cells

B6D2F1 mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Four- to 6-week-old females were used in the present study. The animal facility is approved by the American Association for Accreditation for Laboratory Animal Care and is under the care of Dr. J. Pick. Py-4-1 is a hemangioma-derived endothelial cell line isolated from a transgenic mouse carrying the polyoma virus early region as described previously.[9] Cells were maintained in Dulbecco's modified Eagle's medium supplemented with newborn calf serum at 37 C in a humidified atmosphere of 10% CO_2 in air. Cells have been continuously passaged at confluence after treatment with trypsin-ethylenediaminetetraacetic acid buffered solution for over 100 passages.

Py-4-1 Cell Inoculation

Inoculation of 3×10^6 Py-4-1 cells in 100 µl phosphate-buffered saline (PBS) was made through a 25G 5/8 needle into the subcutaneous tissues of the thigh of 40 B6D2F1 mice or in the peritoneal cavity of five B6D2F1 mice. Fifteen control mice of the same genetic background were injected with 100 µl PBS at the same site. Mice were sacrificed by cervical dislocation at the times indicated.

Histology

Tissue specimens from the site of inoculation and various organs were fixed with Bouin's fixative, embedded in paraffin, and sectioned on a microtome. Specimens were stained with hematoxylin and eosin and observed by light microscopy.

Immunohistochemical Analysis

Deparaffinized sections of tissue specimens fixed with Bouin's fixative were pretreated with 3% H_2O_2 in methanol for 10 minutes, washed in PBS and incubated with 0.05% trypsin (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at 37 C. Sections were then blocked with Dulbecco's modified Eagle's medium containing 10% (v/v) goat serum, 1% (w/v) bovine serum albumin, 50 mmol/L Hepes (pH 7.2), and incubated for 2 hours at 37 C with rabbit anti-human vWF, 1:100 (Dakopatts, Carpinteria, CA) in PBS containing 1% bovine serum albumin. Slides were then washed twice with PBS, incubated with biotinylated goat anti-rabbit immunoglobulin G (Zymed Laboratories Inc., San Francisco, CA) for 10 minutes at room temperature. Sections were developed by treatment with a Streptavidin-Biotin Amplified System (Histostain-SP kit, Zymed Laboratories Inc., San Francisco, CA). Sections were then counterstained with hematoxylin and mounted using standard procedures.

Electron Microscopy

Ultra-thin sections were made according to standard procedure. Briefly, tissue specimens were cut into small pieces, fixed with 3% glutaraldehyde for 3 hours at room temperature, postfixed in 1% osmium tetraoxide, dehydrated, and embedded in Araldite-502. Ultrathin sections were cut at 70 nm, stained in 4% aqueous uranyl acetate and 0.5% lead citrate, and observed with a Zeiss 10A transmission electron microscope at a voltage of 80kV.

Blood Studies

Blood samples were obtained by heart puncture and collected in vacutainer (Becton-Dickinson Vacutainer Systems, Rutherford, NJ). Hematocrits were measured using nonheparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Blood-filled capillary tubes were sealed with a tube-end sealer, and spun for 3 minutes using an IEC MB centrifuge (International Equipment Co., Boston, MA). Ratio were determined using a micro capillary reader (International Equipment Co., Boston, MA). To measure the hemoglobin level, blood samples were treated with ZAP-OGLOBIN II as the lysing agent for red blood cells (Coulter Diagnostics, Hialeah, FL). Hemoglobin levels were measured in the lysed samples using a Coulter Hemoglobinometer (American Scientific Products, Mc-

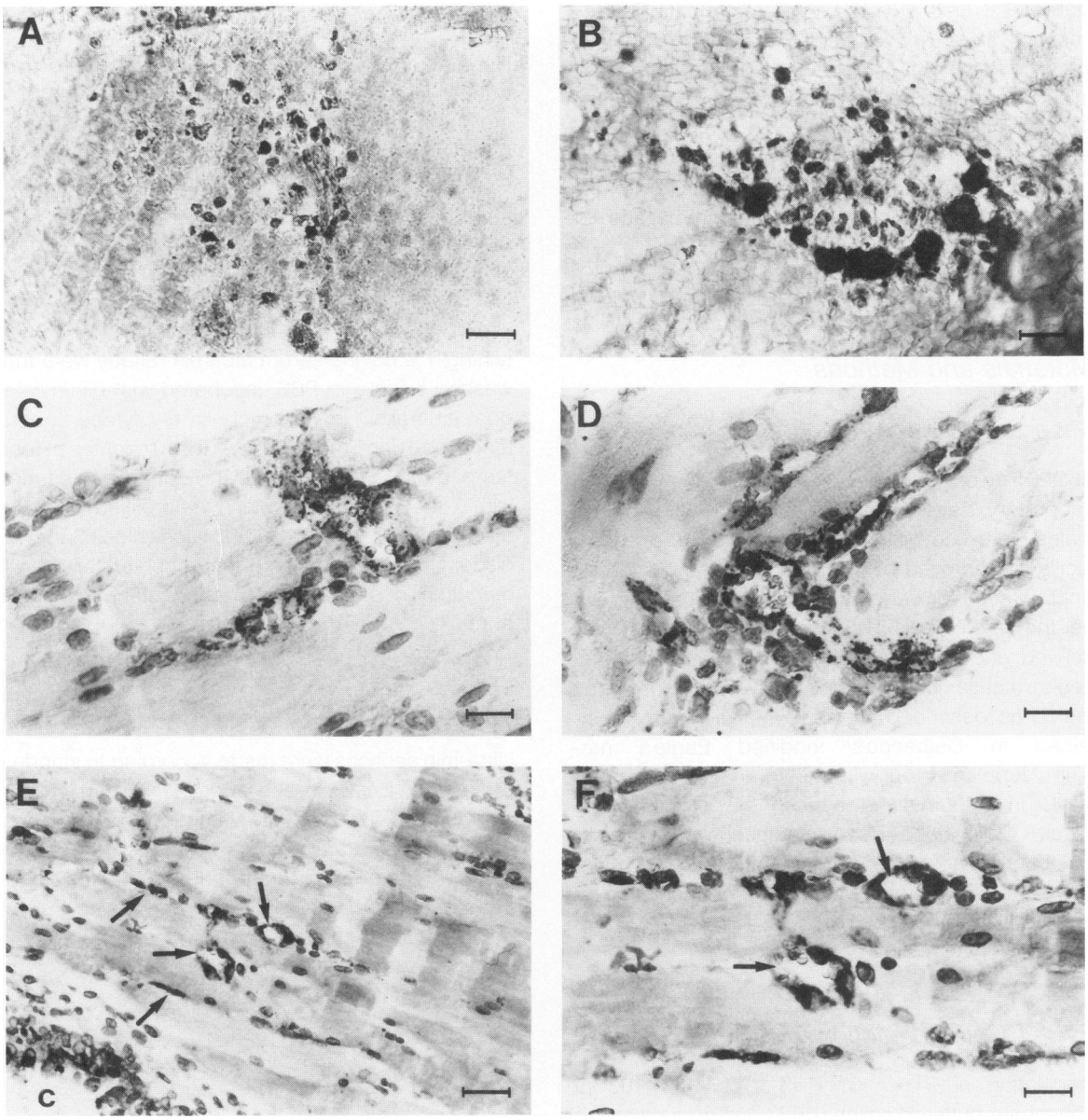

Figure 1. *vWF immunostaining of early stages of Py-4-1 hemangiomas.* **A** *and* **B**: *Injection site 20 hours after Py-4-1 inoculation. Note the aggregates of plump vWF-positive neoplastic cells (scale bar = 75 μ and 50 μ respectively).* **C** *and* **D**: *Hemangioma on day 3. The neoplastic endothelial cells organized into lumen-containing structures filled with erythrocytes (scale bar = 75 μ).* **E**: *Hemangioma on day 6. Endothelial cells (arrows) are seen in the muscle tissue surrounding the cystic cavity (c) (scale bar = 150 μ).* **F**: *Higher magnification of* (**E**). *The neoplastic cells form a loose lumen filled with erythrocytes (arrows) (scale bar = 75 μ).*

Gaw Park, IL). Red blood cell (RBC) counts were determined using a Coulter Counter model ZBI (Coulter Electronics Inc., Hialeah, FL). White blood cell counts were determined manually using a Unopette micro collection system (Becton-Dickinson). Blood smears were obtained by drawing a drop of blood across a slide and air-drying. For bone marrow smears, femurs were dissected and cut at one edge. A drop of bone marrow was then deposited on a slide and drawn across. Blood and bone marrow smears were then stained with Diff Quick Stain Set, a modification of Wright–Giemsa stain (Baxter Health Care Corp., McGaw Park, IL). Platelet counts were performed manually using a Unopette micro collection system (Becton-Dickinson). Reticulocyte determination was performed manually with the Unopette test 5821 procedure using new methylene blue N as the stain (Becton Dickinson). Blood cell analysis were performed using phase contrast microscopy.

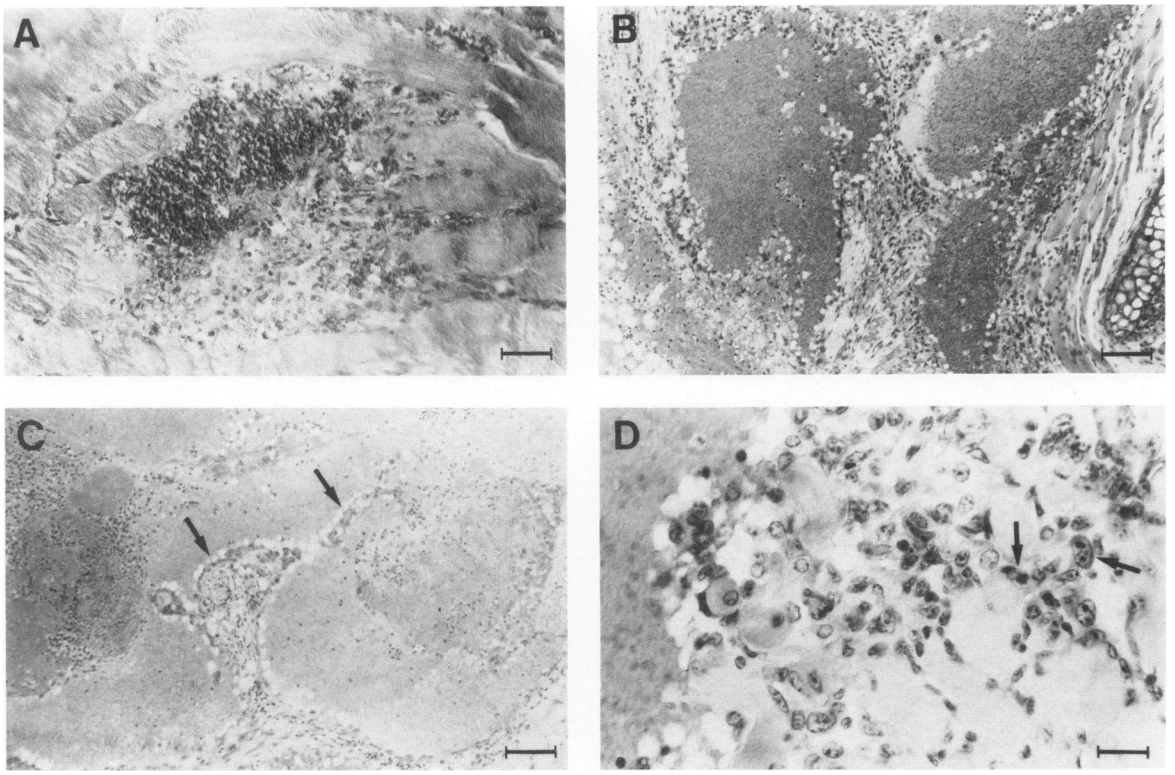

Figure 2. *Histological study of the early stages of Py-4-1 hemangiomas.* A: *Histology on day 5. The tumor has masses of neoplastic cells without recognizable space formation (bottom), and vascular spaces filled with erythrocytes (top) (scale bar = 150 μ).* B: *Histology on day 7. Spongiform structures with various sizes of cysts are seen. The cysts have thick walls composed of neoplastic cells (scale bar = 300 μ).* C: *Histology on day 7. Cells are seen in the cystic cavity arranged in septumlike cords or aggregates (arrows) (scale bar = 300 μ).* D: *Histology on day 7. Neoplastic cells with prominent nuclei and numerous mitotic figures are seen (arrows) (scale bar = 75 μ).*

Results

General Description

Hemangiomas developed in the subcutaneous tissue in 100% of the 40 mice injected with Py-4-1 cells, whereas the 15 mice inoculated with PBS did not show any abnormal features at the site of injection. In Py-4-1-injected mice, tumor growth appeared as a single mass at the site of injection. Hemangiomas developed rapidly, and typically, a bulky mass 1 cm or more in diameter was palpable within 2 to 3 weeks after injection. The tumors were partially encapsulated and were composed of soft dark-red tissues that exuded dark blood upon sectioning. Hemorrhage into the tumor was a prominent gross feature. Metastases were not found in any of the tumor-bearing mice. The tumor-bearing animals died spontaneously 3 to 5 weeks after injection of the Py-4-1 cells. When Py-4-1 cells were injected intraperitoneally, multiple small tumors of similar appearance resulted. The tumor nodules involved the peritoneal surface but did not penetrate deeply into the organs or spread outside the peritoneal cavity. Animals of this group died spontaneously 1 to 2 weeks after inoculation. Hemangioma development in subcutaneously injected mice was further studied because hemangiomas appeared at a single cutaneous site and thus are more likely to mimic hemangioma formation in humans.

Histological Findings

Histological sections stained with hematoxylin and eosin or immunostained for vWF, an endothelial cell marker, were prepared from mice sacrificed at different times after subcutaneous injection of Py-4-1 cells. Py-4-1 cells were present in small aggregates at the site of injection immediately after inoculation (Figure 1A). The neoplastic cells had a plump appearance as opposed to normal flat endothelial cells (Figure 1B). Endothelial cells formed lumenlike structures containing erythrocyte-filled spaces as soon as 3 days after injection (Figure 1, C and D).

On day 5 to day 7 after injection of Py-4-1 cells, the tumor was composed of very immature endothelial cells with extensive atypia. In some areas, masses of endothelial cells occurred without recognizable

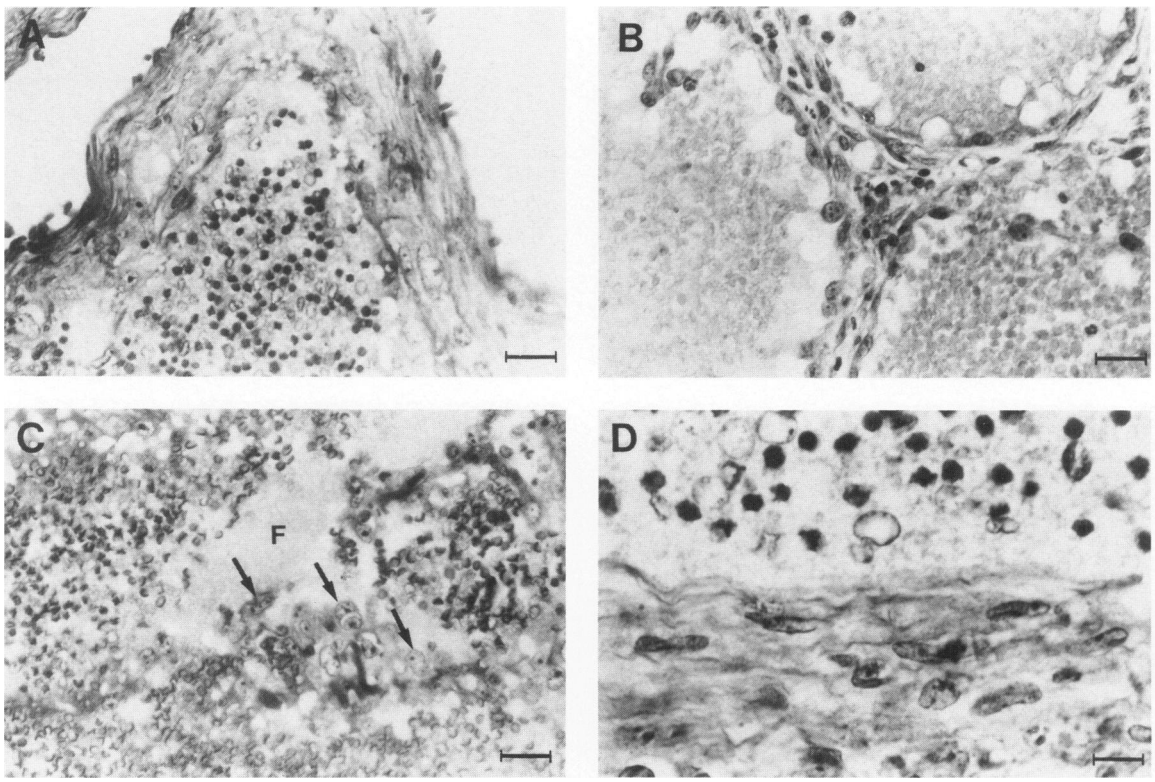

Figure 3. *Histological study of the late stages of Py-4-1 hemangiomas.* A: *Histology at day 15. Thick cyst walls composed of a multilayer of tumor cells are seen (scale bar = 75 μ).* B: *Histology on day 15. A wall of neoplastic cells separating three cystic cavities filled with erythrocytes is shown (scale bar = 75 μ).* C: *Histology on day 17. Cavities show a large collection of blood and fibrin (F = fibrin). Cyst walls are disrupted in this area and show tumor cells (arrows) surrounded by blood cells (scale bar = 75 μ).* D: *Histology on day 17. Numerous immature forms of erythrocytes including reticulocytes are surrounded by fibrin deposits in the tumor cavity (top) (scale bar = 30 μ).*

space formation (Figure 2A). In other places, endothelial cells formed a spongiform structure composed of various sizes of endothelial lined cysts (Figure 2B). In some areas, tumor cells apparently proliferated into the cystic cavity, producing septumlike cords or aggregates that occasionally contained lumenlike spaces (Figure 2C). The neoplastic endothelial cells showed prominent nuclei and numerous mitotic figures (Figure 2D). In some areas, the tumor cells seemed to invade the muscle tissue surrounding the cystic cavities by infiltrating between the muscle bundles (Figure 1, E and F). In the muscle, they formed a lumen containing erythrocytes (Figure 1F). Numerous erythrocytes and a few leukocytes seemed to be trapped in the tumor cavities and between the neoplastic cells.

On days 14 to 17 after injection of Py-4-1 cells, the size of the hemangioma had reached approximately 1 cm in diameter, and it was composed of various sizes of cysts. Walls of the cysts were thicker and consisted of a multilayer of cells with giant nuclei (Figure 3, A and B). Tumor cavities contained a large collection of blood cells including erythrocytes and a few neutrophils. Occasionally, cyst walls were disrupted, revealing endothelial cells surrounded by blood cells (Figure 3C). A light pink color indicated deposits of fibrin in the cavities (Figure 3C). Numerous immature and abnormal forms of erythrocytes as well as reticulocytes were observed in the tumor cavities (Figure 3D). On day 21 after Py-4-1 cell injection, the septation between cystic cavities had often disappeared, and the former cysts were coalesced into a single cyst (data not shown). A large collection of blood with many inflammatory cells was observed in the cavity.

Electron Microscopy Findings

The vascular tumors were examined by electron microscopy at day 5 after injection (Figure 4). The neoplastic endothelial cells lining vascular spaces were very irregular (Figure 4A) and often had interdigitations and irregular microvilli projecting into the blood space (Figure 4B). The endothelial cells had a plump appearance with signs of intracellular activity, such as convoluted nuclear membranes, swollen mitochondria, and rough endoplasmic reticulum (Figure 4, B

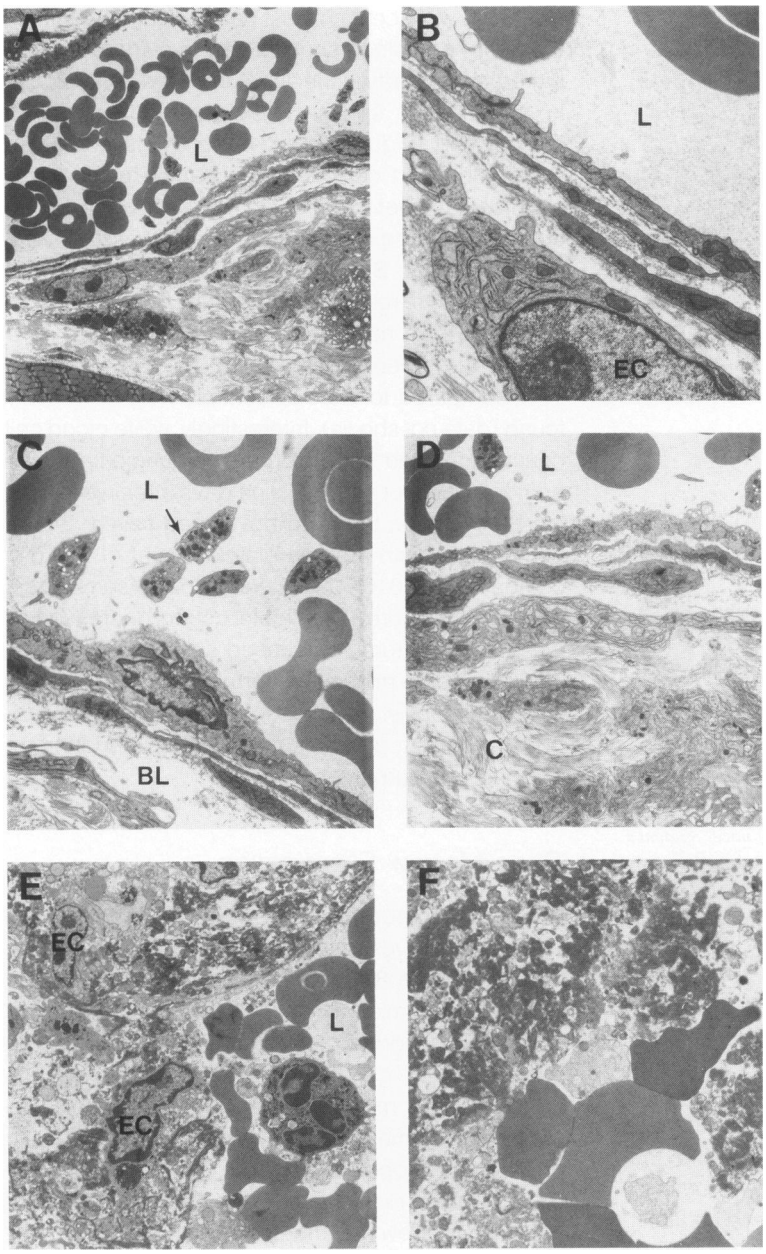

Figure 4. *Electron microscopy study of Py-4-1 hemangioma on day 5* (**A** *to* **D**), *and day 21* (**E** *and* **F**). **A:** *Hemangioma cavity with lumen* (*L*) *filled with erythrocytes. The walls of the hemangioma were a multilayer of tumoral endothelial cells* (*1,700×*). **B:** *Irregular microvilli of neoplastic endothelium projected into the lumen* (*L*) *of vascular space. Note the plump endothelial cell* (*EC*) *with sign of intracellular activity* (*10,100×*). **C:** *Numerous platelets* (*arrow*) *were present in the lumen* (*L*). *Neoplastic cells had a multilayered irregular basal lamina* (*BL*) (*5,000×*). **D:** *Deposits of collagen fibers* (*C*) *were formed on the basal side of the cells* (*3,200×*). **E:** *At day 21, the vascular lining was poorly delimited. Plugs of fibrin fibers, platelet aggregates, erythrocytes, and cell debris were present in the lumen* (*L*) *as well as infiltrating between the tumor walls composed of endothelial cells* (*EC*) (*3,200×*). **F:** *Higher magnification of material found in the lumen of the poorly organized vascular space at day 21* (*6,400×*).

and C). They showed poorly developed intercellular junctions and were frequently surrounded by a basal lamina irregular in thickness (Figure 4, C and D). Interstitial components such as collagen fibers were abundant between the neoplastic cells of the thick walls of the tumor (Figure 4D). Pericytes or smooth muscle cells were not detected. Platelet aggregates and erythrocytes were abundant in the lumen (Figure 4C). At day 21 after injection, the lumina of the hemangioma cavities were poorly delimited (Figure 4E). Abundant fibrin deposits, platelet aggregates, RBCs, and cell debris were found in the lumen (Figure 4, E and F). A striking feature was the extensive distortion of erythrocytes (Figure 4, E and F).

Observation of Other Organs

There was no difference in size and histology of heart, lungs, liver, adrenals, and kidneys (data not shown). However, splenomegaly was a characteristic finding in the mice with tumors (Figure 5A). The mean splenic weight at day 21 was 0.5 g compared to 0.07 g in control mice ($P < 0.01$) (Figure 5B). Histological ex-

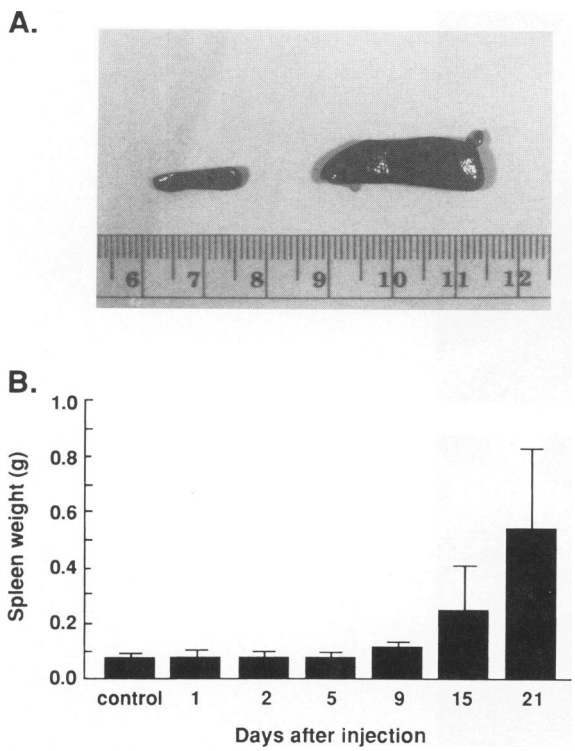

Figure 5. *Spleen size analysis in tumor-bearing mice.* **A:** *Size comparison between a normal spleen (left) and the spleen of a mouse with a subcutaneous hemangioma at 25 days postinjection (right).* **B:** *Time course of the spleen weight in tumor-bearing mice. Student's* t*-test compared to control: day 9,* P < *0.001; day 15,* P < *0.01; day 21,* P < *0.01.*

amination of the spleen from mice with tumors showed no abnormal features (data not shown).

Blood Analysis

As the hemangioma increased in size, the mice developed thrombocytopenia and anemia. Each of the circulating blood parameters studied, RBC count, hemoglobin concentration, hematocrit and platelet count decreased as the tumor size increased (Figure 6). One to 3 days after injection of the Py-4-1 cells, the mice had a significant decrease in RBC count ($P <$ 0.01) (Figure 6A). Two weeks later, the tumor-bearing mice showed a pronounced anemia with a RBC count one-third of the normal count ($P < 0.001$) (Figure 6A). At the terminal stage, the RBC count was as low as 1 million per μl in some mice. The hemoglobin level decreased severely during the course of hemangioma development and correlated with the decrease in RBCs (Figure 6B). The mean hematocrit value in the tumor mice reduced significantly 14 to 17 days after injection ($P < 0.003$) and was 18% compared to 30% in controls (Figure 6C). As the tumor increased in size, the anemia became more severe and hematocrit values of 5% were common just before death. Smears of the peripheral blood showed visible evidence of acquired abnormality in the structure of the erythrocytes in the form of spherocytosis, poikilocytosis, and anisocytosis (Figure 7). Fifteen to 21 days after injection of Py-4-1 cells, numerous abnormal forms of erythrocytes were observed in peripheral blood smears including crenated erythrocytes and poikilocytes (Figure 7, B and C). Blood smears stained for reticulocytes revealed that the anemia was also characterized by a marked reticulocytosis (Figure 7, D and F). At the terminal stage, reticulocyte counts of 80% compared to 2.5% in controls were commonly found (data not shown). Interestingly, white blood cell counts remained normal during hemangioma development (data not shown). Nine days after injection of the Py-4-1 cells, the mice developed severe thrombocytopenia with a mean platelet count of 269,000 compared to 1,016,000 in controls of the same strain ($P < 0.001$) (Figure 6D). Platelet counts decreased as the size of the tumor increased and were as low as 44,000 in some mice at the terminal stage. These results are consistent with the diagnosis of hemangioma associated with hemolytic anemia and thrombocytopenic purpura.[3–6,12]

Bone Marrow Examination

Bone marrow sections and bone marrow smears were examined to investigate the origin of the thrombocytopenia (Figure 8). Bone marrow examination from tumor-bearing mice 21 days after injection revealed an essentially normal marrow except for the presence of numerous megakaryocytes (Figure 8, A and B). In addition, bone marrow smear analysis showed the presence of immature megakaryocytes characteristic of thrombocytopenic purpura (Figure 8, C and D).[13–14] These results suggested that suppression of the bone marrow was not a likely cause for the decrease in circulating platelets.

Discussion

The present study presents a murine model of hemangioma development. Py-4-1 endothelial cells injected into histocompatible mice rapidly organized to form vascular tumors at the site of injection with 100% frequency. The histological and hematological features of Py-4-1-derived vascular tumors during the course of development were as follows. Immediately after inoculation, Py-4-1 cells formed small aggregates that soon organized into vascular spaces filled with erythrocytes. Gradually, the spaces evolved into

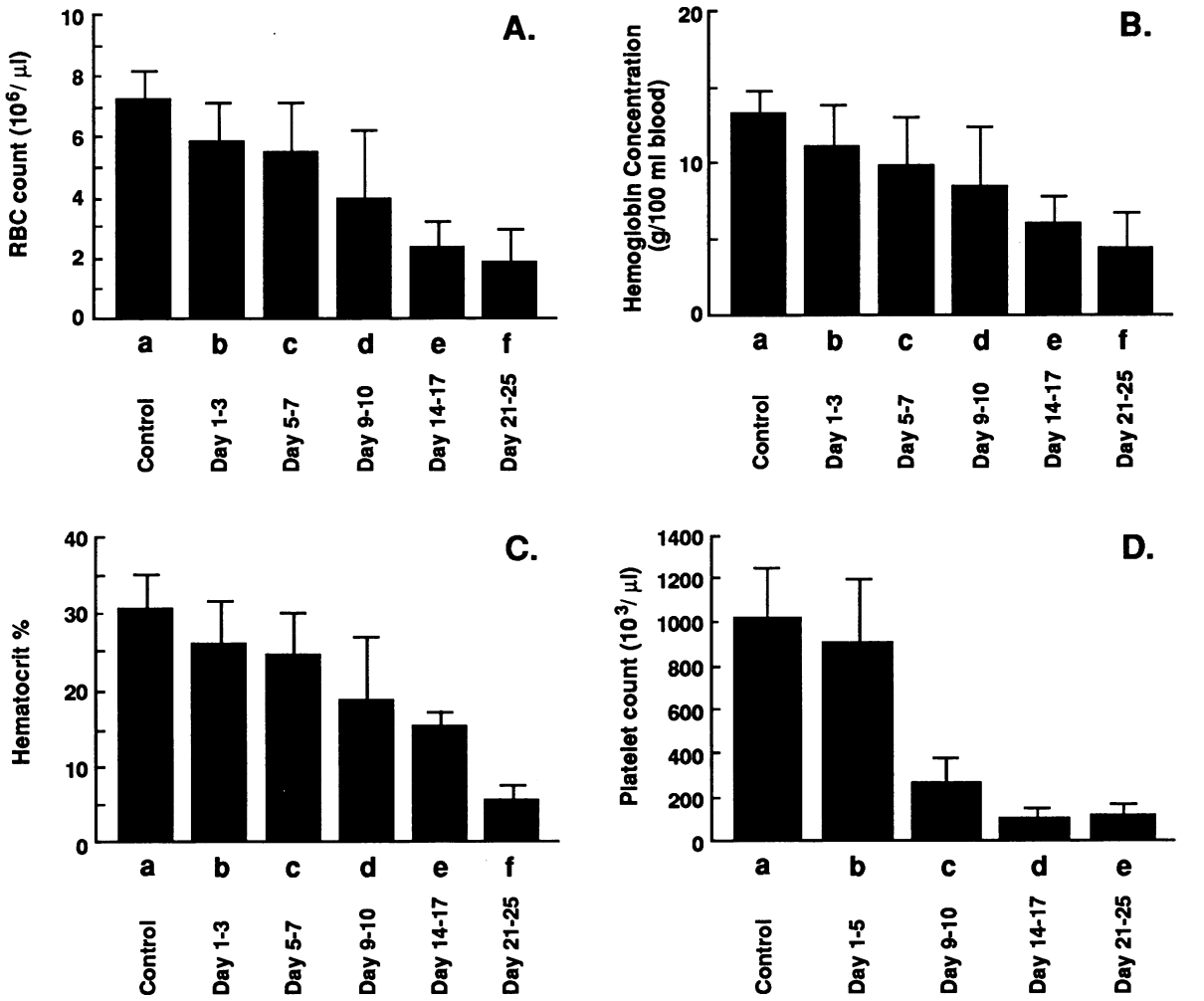

Figure 6. *Time course of RBC count* (A), *hemoglobin concentration* (B), *hematocrit* (C), *and platelet count* (D) *in tumor-bearing mice compared to controls. Student's* t *test compared to control:* (A) *b*, P < *0.01; c*, P < *0.05; d*, P < *0.008; e*, P < *0.001; f*, P < *0.001.* (B) *b*, P < *0.02; c*, P < *0.09; d*, P < *0.02; e*, P < *0.0001; f*, P < *0.0001.* (C) *b*, P < *0.1; c*, P < *0.1; d*, P < *0.02; e*, P < *0.003; f*, P < *0.004.* (D) *b*, P < *0.5; c*, P < *0.001; d*, P < *0.002; e*, P < *0.0001.*

cystic cavities of different sizes. It is not clear whether cystic cavity formation is due to the fusion between adjacent spaces and/or to individual space expansion. Simultaneous to cystic cavity formation, the endothelial cells lining the vascular spaces proliferated extensively and organized into a multilayer lining. At the later stages, numerous large cystic cavities were present and proliferating endothelial cells remained to constitute the cyst walls. These cells, however, showed a poor organization with fewer intercellular junctions. On the basis of these histological features, the Py-4-1 tumors were previously categorized as hemangioendotheliomas or simply as hemangiomas.[15] As the hemangioma increased in size, blood parameters including RBC count, hemoglobin concentration, hematocrit and platelet count decreased significantly. This in turn lead to severe anemia and thrombocytopenia that contributed to the mortality of the mice. These hematological changes are similar to those found in the Kasabach–Merritt syndrome in human beings.[3–6,14] The anemia in these mice may have resulted from several factors such as sequestration of the red blood cells in the tumor or bleeding following rupture of the vascular channels within the hemangioma. Several theories have been proposed to explain the relationship between the hemangioma and the thrombocytopenia in the Kasabach–Merritt syndrome including: 1) trapping or utilization of platelets in the hemangioma, 2) increased peripheral destruction of platelets, and 3) decreased production of platelets in the bone marrow. In this mouse model, the major apparent cause of thrombocytopenia is the trapping or utilization of platelets in the tumor. Observation of numerous plate-

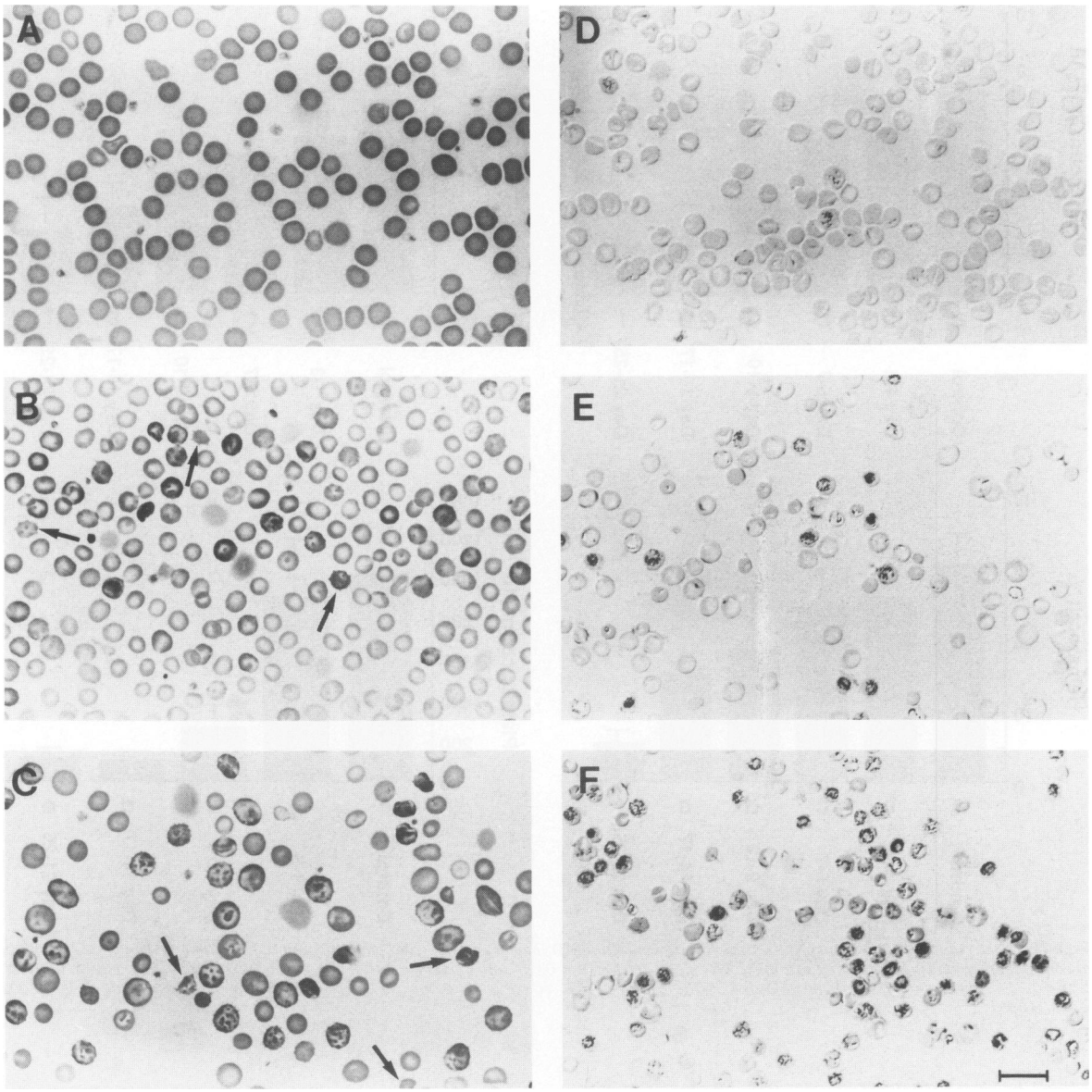

Figure 7. *Peripheral blood smear* (A *to* C) *and reticulocyte analysis* (D *to* F) *during the time course of hemangioma formation. Peripheral blood smears from mice 1 day* (A), *15 days* (B), *and 21 days* (C) *after subcutaneous injection of Py-4-1 cells. Note the presence of numerous crenated erythrocytes or "Burr cells" (arrows in* B) *and sharp-angled "helmet" cells or poikilocytes (arrows in* C). *Peripheral blood reticulocyte staining from mice 1 day* (D), *15 days* (E), *and 21 days* (F) *after injection of Py-4-1 cells. Note the increasing number of dark staining reticulocytes. Scale bar = 50 μ.*

lets sequestered within the hemangioma, the presence of numerous megakaryocytes in the bone marrow, and low plasma platelet counts provide the primary evidence for this concept.

Objectives of therapy in the Kasabach–Merritt syndrome are twofold: to control the coagulopathy and to eradicate or reduce the size of the lesion. In the past, selected patients had been treated with corticosteroids or cytotoxic drugs, but these treatments had adverse side effects and were inconsistently effective.[16–19] Some patients have benefited from embolization of feeding vessels, surgical extirpation of the hemangioma, and anticoagulation treatment.[6,18,20–25] Radiation therapy is now rarely employed for these patients as the incidence of growth retardation and complications are significant. Recombinant interferon is a relatively new addition to the treatment of selected vascular malformations, and the results in a few patients with aggressive hemangiomas has been encouraging.[26–29] At this time, how-

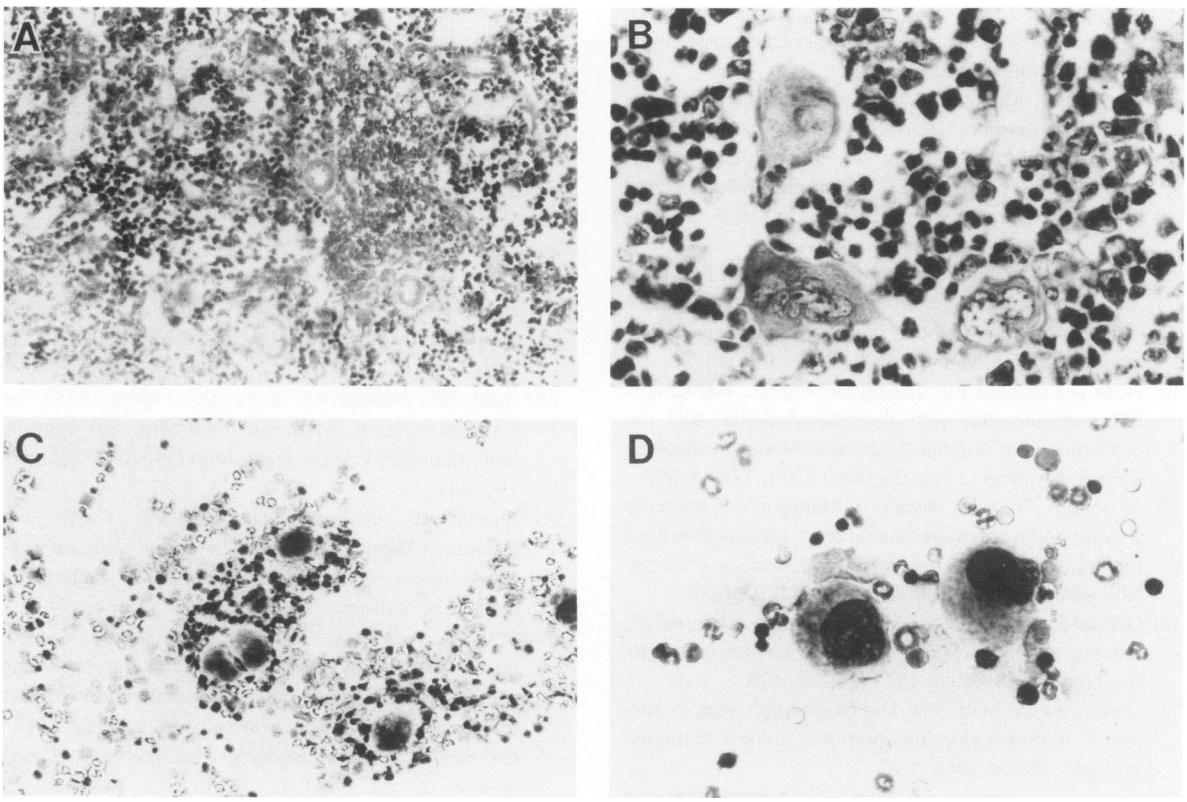

Figure 8. *Bone marrow study 21 days after subcutaneous injection of Py-4-1 cells.* **A:** *Bone marrow section (400×).* **B:** *Bone marrow section showing the presence of numerous megakaryocytes (800×).* **C** *and* **D:** *Bone marrow smears revealing the presence of immature megakaryocytes* (**C:** *400×;* **D:** *800×*).

ever, the mechanisms of recombinant interferon action in these patients are entirely unknown, as are the appropriate dosage, interval, and duration. This murine model provides a good system to study the effects of recombinant interferon and other angiogenesis inhibitors on hemangioma formation and endothelial cell proliferation.

In conclusion, Py-4-1 cells injected into histocompatible mice present a novel animal model to study vascular tumors. This model is particularly convenient to investigate vascular endothelial cell tumorigenesis as well as to perform therapeutic studies because it is easily manipulable, reproducible at 100% frequency, and blood parameters can be followed to determine if experimental treatment will influence tumor progression. In addition, this model is suitable for manipulating Py-4-1 cells *in vitro* before inoculation. For example, the cells could be transfected with a gene construct encoding interferon-α. The effects of interferon-α production by Py-4-1 cells on hemangioma formation and endothelial cell proliferation could then be followed *in vivo*.

Acknowledgments

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