Role of Angiogenesis in Chronic Lymphocytic Leukemia

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Angiogenesis is a physiologic process of new blood vessels formation mediated by various cytokines called angiogenic and angiostatic factors. Although its potential pathophysiologic role in solid tumors has been extensively studied for more than 3 decades, enhancement of angiogenesis in chronic lymphocytic leukemia (CLL) and other malignant hematological disorders has been recognized more recently. An increased level of angiogenesis has been documented by various experimental methods both in bone marrow and lymph nodes of patients with CLL. Although the role of angiogenesis in the pathophysiology of this disease remains to be fully elucidated, experimental data suggest that several angiogenic factors play a role in the disease progression. Biologic markers of angiogenesis were also shown to be of prognostic relevance in CLL. The current findings provide the rationale for investigating antiangiogenic agents in CLL. In the current review angiogenesis in CLL is discussed and its potential diagnostic and therapeutic applications. ***Cancer* 2006;107:925–34.** © *2006 American Cancer Society.*

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Development of tumors is a highly complex process in which several molecular events are required for tumor cells to achieve independent growth.[1] One such event is the enhancement of angiogenesis. Tumor hypervascularity was initially thought to reflect vasodilatation of preexisting vessels that occurred because of inflammatory processes induced by tumor metabolites and necrosis.[2] Folkman et al.[3] anticipated more than 30 years ago a more active role for newly formed blood vessels in tumor growth and metastasis and the possible therapeutic implications of such events.

Because the bone marrow and lymphatic organs are predominant sites of tumor accumulation in hematologic malignancies, it was initially believed that angiogenesis would not be as relevant in these disorders ("liquid tumors") as it is in solid tumors.[4] One of the first discoveries in this field came from Perez et al.,[5] who studied bone marrow biopsy specimens from children with untreated acute lymphoblastic leukemia and compared them with biopsy specimens from healthy subjects. A significantly higher bone microvessel density and a higher level of urinary basic fibroblast growth factor (bFGF) were found in patients with acute lymphoblastic leukemia. Many subsequent studies showed a relation between various hematologic malignancies and changes in the angiogenic profile.[6,7]

In this review, we discuss the prognostic significance and therapeutic implications of angiogenesis in chronic lymphocytic leukemia (CLL).

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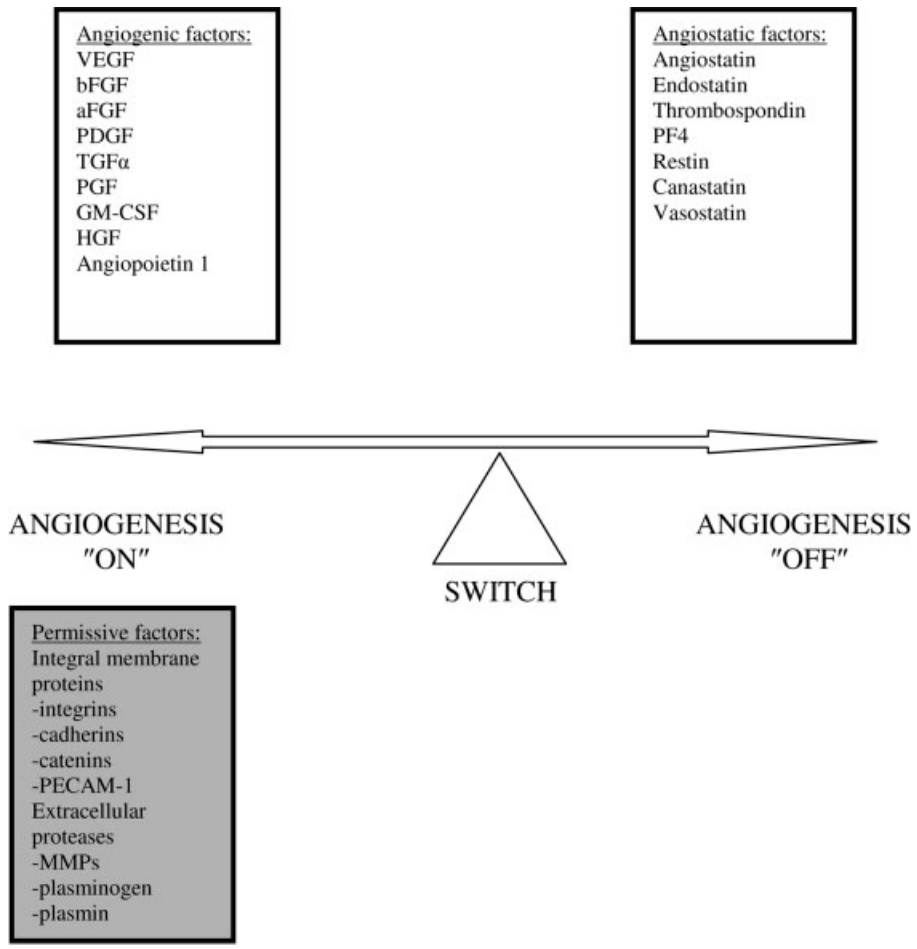

FIGURE 1. Angiogenesis switch model and permissive factors of angiogenesis. VEGF indicates vascular endothelial growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor: PDGF, platelet-derived growth factor, TGF-α, transforming growth factor-α; PGF, placental growth factor; GM-CSF, granulocyte-macrophage–colony-stimulating factor; HGF, hepatocyte growth factor; PF4, platelet factor-4; PECAM-1, platelet endothelial cell adhesion molecule-1; MMPs, matrix metalloproteinases.

Physiologic Role and Key Players in Angiogenesis

Angiogenesis is the development of new blood vessels from existing ones, as opposed to vasculogenesis, which refers to the de novo formation of blood vessels.[8] The induction and rate of angiogenesis depend on the balance of two functionally opposing groups of cytokines called angiogenic and angiostatic (or antiangiogenic) factors. Ellis et al.[9] in the "angiogenesis switch model" summarized this interplay. Figure 1 illustrates the factors involved in the angiogenic switch model. The process of angiogenesis also depends on cell-to-cell and cell-to-extracellular matrix interactions. Integral membrane proteins and extracellular proteinases are involved in these interactions (see Fig. 1).[10–12]

Methods to Assess Angiogenesis in Hematologic Malignancies

Angiogenesis can be assessed by direct or indirect methods (Table 1). When using direct methods, investigators estimate the number of blood vessels in a specimen (usually bone marrow in hematologic malignancies). To estimate the blood vessel count, one has to visualize a vessel, which is accomplished by immunohistochemical staining, using antibodies targeted to the specific antigens found on the surface of endothelial cells. The most frequently used antibodies are those directed against CD34, CD31, factor VIII, von Willebrand factor, thrombomodulin, and UEA-1.[13] After analysis of the immunohistochemical staining the results are usually expressed as microvessel density or microvessel surface area either by using standard methods developed by Weidner

TABLE 1
Methods of Angiogenesis Measurement

Methods	
Direct methods	**Indirect methods**
Assesment of microvascular density:	Measurement of:
Immunohystochemically	Angiogenic factors (ELISA)
PET	Angiostatic factors (ELISA)
Enhanced dynamic MRI	Angiogenic factor receptors(usually Western blot)
Infrared thermography	Circulating EC (FC)
	Assesment of biological activity:
	Chick chorioalantoic mebrane assay
	HUVEC assay

ELISA indicates enzyme-linked immunoadsorbent assay; PET, positron emission tomography; MRI, magnetic resonance imaging; EC, endothelial cell; FC, flow cytometry; HUVEC, human umbilical vein endothelial cells.

et al.[14,15] or by using computerized techniques.[16] When immunohistochemically stained, blood vessels can be evaluated morphologically as well. In contrast to normal vessels, tumor vessels are tortuous and dilated with excessive branching and shunts. Ultrastructurally these vessels have widened interendothelial junctions and a discontinuous or absent basement membrane. Endothelial cells are abnormal in shape, growing on top of each other and projecting into the lumen, making these vessels leaky.[2] Chang et al.[17] provided evidence that tumor blood vessels may be mosaics in which both endothelial and tumor cells form the luminal surface. Finally, smooth muscle cells surrounding tumor vessels do not function as normal contractile elements.[2,18] An invasive procedure, such as a bone marrow or lymph node biopsy, is required to collect the specimen for the various direct methods. Novel noninvasive techniques such as enhanced dynamic magnetic resonance imaging, positron emission tomographic imaging,[19] and infrared thermography[20] are being developed. These techniques can be easily reproduced and are very promising. An advantage of the direct methods is the ability to allow quantification as well as morphologic evaluation of the blood vessels. The two major disadvantages of these methods are the need of an invasive procedure to obtain the sample and the risk of sampling errors because the microvessel density in the sample obtained may not reflect the one of the whole neoplasm.

Indirect methods for estimating the number of blood vessels measure the levels of various cytokines and their receptors (i.e., angiogenic or angiostatic factors or other previously mentioned molecules important for adequate angiogenesis). The levels of angiogenic factors are measured by commercially available systems based on enzyme-linked immunosorbent assays.[21] These cytokines can be measured in serum, plasma, bone marrow, or purified lysates of tumor cells. Some authors recommend the use of plasma rather than the use of serum to measure these levels, because the levels of certain angiogenic cytokines are significantly higher in serum than in plasma as a consequence of their release during the clotting process.[22] Conversely, other authors have shown that serum levels can be used as an indirect estimate of solid tumor angiogenic factor expression.[23] Recently, levels of free cytokines have been measured; free circulating receptors may bind to circulating cytokines and reduce their availability.[24] Indirect methods can be used to measure the effects of either malignant cells or their products in well-established biologic models such as a chick chorioallantoic membrane cell assay[25] or a proliferation assay using human umbilical vein endothelial cells. Evaluation of numbers of circulating endothelial cells by flow cytometry in peripheral blood is also used as a marker of angiogenesis. Circulating endothelial cells are believed to serve as a building material that is used when angiogenesis is enhanced. Their number is increased under the influence of angiogenic factors, which was shown in patients with B-CLL[26] and other hematologic malignancies.[27,28] Chemotherapy and antiangiogenic therapy were found to decrease the number and viability of circulating endothelial cells.[26,29] Indirect methods have the advantage of being less invasive; however, when their results are compared with angiogenesis measured by direct methods, the correlation appears poor.[26,27,30]

Other approaches such as fluorescence in situ hybridization and molecular techniques are being used to focus on abnormalities in angiogenesis-relevant genetic loci such as the fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) genes.[31]

Role of Abnormal Angiogenesis in CLL

Solid tumors with their organ-like structure are highly dependent on an adequate vascular network to provide them necessary oxygen and nutrients, allowing them to grow. In CLL, bone marrow and lymphatic organs are predominant sites of disease.[32] Therefore, the possible benefit of additional blood vessels in CLL, as well as in other hematologic malignancies, was initially hypothesized to be less relevant than in solid tumors. Current data indicate at least two possible advantages that enhanced angiogenesis could give to malignant cells.

First, tumor growth in CLL and other hematologic malignancies could be strongly influenced by angiogenesis, primarily through powerful cytokine interactions, both paracrine and autocrine. These interactions are found between at least three subsets of cells found in bone marrow. One subset, endothelial cells, produces

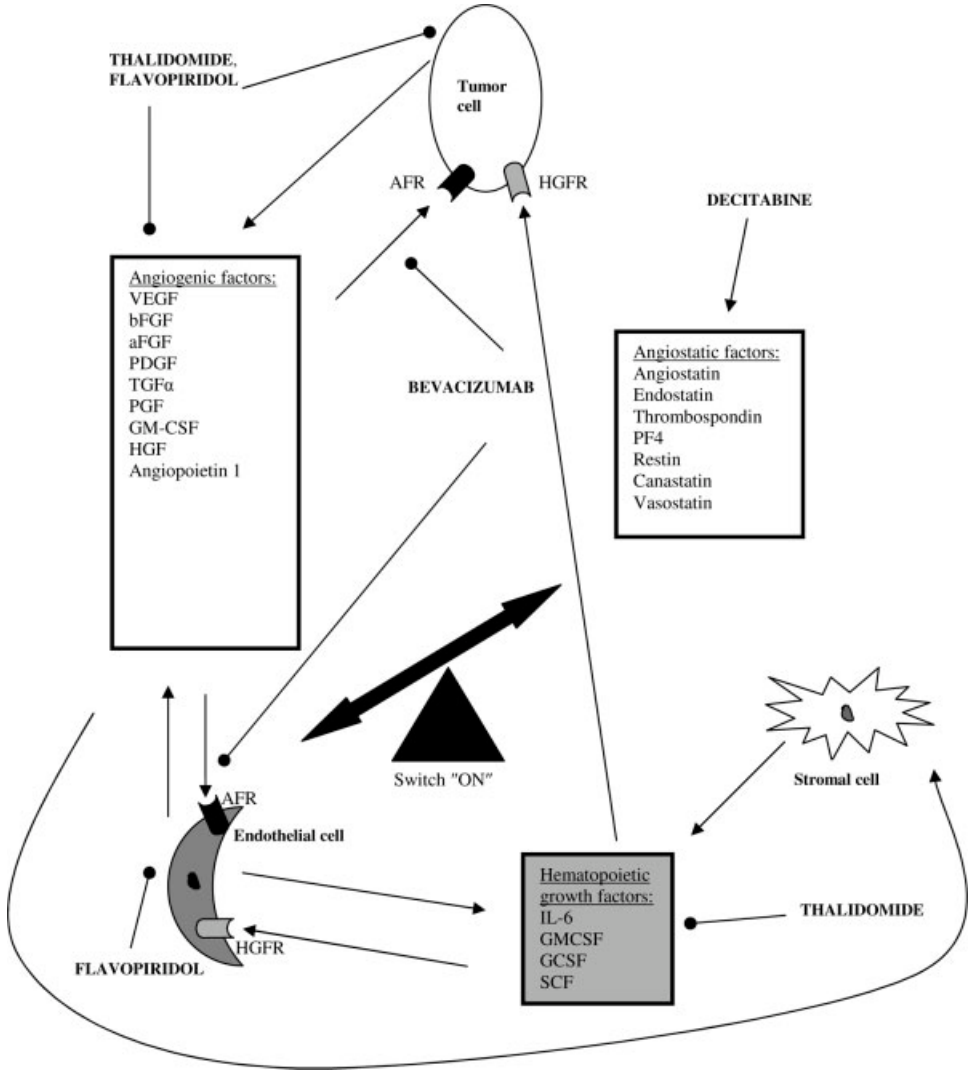

FIGURE 2. Chronic lymphocytic leukemia angiogenesis. Illustration of autocrine and paracrine loops and mechanisms of action of antiangiogenic agents used in current clinical trials. VEGF indicates vascular endothelial growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor: PDGF, platelet-derived growth factor, TGF-α, transforming growth factor-α; PGF, placental growth factor; GM-CSF, granulocyte-macrophage–colony-stimulating factor; HGF, hepatocyte growth factor; PF4, platelet factor-4; IL-6, interleukin-6; GCSF, granulocyte–colony-stimulating factor; SCF, stem cell factor; HGFR hematopoietic growth factor receptor; AFR, angiogenic factor receptor. Line with arrow indicates stimulation or secretion; line with circle, inhibition.

hematologic growth factors. When exposed to an angiogenic mitogen (e.g., VEGF or basic FGF [bFGF]), endothelial cells increase their expression of mRNA of various hematopoietic growth factors (e.g., granulocyte–colony-stimulating factor [G-CSF], granulocyte-macrophage–colony-stimulating factor [GM-CSF], stem cell factor [SCF], and interleukin-6 [IL-6]). These hematopoietic cytokines then have an autocrine effect (i.e., they stimulate the proliferation and migration of endothelial cells), which express specific receptors for those cytokines.[33,34] The second subset, malignant CLL cells, produces strong angiogenic factors such as VEGF and bFGF,[35] which have an antiapoptotic effect on the CLL cells[36,37] and correlate positively with expression of bcl-2, a well-established antiapoptotic gene.[38,39] This interplay of cytokines and their functions is further expanded by the active role of nearby stromal cells (the third subset) that produce hematopoietic growth factors under the influence of angiogenic factors.[40] The possible interactions of various cytokines and cell types found in the bone marrow microenvironment and the possible interference of antiangiogenic agents are shown in Figure 2.

One explanation for the pro-angiogenic environment in CLL could be that it is merely a reflection of

the greater number of leukemic cells present, which would naturally produce larger amounts of angiogenic factors that would lead to increased angiogenesis without playing any significant role in the pathogenesis of the disease. This argument, however, is contradicted by the fact that when the level of angiogenesis is adjusted by the cellularity of the bone marrow, it is still higher in the bone marrow of patients with CLL than in the bone marrow of control subjects.[41]

Another possible advantage of increased angiogenesis in malignant hematologic diseases is the facilitation of its dissemination throughout the body. Newly formed blood vessels, whose permeability is increased under the influence of VEGF, could be ideal for releasing leukemic cells into systemic circulation. A recent study showed that CLL cell motility on and through the endothelium seems to depend on autocrine VEGF and $\alpha 4\beta 1$ integrin. This process is believed to be important for the invasion of lymphoid tissues (i.e., progression of the disease).[42] Increased expression of CD38 and CD31 (also known as platelet endothelial cell adhesion molecule 1) on B-CLL lymphocytes in lymphoid organs[43] and the interaction with CD31 on endothelial cells, which is the only known ligand for CD38,[44] may also promote B-CLL progression by providing important signals through the CD38 signaling pathway.[45]

Prognostic Relevance of Angiogenesis in CLL

The first report of increased bone marrow angiogenesis in B-CLL came from Peterson et al.,[46] who evaluated 12 patients with CLL and showed that the microvessel count per high-power field in the bone marrow was higher than in healthy control subjects. They also found a positive correlation between the microvessel count and the clinical stage (according to the Rai system), meaning that patients with higher microvessel counts were more likely to have advanced disease. Patients with higher microvessel counts had a higher percentage of their bone marrow involved by the disease. In another study, conducted on 45 patients with early-stage CLL (Binet Stage A), the microvessel area was found to be larger than in the control population. These investigators selected the 75th percentile of microvessel area as a cutoff value and showed that patients above the cutoff had shorter progression-free survival than patients below the cutoff (12 months vs. 40 months). Similar results were obtained when patients were grouped according to the Rai staging system (i.e., patients within the same Rai stage had significantly shorter progression-free survival if their microvessel area was above the 75th percentile). Morphologic differences between the two groups were observed. Patients with a microvessel area above the cutoff had tortuous, arborized, and extremely dilated vessels, as opposed to patients with less microvessel area, whose microvessels displayed morphologic features similar to those seen in the control group.[47,48] These studies were in contrast to the study by Aguayo et al.,[30] which did not show a statistically significant difference in microvascular densities between 23 patients and controls. Increased vascular density was not seen only in the bone marrow. In the lymph nodes of 3 patients with CLL, microvessel density was higher than in the lymph nodes of healthy control subjects.[49]

Expression of Angiogenic Factors in B-CLL

The first proof of increased expression of angiogenic factors in patients with CLL came from Duensing and Atzpodien,[50] who evaluated 18 patients and found elevated levels of bFGF in both plasma and lymphocytes of patients. When patients were stratified by a cutoff value of plasma bFGF, those with higher levels were found to be in more advanced stages of the disease, according to the Rai staging system, and tended to have higher lymphocyte counts. A possible correlation of increased levels of serum bFGF and advanced stages of CLL was also shown by the work of Gora-Tybor et al.,[51] who showed that Rai Stage III-IV patients had significantly higher levels of serum bFGF than patients in Rai Stage 0-II. They also found lower levels of the antiangiogenic cytokine endostatin and transforming growth factor beta 1 in patients with more advanced stages of the disease. Conversely, elevated levels of intracellular bFGF were found to correlate with the risk profile of disease in a given patient (i.e., patients with high-risk disease had higher median levels of intracellular bFGF than patients with low-risk disease had [with risk stratification performed according to the modified Rai classification]). In vitro analysis showed that CLL cells with elevated intracellular levels of bFGF were more resistant to fludarabine-induced apoptosis than were cells with lower levels.[52] The antiapoptotic action of bFGF was shown in culture studies in which it up-regulated the production of bcl-2 protein in an experimental model of B-CLL.[53] Similarly, production of the bcl-2 protein was found to positively correlate with serum bFGF and negatively with cellular VEGF levels.[39] Contrary to what was seen in an in vitro study, CLL cells did not bind strongly to exogenous bFGF, and application of bFGF did not increase phosphorylation (i.e., activation) of well-established proliferation kinases and did not have any effect on the viability of B-CLL cells in 5-day cultures.[35] Several researchers have reported on the ability of CLL cells to produce bFGF.[54,55]

VEGF was also found to be overexpressed in patients with CLL,[56] and CLL cells were found to pro-

duce VEGF and to exert angiogenic biologic effects.[49] In a study by Molica et al.[56] of 81 patients with CLL, serum VEGF was shown to positively correlate with substages of the disease, lymphocytosis, bone marrow histology, and beta2-microglobulin. Patients with VEGF levels above the median had a 3 times higher risk of disease progression than those with below-median levels, as shown in a univariate analysis. When VEGF levels and beta2-microglobulin were examined in combination, the highest prognostic power was obtained. Patients with elevation of both markers had the worst prognosis (i.e., median progression-free survival of 13 months), whereas patients with both factors negative had not yet reached their median progression-free survival after 40 months. Intracellular levels of VEGF were shown to have quite opposite effects on the biology of CLL. In a study conducted on samples collected from 225 patients, lower intracellular levels of VEGF were associated with shorter survival times in a subgroup of CLL patients with a good prognosis or early-stage disease.[57] Another angiogenic factor called angiogenin was also investigated. Angiogenin was the first human tumor-derived protein with in vivo angiogenic activity that was isolated.[58] It was shown to enhance endothelial cell adhesion to the components of the extracellular matrix,[59] but its in vitro activity on endothelial cell proliferation and migration was never proven.[60] In a study by Molica et al.[61] of 77 patients with previously untreated early-stage B-CLL, no difference in serum angiogenin levels between patients and healthy controls was found. When patients were stratified using the median level of serum angiogenin as the cutoff, surprisingly, patients with higher levels had better 5-year progression-free survival rates (85% vs. 51.5%). Angiogenin levels retained their prognostic significance when patients were staged according to the Rai system.

Expression of Angiogenesis Factor Receptors in B-CLL

VEGF exerts its biologic activities through three known types of tyrosine kinase receptors. All three types are expressed on the surface of B-CLL cells.[62,63] A study on 216 patients showed that those who had higher concentrations of VEGF receptor 2 (VEGFR-2) in their peripheral blood lymphocytes had significantly shorter survival. VEGFR-2 levels also negatively correlated with hemoglobin levels and positively correlated with lymphocyte counts.[64] In another study, VEGF receptor 1 (VEGFR-1) expression in peripheral blood lymphocytes collected from 231 patients with CLL was analyzed. The expression of this receptor and Tie-1, another angiogenesis-related receptor, was significantly higher than in healthy control donors. A negative correlation between VEGFR-1 levels and the duration of the disease was found, which showed that the level of the receptor decreases throughout the course of the disease. Patients with Rai Stages 0-II disease had significantly shorter survival if they had high levels of the Tie-1 receptor, indicating a possible role of Tie-1 in disease progression in early-stage patients.[65]

Expression of Matrix Metalloproteinases in B-CLL

Matrix metalloproteinases (MMPs) have important roles in angiogenesis. Bauvois et al.[66] found significantly higher levels of MMP-9 in patients with CLL than in healthy controls. MMP-9 was found in B-CLL cells by immunostaining, and antibodies directed against VEGF and tumor necrosis factor alpha blocked its expression. Reduced expression of MMP-9, on both the mRNA and protein levels, was shown with type I and type II interferons. The level of MMP-9 in cells was significantly higher in patients with Binet Stage C than in those with Stages A and B. When patients were stratified by an arbitrary cutoff value, patients with higher MMP-9 levels had significantly shorter survival probabilities. In vitro evidence showed that MMP-9 inhibition resulted in reduced B-CLL cell migration through collagen-coated membranes and endothelial monolayers. Tissue invasion by the malignant cells, on the other hand, was associated with the rate of production of MMP-9.[67] In another study conducted on 62 patients with Binet Stage A B-CLL, using the 33rd percentile of serum concentration of MMP-9 as the cutoff value, the median progression-free survival was 30 months for patients with higher concentrations. Patients with lower concentrations had not reached their median survival after 50 months. Among patients with Rai Stages I and II disease, 4-year progression-free survival also significantly differed. Those who had MMP-9 concentrations above the cutoff value had significantly shorter 4-year progression-free survival rates (13.6% vs. 80%).[68]

Antiangiogenic Therapy in CLL

Evidence of a potential pathophysiologic role of angiogenesis in CLL supports investigations of antiangiogenic drugs in the treatment of patients with CLL.[69] Antiangiogenic agents can be further categorized according to their mechanism of action: those that prevent formation of new vessels (antiangiogenic) and those that destroy existing vessels (vascular-targeting).[70] Thalidomide, one of the oldest antiangiogenic drugs, probably acts by inhibiting the synthesis of various cytokines (e.g., IL-6, tumor necrosis factor-α [TNF-α], and VEGF) that play important roles in angiogenesis.[71] In one reported case, thalidomide was used in a patient with both myeloma and CLL.[72] Thalidomide has shown single-agent activity in CLL: it induced a median 55% decrease in lymphocytosis in treated patients.[73] In a recently reported experience, thalidomide was used in combination with

TABLE 2
Antiangiogenic Agent Clinical Trials

Agent	Mechanism of action	Disease	Phase
Thalidomide with or without fludarabine	Inhibition of synthesisof angiogenic factors	Fludarabine-refractory CLL or SLL	II
Bevacizumab	Anti-VEGF antibody	Recurrent or refractory CLL	II
Flavopiridol	Endothelial cell apoptosis and inhibition ofinduction of VEGF	Metastatic or unresectable solid tumors orhematologic malignancies	I
Decitabine	Demethylation of angiostatic factor genes and othertumor supressor genes	Recurrent or refractory leukemia,myelodispastic syndromes or myeloproliferative disease	II

CLL indicates chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; VEGF, vascular endothelial growth factor.

fludarabine in 20 previously untreated patients. The overall response rate was 100% and complete responses or nodular partial responses were achieved in 65% of the patients.[74] Lenalidomide, a thalidomide analog with preferential TNFα inhibitory properties in addition to some retained antiangiogenic activity, is undergoing clinical evaluation in CLL and recent data in previously treated patients have shown encouraging activity.[75]

Farnesyl transferase inhibitors are compounds shown to have antiangiogenic activity in vitro.[76] These drugs inhibit farnesylation of various proteins, including *ras,* thereby blocking a critical step in signal transduction, growth, and angiogenesis. Dasatinib (Bristol-Myers Squibb, Princeton, NJ), a dual Abl/src and c-kit inhibitor, induced apoptosis ex vivo in lymphocytes from 18 patients with CLL, including some cases that were fludarabine-resistant.[77] Flavopiridol is another antiangiogenic agent being investigated in patients with CLL. Flavopiridol is an inhibitor of cyclin-dependent kinases that induces endothelial cell apoptosis and inhibits hypoxic induction of VEGF.[78] In addition to its action on endothelial cells, it exerts a clear antiapoptotic effect on CLL cells that circumvents the bcl-2 pathway.[79,80] When used as single agent in patients with previously treated CLL, it showed only modest activity,[81,82] but when used in combination with fludarabine and rituximab, it demonstrated significant clinical activity (overall rate response 90% and complete remission 71%) in a small group of patients with indolent B-cell lymphoproliferative disorders (including 8 patients with CLL); however, administration of therapy was limited by cytopenias.[83] Table 2 summarizes clinical trials involving antiangiogenic therapy in CLL.

Conclusions

The available data suggest that angiogenesis plays an important role in the pathogenesis of CLL. What can be learned from these results, how these studies can be expanded, and how these data can help us manage patients with CLL are important questions. It appears that angiogenesis-related studies have a future in both the diagnostic and therapeutic arenas.

The diagnostic and prognostic implications of measurements of angiogenesis seem quite reasonable, particularly using it as a prognostic tool that allows us to further classify patients within the same Rai or Binet stage. As shown previously, patients within the same Rai stage differ significantly in their prognosis in relation to various parameters of their angiogenic profile. Some insight into changes in the angiogenic profile through the course of the disease was given by the work of by Shanafelt et al.,[84] who have shown that the ratio of bFGF to thrombospondin correlates with time to treatment as a continuous variable (i.e., in sequential samples). Furthermore, we hypothesize that changes in levels of angiogenic parameters can be predictors of clinical response to a specific therapeutic approach or can be early indicators of recurrence. Numerous other possibilities await further studies.

A potential shortcoming of antiangiogenic therapy could come from redundancy of proangiogenic factors. Currently used antiangiogenic substances typically block 1 pathway (that is believed to be predominant) involved in angiogenesis. When a predominant pathway is suppressed, other pathways that had appeared to be insignificant could become more active. Antiangiogenic therapy also might induce the selection of highly malignant clones that are less vulnerable to hypoxia. Because increased microvessel density may persist even after successful therapy, a phenomenon that has been observed after both conventional and antiangiogenic therapeutic protocols, a lack of surrogate markers for therapeutic efficacy of antiangiogenic therapy is another potential problem.[85]

In conclusion, angiogenesis plays an important role in CLL. Future studies are expected to further elucidate its pathophysiology and should provide novel therapeutic options in CLL.

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