High Angiogenic Activity in Cells Isolated From Cystic Hygroma

Role of bFGF

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Background: Cystic hygromas are characterized by a proliferation of small vessels and lymphatics with intervening fibrous tissue. Studies have shown malignant tumors and some benign neoplasms are dependent on angiogenesis, the induction of new capillaries from preexisting vessels. Growth and progression of these tumors are associated with a disturbance in the balance of angiogenic inducers and inhibitors. We have postulated that cells derived from cystic hygromas are angiogenic due to secretion of higher levels of angiogenic inducers that promote vascular proliferation.

Design: A large cystic mass was surgically removed and a portion of the sterile tumor was immediately placed in the medium. The tissue was minced, washed in phosphate-buffered saline, and grown to near confluence. Conditioned medium was collected under serum-free conditions after 48 hours. Secreted proteins were concentrated, quantitated, and analyzed in an in vitro endothelial cell migration assay and by Western blot. Antibody to factor VIII-related antigen was performed to confirm endothelial cell origin of the cultured cells.

Main Outcome Measures: In vitro angiogenic activity of secreted proteins in a capillary endothelial migration assay was tested by using blocking antibodies to angiogenic inducer, basic fibroblast growth factor, and angiogenic inhibitor, thrombospondin-1. Total protein levels of thrombospondin-1 were determined by Western blot.

Results: Cells isolated from cystic hygroma are angiogenic in vitro and this angiogenic activity is due to secretion of high levels of angiogenic inducer, basic fibroblast growth factor, and lower levels of naturally occurring angiogenic inhibitor, thrombospondin-1.

Conclusions: Cystic hygromas may represent another neoplasm dependent on angiogenesis. The angiogenic activity is due in part to elevated levels of potent angiogenic inducer, basic fibroblast growth factor. Antiangiogenic therapy directed at the endothelial cell may help suppress the growth of cystic hygromas.


Cystic hygromas are benign, locally aggressive lesions that can be difficult to manage because of recurrence following surgery and the limited efficacy and high toxicity associated with current pharmacological interventions. Cystic hygromas are characterized histologically by a proliferation of blood vessels and lymphatics with intervening fibrous tissue and lymphoid aggregates. Studies have shown that tumors and some benign neoplasms are dependent on angiogenesis. Angiogenesis, the induction of new capillaries from preexisting vessels, is regulated by a balance of angiogenic inducers and naturally occurring inhibitors. Tumor growth and progression is favored when angiogenic proteins such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) predominate locally or systemically. We postulated that the growth of cystic hygromas results from the dysregulation of angiogenesis secondary to proteins secreted by cells derived from this lesion that help promote neovascularization and tumor progression.

RESULTS

The patient was a 16-year-old white boy who presented to Children’s Memorial Hospital Outpatient Clinic (Chicago, Ill) with a 1-month history of an enlarging mass located in the right supraventricular area. The mass was first noted to occur after a viral illness. The patient did not experience any other symptoms. On physical examination, the mass appeared cystic and measured 8 x 10 cm. Results of evaluation with a computed tomographic scan evaluation were consistent with cystic hygroma and surgical excision was recommended. Surgery was performed; the
MATERIALS AND METHODS

CELL CULTURE AND COLLECTION OF CONDITIONED MEDIUM

One gram of fresh tumor tissue from the patient was minced and rinsed 3 times with the nutrient tissue culture medium. Dulbecco modified Eagles medium (DMEM; GIBCO, Grand Island, NY) without serum. Twenty milliliters of collage-nase (3 mg/ml with 1-mmol/L calcium chloride, Sigma, St Louis, Mo) was then added and the sample was incubated for 20 minutes at 37°C with agitation. The suspension was filtered through nylon gauze (mesh size, 60 µm), washed twice with Gey Balanced Salt Solution, and the suspension layered onto a cushion with polysucrose and sodium diatrizoate (Histo-Paque R-1077, density 1.077 g/ml, Sigma) at 1500 relative centrifugal field measured in measured in acceleration due to gravity. The layer of cells was harvested, washed twice in DMEM, diluted in the same medium containing 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of fungizone, and plated in flasks and incubated at 37°C with 5% carbon dioxide. When the cells were 70% to 80% confluent, the cells were rinsed 4 times with DMEM without serum and cultured with the serum-free medium for 4 hours, then rinsed 3 times again to wash out the protein adherent to the surface of the cells. Twenty milliliters of DMEM without serum was added into the flask after the washes. The cells were cultured at 37°C with 5% carbon dioxide for 48 hours. The medium was collected, concentrated, protein levels calculated, and stored at −70°C until processed.

CAPILLARY MIGRATION ASSAY

Bovine adrenal capillary endothelial cells were grown in DMEM containing 10% donor calf serum (GIBCO) and 100 µg/mL L-endothelial cell mitogen (Biomedical Technologies, Stoughton, Mass) and used between passages 13 and 15. To measure migration, cells were starved overnight in DMEM containing 0.1% bovine serum albumin. The cells were harvested, suspended in DMEM with 0.1% bovine serum albumin at 1 × 10^6/mL and 28 µL of cell suspension loaded into each well of a Boyden chamber, covered with a gelatinized 5.0-µm filter (Nucleopore Corporation, Pleasanton, Calif) in an inverted position and incubated 1 to 2 hours at 37°C, during which time cells adhered to the membrane. The chamber was reverted to an upright position and the test-conditioned media (1 to 2 µg/well) from CS were added to each well and incubated for 3 to 4 hours at 37°C. The chambers were dis-assembled, the membranes fixed and stained, and the number of cells that migrated to the top of the membrane in 10 high-power fields counted. The DMEM containing 0.1% bovine serum albumin was used as the negative control, and 10 ng/mL of bFGF as the positive control.

WESTERN ANALYSIS FOR THROMBOSPONDIN-1 (TSP-1)

Ten micrograms of total protein from conditioned medium of CS was loaded into a 5% to 8% sodium dodecyl sulfate–polyacrylamide gel. The protein was transferred to nitrocellulose that was blocked with 5% milk in phosphate-buffered saline for 1 hour, then incubated with antihuman TSP-1 monoclonal antibody (clone 1A4, GIBCO) for 1 hour. After applying the second antibody conjugated with horseradish peroxidase, the membrane was washed, treated with chemiluminescence reagent (Amersham Corporation, Arlington Heights, Ill), the film was explored, developed, scanned, and the data quantified with a densitometer. Human recombinant protein (GIBCO) was used simultaneously as the positive control.

mass was identified at the posterior triangle of the neck and was excised without incident. The specimen was delivered for surgical pathologic examination for further analysis, and the diagnosis of cystic hygroma was confirmed. Histological sections of the tumor revealed a proliferation of lymphatics, arterioles, and loose fibrous tissue. Small aggregates of lymphocytes were present in the interstitial fibrous matrix.

To determine if the tumor cells derived from the cystic hygroma secreted proteins with high angiogenic activity in vitro, cells were grown in culture and media was collected under serum-free conditions. Using an in vitro angiogenesis assay, media conditioned by the tumor cells or the secreted proteins had significantly higher angiogenic activity when compared with the negative control (compare bovine serum albumin or negative control column to CS media alone in Figure 1). Adding a blocking antibody to bFGF relieved most, but certainly not all, the angiogenic activity. This finding suggests that bFGF is not the only angiogenic mediator in the media, and other known factors, such as VEGF, may also play a role in the growth of this neoplasm.

To assess the level of angiogenic inhibitor activity in the tumor cell–conditioned media, bFGF was added and the expected angiogenic response was only partially blocked, suggesting only a modest level of angiogenic inhibitors. Identification of the secreted inhibitory substance was determined by Western blot using an antibody to a naturally occurring inhibitor, TSP-1 (Figure 2). Although TSP-1 protein was identified, the in vitro assay clearly demonstrated that it was not secreted in sufficient quantities to overcome the strong angiogenic activity in the media.

The causative factors responsible for the vascular and lymphatic proliferation in cystic hygromas are not well defined. It has been debated whether these vascular neoplasms are developmental in origin or represent true hamartomatous lesions. Their tendency to be locally aggressive and destructive to native tissues are features that lead to disfigurement and difficulty in obtaining a complete surgical resection. Our study investigated the possibility that the growth of cystic hygromas, similar to other tumors, is dependent on angiogenesis. Identifying the tumor cell–derived factors responsible for the angiogenic activity could assist in our understanding of
the pathophysiological features of these tumors and open a new avenue of therapy using antiangiogenic agents.

In this study, tumor cells derived from a large cystic hygroma located laterally in the neck of a child secreted proteins with high angiogenic activity as measured by an in vitro angiogenic assay. A significant amount of this angiogenic activity could be attributed to bFGF and the level in the conditioned media was sufficient to overcome the inhibitory effect of TSP-1. Since all the angiogenic activity could not be relieved by a blocking antibody to bFGF, it is possible that other angiogenic mediators such as VEGF may play a role in the neovascularization seen in cystic hygromas.

The importance of angiogenic mediators in tumor growth and metastases is well established, including studies involving cancer of the head and neck region. Studies directly evaluating angiogenesis in cystic hygromas have not been previously reported. The potent angiogenic protein, bFGF, has been implicated in various types of tumors and recovered from both the serum and urine of patients with cancer. Its receptors have been shown to be up-regulated in the vasculature and tumor cells in vitro. Blocking antibodies to bFGF have proved to be beneficial in some animal tumor models.

Thrombospondin-1 is a homodimeric 450-kd glycoprotein that is a member of a small group of naturally occurring inhibitors of angiogenesis found in normal tissue, including fibroblasts. It is able to block migration and mitogenesis of capillary endothelial cells in vitro. The local level of this inhibitor is critical in stabilizing neovascularization, especially during the time when tumors are secreting potent angiogenic mediators. In addition, recent evidence suggests that TSP-1 may be important in the regulation of programmed cell death or apoptosis. It is possible that the level of TSP-1 secreted by cystic hygromas is inadequate to induce endothelial cell apoptosis within the highly vascular tumor, thereby promoting further growth and neovascularization.

Although VEGF was not tested in this study, it is another important angiogenic mediator in premalignant and malignant neoplasms. Several studies have found that VEGF may not only be a mediator of angiogenesis but may also act as a growth factor promoting the proliferation of lymphatics. Vascular endothelial growth factor C appears to be the first angiogenic mediator that is specific for the lymphatic system. The selectivity of VEGF-C was recently confirmed when hyperplasia of the lymphatic system was demonstrated in VEGF-C transgenic mice. This finding is especially intriguing in the context of cystic hygromas since the lymphatic component of this tumor can be the predominant pathological feature in primary tumors and recurrences. Selective blockade of a "lymphatic-specific" growth factor may provide a new pharmacological modality to suppress local growth of these lesions.

This study demonstrates that media conditioned by tumor cells cultured from a large cystic hygroma have high angiogenic activity. Most, but not all, of the activity can be attributed to bFGF. The cells also secreted an angiogenic inhibitor, TSP-1, although not in adequate quantities to block the angiogenic activity. These findings suggest that the growth of these benign neoplasms may be dependent on excessive angiogenesis. Further testing is required on a large group of cystic hygromas or lymphangiomas to determine the relative contribution of bFGF or VEGF to the neovascularization, to investigate the cellular sources of these proteins, and, eventually, to test the efficacy of antiangiogenic therapy to control tumor growth.

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REFERENCES


