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Retrovirus-transformed erythroleukemia cells induce central nervous system failure in a new syngeneic mouse model of meningeal leukemia

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Abstract

Lack of suitable mouse models for central nervous system (CNS)-associated leukemias has hindered mechanism-guided development of therapeutics. By transplanting retrovirus-transformed mouse erythroleukemia cells into syngeneic mice, we developed a new animal model of meningeal leukemia associated with rapid paralysis. Necropsy revealed massive proliferation of the leukemic cells in the bone marrow (BM) followed by pathological angiogenesis and invasion of the leukemic cells into the meninges of the CNS. Further analysis demonstrated that the erythroleukemia cells secreted high levels of VEGF and preferentially adhered in vitro to fibronectin. This unique animal model for meningeal leukemia should facilitate studies of engraftment and proliferation of leukemic cells in the BM and their invasion of the CNS as well as pre-clinical evaluation of experimental therapeutics for CNS-associated leukemias.

Keywords

Animal Model; Meningeal leukemia; Murine erythroleukemia cells; Cell adhesion Angiogenesis; PU.1; Friend SFFV

1. Introduction

Central nervous system (CNS) leukemia, such as meningeal leukemia, is a morbid complication of myeloid and lymphoid leukemias [1] that is generally detected in the cerebral spinal fluid (CSF) or CNS tissues as relapsed disease following systemically-successful therapy [2] or upon initial presentation of individuals experiencing neurological symptoms [3]. CNS relapse was associated with a 2.2-fold diminished survival rate in a study involving 690 patients with acute myeloid leukemia (AML) that entered remission

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Contributions: GRM designed, performed and analyzed the majority of the experiments and drafted the manuscript; CAH and DMT provided critical technical support for these studies; CMP carried out all *in vivo* studies; JLC contributed to the design and interpretation of these studies and helped write the final manuscript; and SKR contributed to the conception and design of the study, directed the research and wrote the final manuscript.

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following a course of chemotherapy [4] and was the highest in very young children [4,5]. CNS directed therapy for relapsed leukemia is invasive, involving radiotherapy and repeated lumbar punctures for chemotherapy or CNS irradiation, procedures that in children are associated with complications ranging from cognitive delay to secondary neoplasms [6–9]. Nevertheless, in the absence of CNS-directed prophylaxis, neurological damage can result when proliferating leukemic blasts transit the blood-brain barrier and/or blood-CSF barrier that separates the bone marrow from the meninges and invade the meningeal spaces and CSF. Safer therapeutic agents to treat CNS leukemia are needed, as are new preclinical animal models in which to test them. Current animal models for CNS leukemia involve transplantation of human leukemic cells into immunodeficient rodents (10, 11). Unfortunately, the lack of a normal immune system in these animals complicates the study of disease mechanism.

Friend spleen focus-forming virus (SFFV) causes a multi-stage erythroleukemia in mice that parallels in many respects the biphasic development of myeloid leukemias in humans [12,13]. In the first stage of SFFV-induced leukemia, virus-infected erythroid cells proliferate and differentiate in the absence of erythropoietin (Epo). This is the result of expression of the SFFV envelope protein, which interacts with the Epo receptor and the receptor tyrosine kinase sf-Stk to cause constitutive activation of signal transduction pathways needed for erythroid cell proliferation and differentiation. In the second stage, SFFV-infected erythroid cells become transformed and can be grown as erythroleukemia cell lines (SFFV-MEL). This is due to SFFV integration into the *Sfpi1* locus, leading to non-physiological levels of the myeloid transcription factor PU.1 and a block in erythroid cell differentiation. In this study we demonstrate that intravenous transplantation of transformed SFFV-MEL cells to syngeneic mice results in massive proliferation of these cells in cranial and vertebral bone marrow and infiltration of the meninges, representing a new mouse model of meningeal leukemia. Further studies shed light on changes that have occurred in these cells that might favor their proliferation and retention in the BM and support invasion of the CNS. This animal model should be valuable not only for determining the genetic changes occurring during leukemic cell progression that are necessary for the development of meningeal leukemia but also for pre-clinical evaluation of experimental therapeutics for hematopoietic malignancies with CNS involvement.

2. Materials and Methods

2.1. Cell lines and mice

SFFV-spl cells were isolated from the enlarged spleens of BALB/c mice infected as adults with Friend MuLV/SFFV and maintained for several days in Iscove's-modified Dulbecco's minimal essential medium (IMDM) supplemented with 30% fetal calf serum (FCS) and 500 mM β -mercaptoethanol. NP1, NP4, NP5 and NP7, murine erythroleukemia cell lines (SFFV-MEL) generated from BALB/c mice infected with helper-free SFFV [14], were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. To induce meningeal leukemia, SFFV-MEL cells (5×10^6 in 0.1 ml DMEM) were injected into the tail veins of adult BALB/c mice. For comparison, mice were injected intravenously with an equivalent number of SFFV-spl or DMEM.

2.2. Protein analysis

Western blot analysis was carried out on tissues harvested from mice during necropsy or cells in culture as previously described [15] using an anti-SFFV gp55 monoclonal antibody (7C10) [16] as well as antibodies to PU.1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), ERK (New England Biolabs, Inc., Beverly, MA) or β -tubulin (Sigma, St. Louis, MO).

2.3. Histopathology and Immunohistochemistry

Tissues collected following inter-cardiac perfusion of mice were fixed and stained with hematoxylin and eosin. For CD31 immunohistochemistry, mouse femurs were fixed in 10% neutral buffered formalin and then decalcified in formic acid for 48 hrs at room temperature before sectioning. 2% normal rabbit serum in PBS was used for blocking and goat anti-mouse CD31 (Santa Cruz Biotechnology) applied for 30 minutes at room temperature. 1% biotinylated rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA) and streptavidin were used for secondary detection. For osteoclast detection, bones were decalcified in 19.5 M EDTA/31.9 M sucrose for 4 weeks, replacing the solution four times. Slides were prepared and immersed in tartrate resistant acid phosphatase (TRAP) solution for 2 hours at 37°C. Sections were washed and counterstained in hematoxylin for 4 minutes followed by a brief staining with saturated lithium carbonate and a 5 minute wash in running tap water.

2.4. Microarrays and Analysis

Total RNA was extracted from replicate SFFV-MEL or SFFV-spl cell pellets. cDNA synthesis and cRNA labeling for each RNA sample was performed using 1 µg of total RNA and the One-Cycle Target Labeling protocol (Affymetrix, Santa Clara, CA). Subsequent hybridization of biotinylated RNA to GeneChip® microarrays (representing 14,000 mouse genes) was performed according to the manufacturer's instructions (Affymetrix). Data storage, statistical analysis and data filtering were performed using the National Cancer Institute's Microarray Database and Statistics tool ("mAdb"; <http://madb-nci.cit.nih.gov>). Multiple array hybridizations with cRNA were filtered to exclude those arrays that yielded signal in less than 50% of the array. For the remaining arrays, comparison of mean signal intensities between groups was performed by pooled *t*-test with equal variance, and results filtered to exclude genes whose fold expression level difference between groups was less than 3, and with *p*-values greater than 0.01. The resulting gene set was categorized into pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.5. Matrix adhesion assay

SFFV-MEL or SFFV-spl cells (1×10^6) were seeded in triplicate wells of tissue culture plates coated with fibronectin, type I collagen, type IV collagen or poly-D-lysine (BD Biosciences, San Jose, CA). After 16 hours, media was removed and the wells were subjected to two PBS washes. Adherent cells were released by trypsinization into PBS and counted, as were non-adherent cells from the media and both wash steps. In some cases, cells (1×10^6 in 100 µl medium) were pre-incubated with 2 µg of antibodies specific for mouse integrin $\alpha_5\beta_1$ (Chemicon, Temecula, CA), mouse Epo receptor (Santa Cruz Biotechnology) or with a mouse IgG2bκ isotype control antibody (Abcam, Cambridge, MA) for 45 minutes at 37 °C prior to incubation in matrix-coated wells. Statistical comparison of matrix binding affinities was by unpaired *t*-test (Graphpad Prism Software, San Diego, CA).

2.6. VEGF ELISA

VEGF levels in conditioned media of 72 hr cultures of SFFV-MEL cells were determined using a VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Conditioned medium from confluent 72 hr cultures of NIH3T3 cells was used as a control.

3. Results

3.1. Mice transplanted with SFFV-MEL cells develop meningeal leukemia and CNS failure

To determine the metastatic potential of SFFV-transformed erythroleukemia cell lines (SFFV-MEL), 5×10^6 cells were injected via tail vein to normal, syngeneic BALB/c mice. The SFFV-MEL cells used for this study were prepared using helper-free SFFV and do not release infectious virus. Thus, pathological changes that occur are the result of the transplanted cells and not virus infection of host cells. Southern analysis of the proviral genomic integration patterns obtained from soft agar clones of the SFFV-MEL cell lines verified their clonal nature (data not shown). Control mice received DMEM or 5×10^6 primary, non-transformed erythroid cells from the enlarged spleens of F-MuLV/SFFV-infected mice (SFFV-spl cells). SFFV-spl cells release infectious virus, so pathological changes that occur can be the result of the transplanted cells or virus infection of host cells. Within 17 days of transplantation, 100% of the SFFV-MEL transplanted mice experienced neurological failure characterized by lack of grooming, ataxic gait, tremor and labored breathing that progressed to hind limb paralysis and death over a period of 12 hours (Table 1). This protocol was repeated multiple times, using 4 different SFFV-MEL cell lines, with identical results. Mice given subcutaneous injections of SFFV-MEL cells developed solid tumors but no paralysis (data not shown). Necropsies of the SFFV-MEL transplanted mice revealed slightly enlarged spleens (0.2–0.4 gm) and reduced brain integrity, with “mushy” hindbrain consistency compared with control mice. Mice transplanted with SFFV-spl cells showed greatly enlarged spleens (1–2 gm) within 2–3 wks but no paralysis (Table 1). Mice injected with DMEM remained healthy throughout the experiment. As shown in Fig. 1, cell lysates prepared from BM (lane 1), liver (lane 4) and spleen (lane 5) of mice transplanted with SFFV-spl cells expressed high levels of the SFFV envelope protein (SFFV gp55) as the result of spread of infectious SFFV from the SFFV-spl cells in these mice. In contrast, mice transplanted with SFFV-MEL cells showed very low expression of SFFV gp55 in the spleen and liver (lanes 7 and 9) but very high levels in the BM (lane 10). No SFFV gp55 could be detected in the brains of any transplanted mice (Fig. 1, lanes 2 and 12). Since SFFV-MEL cells do not release infectious virus, the cells expressing SFFV gp55 in the BM of SFFV-MEL transplanted mice are the transplanted SFFV-MEL cells. Curiously, unlike the original SFFV-MEL cells injected, the cells growing in the BM failed to express PU.1 (Fig. 1, lane 10). However, when these cells were put into culture, they rapidly grew as immortal cells and expressed SFFV gp55 as well as PU.1 (Fig. 1, lane 11).

Although mice transplanted with SFFV-MEL cells became paralyzed, the transplanted cells could not be detected in the brains of the diseased mice. To better understand why transplantation of SFFV-MEL affected the CNS, we carried out histological analyses on tissue sections through the skull and brain as well as the vertebrae and spinal cord. As shown in Fig. 2, SFFV-MEL transplanted mice revealed replacement of cranial (Fig. 2A and B) and vertebral (Fig. 2C) BM with malignant cells and extensive involvement of the adjacent meninges. Putative sites of meningeal invasion were identified in vertebral and cranial bone (see white arrows in Fig. 2A and C). Meningeal expansion appeared to induce compression necrosis of nervous tissue in the cerebellum and spinal cord. Dramatic change in cerebellar shape was evident in some SFFV-MEL transplanted mice at the end-stage of disease (Fig. 2B). Foci of spinal cord hemorrhage were also identified (data not shown). Leukemic cells were not detected in the tissues of the brain or spinal cord. Rather, extensive population of the meningeal spaces separating the BM from the CNS was observed (Fig. 2). Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts showed evidence of osteoclastogenesis at putative invasion sites of SFFV-MEL from the BM into the meninges of the spinal cord (Fig. 2D).

3.2. Differential Expression of Genes Between SFFV-MEL and SFFV-Spl cells

To identify genes whose expression levels were altered during the progression from the hyperplastic stage of SFFV-induced disease (represented by SFFV-spl cells) to the malignant stage that was capable of causing meningeal leukemia (represented by SFFV-MEL cells), we carried out microarray analysis. Group comparison by pooled *t*-test with equal variance, and statistics results filtering to exclude genes whose mean fold expression difference between groups was less than 3 or with *p*-values greater than 0.01, produced a dataset of 877 genes. The majority of differentially expressed genes fell into 17 pathways represented in the KEGG database (supplementary Table 1). Ten genes up-regulated in SFFV-MEL cells compared with SFFV-spl cells and 8 down-regulated genes were chosen for discussion based on potential relevance to leukemic cell invasiveness, BM homing and/or metastasis (Table 2). As previously reported, the *Sfpi1* gene, which encodes PU.1, is overexpressed in SFFV-MEL cells [17] and, thus, serves as an internal control. Up-regulated genes in the SFFV-MEL cells included those encoding extracellular matrix proteins (*Olfn-1*; *THBS-2*), oncogenes (*Sfpi1*; *Kit*), angiogenic growth factors (*VEGF-A*; *FGF-11*; *FGF-13*), a metalloproteinase (*ADAMTS-7*) and an integrin (*ITGA-5*). Down-regulated genes in the SFFV-MEL group included those that encode an antiangiogenic factor (*Thbs1*) and its receptor (*CD36*), an MMP-2 inhibitor (*Timp2*), a selenium regulatory protein (*Sepp1*), a tumor suppressor (*Lats2*), a myeloid differentiation marker (*Mnda*) and an erythroid differentiation marker (*Hbb-b1*). Changes in gene expression for all genes shown in Table 2 were validated by quantitative real-time PCR (Q-PCR) (Supplementary Figure 1).

3.3. Pathological angiogenesis in the bone marrow of SFFV-MEL transplanted mice

Myeloid leukemia and multiple myeloma induce pathological angiogenesis in the BM of affected individuals [18]. Accordingly, our gene expression data comparing SFFV-MEL cells to SFFV-spl cells confirmed differential regulation of genes involved in the angiogenic process (Table 2). To determine if pathological angiogenesis occurs in the BM of SFFV-MEL-transplanted mice, we performed CD31 immunohistochemistry to detect vessels. Compared with DMEM-injected control mice (Fig. 3A, top panels; Fig. 3B), the BM of SFFV-MEL transplanted mice at end stage of disease had significantly higher microvessel numbers (Fig. 3B) and these vessels appeared larger, with abundant fragmented neovessels, highly branched and tortuous vessel networks and vessels with collapsed luminae (Fig. 3A, lower panels).

Our microarray and Q-PCR analyses indicated that VEGF mRNA is expressed at higher levels in SFFV-MEL cells than SFFV-spl cell, consistent with the pathological angiogenesis detected in the BM of SFFV-MEL transplanted mice. To determine if SFFV-MEL cells secrete VEGF, conditioned media from these cells was assayed by a VEGF-specific ELISA assay. As shown in Fig. 3C, SFFV-MEL cells secrete high levels of VEGF compared to negative control NIH3T3 cells. SFFV-spl cells were not sufficiently viable to obtain meaningful data on VEGF secretion.

3.4. SFFV-MEL cells bind with high affinity to fibronectin via integrin $\alpha_5\beta_1$

Adherence to matrix proteins is a mechanism of myeloid leukemic cell BM engraftment, conferring a survival advantage to the leukemic cell that promotes chemotherapy resistance and minimal residual disease [19]. We determined the binding affinity of SFFV-MEL or SFFV-spl cells for fibronectin, type I collagen, type IV collagen and poly-D-lysine. As shown in Fig. 4A, significantly higher SFFV-MEL binding affinity was noted for fibronectin versus collagen I (unpaired *t*-test; *p*-value = 0.002) and to a greater extent versus collagen IV or poly-D-lysine substrates. SFFV-spl cells had low binding affinity for all of the substrates tested (Fig. 4A). To determine if SFFV-MEL binding to fibronectin is mediated by the fibronectin receptor integrin $\alpha_5\beta_1$, SFFV-MEL cells were pre-incubated with an antibody

specific for integrin $\alpha_5\beta_1$ and assayed for fibronectin adherence. The integrin $\alpha_5\beta_1$ antibody partially blocked SFFV-MEL interaction with fibronectin, resulting in a statistically significant reduction in fibronectin adhesion compared with cells treated with antibody to an unrelated cell surface protein (Epo receptor) or an isotype control (Fig. 4B). When the SFFV-MEL cells were grown for 2 days on fibronectin, they no longer expressed PU.1 (compare lanes 1 and 2 in Fig. 4C), but PU.1 expression returned after the cells were removed from the fibronectin and grown on untreated tissue culture plates for 2 days (Fig. 4C, lane 3). Exposure to fibronectin did not alter expression of SFFV gp55 (Fig. 4C, lane 2). SFFV-MEL cells grown on fibronectin did not differentiate, as determined by lack of expression of β -globin (data not shown).

4. Discussion

Human myeloid leukemias encountered clinically progress from proliferative and differentiation competent myelodysplastic syndromes (MDS) to malignant, undifferentiated acute leukemias [20] with the capacity to proliferate extramedullarily [1]. Replacement of normal BM with leukemic blasts and infiltration into the CNS is associated with clinical morbidity and poor prognosis particularly in pediatric patients [4]. In fact, the clinical significance of CNS involvement in leukemia has been recognized for more than a century [21], yet attempts to model this phenomenon in mice have not generated a highly-efficient, rapid system in normal, syngeneic mice. Our meningeal leukemia model will be useful to evaluate anti-leukemia drugs and explore the mechanism of leukemia metastasis into the CNS.

Friend SFFV disease occurs in distinctive stages which parallel in many respects the progression in human leukemia from MDS to invasive leukemia. The first stage is characterized by abnormal erythroid progenitor (SFFV-spl) cell expansion with differentiation primarily in the spleen, resulting in death from splenomegaly. In the second stage, proviral insertional activation of the PU.1 gene and the resulting block in differentiation affords full transformation to a subpopulation of erythroid cells in the spleen, which can be isolated and expanded *in vitro* [18, 22–24]. In this study we demonstrate that transplantation of these SFFV-transformed erythroleukemia cells (SFFV-MEL) to syngeneic, normal mice results in their preferential proliferation in the BM as well as extramedullary proliferation in the meningeal spaces of the CNS, resulting in rapid hind limb paralysis and death in 100% of the mice. Histological analysis of CNS tissues from SFFV-MEL transplanted mice showed adherence of the leukemic cells to the meninges, and damage to the CNS was obvious, with marked compression necrosis of the cerebellum and spinal cord appearing to be the major cause of damage.

Using expression of the SFFV envelope protein gp55 as a marker for SFFV-MEL cells, we could detect the leukemic cells primarily in the BM of SFFV-MEL-transplanted mice. No expression of SFFV gp55 could be detected in brain tissue, consistent with the histological data indicating that the SFFV-MEL cells were present only in the meningeal spaces between the bone and brain. Also, little expression of SFFV gp55 was detected in the spleen, consistent with the observation that the spleen did not significantly increase in size after transplantation of SFFV MEL cells. The basis for the acquisition of BM- tropism of SFFV-MEL cells is not known. Adherence to matrix proteins is thought to be an important mechanism by which leukemic cells engraft into the BM [25, 26] and may be facilitated by the interaction between fibronectin and its $\alpha_5\beta_1$ -integrin receptor, the α subunit (*Itga5*) of which is up-regulated in SFFV-MEL cells. Consistent with this hypothesis is the observation that SFFV-MEL cells adhered preferentially to fibronectin in a matrix adhesion assay and this adherence was inhibited with an antibody specific for integrin $\alpha_5\beta_1$. Interestingly, PU.1 expression in SFFV-MEL cells was downregulated when the cells were grown on

fibronectin, as was observed in BM cells from mice transplanted with SFFV-MEL cells. When the SFFV-MEL cells were removed from the fibronectin or when BM cells from SFFV-MEL transplanted mice were grown in suspension culture *in vitro*, PU.1 expression returned to its original level. This data is consistent with previous studies showing that PU.1 expression in human BM cells is down-regulated when the cells are grown on fibronectin [27] and with other data showing that underexpression of PU.1 is linked to human myeloid leukemias [28]. Thus, although PU.1 expression is necessary to transform SFFV-infected erythroblasts by blocking erythroid cell differentiation, its expression is not required, and may be detrimental, for the retention of transformed SFFV-MEL cells in the BM.

Invasion of extramedullary sites by leukemic cells in the BM is a clinical indicator of poor prognosis. When the invasion site is the CNS, chemotherapy resistance and CNS pathology result and disease treatment options become more limiting, complicated and invasive [4, 6–8, 29,30]. Despite its clinical importance, the mechanism of passage of leukemic cells into the CNS is not well understood. [31]. As with solid tumor metastasis [32], the process by which leukemic cells migrate from the BM to other organs involves degradation of the extracellular matrix (ECM), chemotaxis and chemokine-mediated homing to and establishment in a different tissue. A recent study in T-cell acute lymphoblastic leukemia demonstrated that the chemokine receptor CCR7 was necessary and sufficient for CNS invasion [33], although this protein was not involved in CNS invasion by leukemic B-cells. In our study, the CCR7 gene was not differentially expressed in SFFV-MEL cells compared with SFFV-spl cells (data not shown). Rather, gene expression profiling indicated that a number of genes involved in ECM processing (*Olfm1*, *Thbs2*, *Adamts7*) were upregulated in SFFV-MEL cells compared with SFFV-spl cells, while others that inhibit ECM processing (i.e. *Timp2*), are down-regulated. These changes may impact the capacity of the SFFV-MEL cells to engraft in the BM and invade the CNS. There is little published data describing the entry point(s) of leukemic infiltration into the meninges. One possible entry point for meningeal invasion is through bone following osteoclastogenic differentiation of cranial and vertebral osteoblasts, perhaps stimulated by the leukemic cells. In support of such a mechanism is data showing increased osteoclastic bone resorption in patients with adult T-cell leukemia/lymphoma [34]. TRAP staining of CNS pathology sections from mice transplanted with SFFV-MEL cells revealed osteoclastogenesis at putative invasion sites. Osteoclast stimulation by SFFV-MEL cells may involve overexpression of thrombospondin-2 (*Thbs2*), a bone ECM protein [35] whose absence in knockout mice results in increased osteoblastogenesis and decreased bone resorption [36]. Also, SFFV-MEL cells show upregulation of the VEGF-A gene, which, in addition to being an angiogenic factor, is also an osteoclast stimulating factor [37].

Pathological angiogenesis, which is a complication in the BM of patients with myeloid and other leukemias [18, 38, 39], may also play a role in the progression of meningeal leukemia in SFFV-MEL transplanted mice. CD31 immunohistochemistry revealed high microvessel numbers as well as abundant abnormal vessels in the BM of SFFV-MEL transplanted mice compared with vehicle controls. Consistent with increased angiogenesis is upregulation of pro-angiogenic VEGF-A mRNA expression in SFFV-MEL cells and the secretion of high amounts of VEGF from these cells. Also, upregulation of the tyrosine kinase Kit in SFFV-MEL cells may further contribute to angiogenesis since the SCF/cKit pathway has been shown to play a role in angiogenesis associated with malignant glioma [40]. In addition to the upregulation of pro-angiogenic genes, expression of several anti-angiogenesis genes were downregulated in SFFV-MEL cells, such as thrombospondin-1 and the thrombospondin receptor CD36. It is plausible that SFFV-MEL cells invade the meninges of transplanted mice via a process that requires angiogenesis. Thus, it will be of interest to determine the role of angiogenesis in meningeal invasion in our model, and whether it can be inhibited by angiogenesis blockade.

5. Conclusions

Using retrovirus-transformed mouse erythroleukemia cells, we have developed a new mouse model of meningeal leukemia in a non-immunocompromised, syngeneic mouse system. We have also identified several changes that have occurred in the leukemia cells that may be important for CNS invasion, including their ability to bind to fibronectin, which may favor BM engraftment, and to secrete VEGF, leading to pathological angiogenesis. Future studies in this system might lead to the identification of molecular targets for treatment of meningeal leukemia and molecular markers associated with greater risk of CNS relapse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of SFFV gp55 and PU.1 in tissues of mice transplanted with SFFV-MEL or SFFV-spl cells

Western blot analysis using a monoclonal antibody to SFFV gp55 or antiserum to PU.1 was carried out on tissues from mice transplanted with SFFV-spl cells or SFFV-MEL cells. Lane 6: spleen extract from an uninfected mouse; Lane 11: BM from mice transplanted with SFFV-MEL cells, expanded in tissue culture. Antibody to ERK was used as a loading control.

Figure 2. Central nervous system pathology induced by transplantation of SFFV-MEL cells
Adult BALB/c mice injected with SFFV-MEL cells or DMEM (control) were perfused and cross-sections of the skull and brain (Panels A and B) or vertebral bone and spinal cord

(Panel C) were prepared and stained with hematoxylin and eosin. White arrows: sites of bone destruction and SFFV-MEL cell infiltration of meningeal spaces. Panel D: vertebral bone stained positive for the osteoclast marker TRAP (indicated by red staining and blue arrows). Meningeal space (MS); vertebral bone (VB); cranial bone (CB); bone marrow (BM).

Figure 3. SFFV-MEL cells induce pathological angiogenesis in the BM of transplanted mice and secrete high levels of VEGF

A. Blood vessels in the BM of mice injected with SFFV-MEL cells or DMEM (control) were evaluated by CD31 immunohistochemistry. Arrows indicate immature vessels or vessel fragments. **B.** Number of CD31+ microvessels in a 20x field of BM. Data represent the average number of CD31+ microvessels from 5 fields $\pm$ standard deviation. * $p < 0.01$ when compared to either control mouse using student's unpaired t -test. **C.** Conditioned medium from SFFV-MEL cells or NIH3T3 rodent fibroblasts (negative control) were tested for expression of VEGF. Data represent the average level of VEGF from triplicate samples $\pm$ standard deviation.

Figure 4. SFFV-MEL cells bind fibronectin with high affinity *in vitro* via integrin $\alpha_5\beta_1$
A. Cells were seeded in plates previously coated with various matrix proteins and the number of adherent cells determined. The experiment was repeated 3 times; data shown indicate the average number of adherent cells $\pm$ standard deviation. **B.** SFFV-MEL cells were preincubated with antibodies to integrin $\alpha_5\beta_1$, Epo receptor or an isotype control antibody (IgG2b κ) and the number of cells adhering to fibronectin determined. The experiment was repeated 3 times; data shown indicate the average number of adherent cells $\pm$ standard deviation. **C.** Western blot analysis using anti-PU.1 or a monoclonal antibody to SFFV gp55 was carried out on SFFV-MEL cells grown on uncoated plates (lane 1) or fibronectin-coated plates (lane 2). Lane 3 represents cells that were grown on fibronectin for 3 days and then transferred to new, uncoated plates for 3 days. Antibody to β -tubulin was used as a loading control.

Table 1
Mice transplanted intravenously with SFFV-MEL cells develop hind limb paralysis

Adult BALB/c mice were injected i.v. with 5 million cells. Mice were observed for hind limb paralysis over a 3 wk period or until they became moribund. Splenomegaly was determined by spleen weight (0.5 gm or larger) at necropsy.

Cells Transplanted	Splenomegaly	Paralysis
SFFV-spl	10/10	0/10
SFFV-MEL-NP1	0/5	5/5
SFFV-MEL-NP4	0/5	5/5
SFFV-MEL-NP5	0/5	5/5
SFFV-MEL-NP7	0/20	20/20

Table 2

Microarray data showing selected genes differentially expressed between SFFV-MEL and SFFV-Spl cells

Gene	Difference (SFFV-MEL vs SFFV-spl)	Function/Relevance
Upregulated		
<i>Olfm1</i>	8.3	Neuroepithelial extracellular matrix protein
<i>Fgf13</i>	8.0	Putative angiogenic growth factor; overexpressed in acute promyelocytic leukemia
<i>Thbs2</i>	7.3	Bone extracellular matrix protein
<i>Kit</i>	5.4	Receptor tyrosine kinase
<i>Adamts7</i>	4.6	Cartilage-degrading metalloproteinase
<i>Fgf11</i>	4.4	Putative angiogenic growth factor
<i>Vegfa</i>	3.6	Angiogenic growth factor
<i>Itga5</i>	3.3	Mediates leukemic cell interaction with fibronectin in BM
<i>Sfp1</i>	3.3	Friend SFFV proviral integration site encoding PU.1
Downregulated		
<i>Vcam1</i>	8.2	Adhesion molecule on BM endothelial cells
<i>CD36</i>	6.8	Thrombospondin receptor
<i>Sepp1</i>	6.2	Selenium regulating protein; downregulated in tumors
<i>Thbs1</i>	4.7	Anti-angiogenic protein
<i>Timp2</i>	4.2	Inhibits MMP-2-mediated matrix turnover, suppressing metastasis
<i>Lats2</i>	4.0	Tumor suppressor
<i>Mnda</i>	3.6	Expressed in maturing myeloid precursor cells
<i>Hbb-b1</i>	3.5	Iron containing metalloprotein in mature rbc