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Mechanism and Function of Angiogenin in Hematopoietic Malignancy

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Abstract

Angiogenic factors have been widely implicated in the formation and progression of solid tumors. A number of angiogenic mediators have been recently appreciated as having equivalent function in non-solid tumors, such as leukemia. One such factor, angiogenin (ANG), promotes tumor cell growth and angiogenesis in solid cancers; however its precise function(s) in hematological disorders are not fully understood. This review summarizes current knowledge of the function and therapeutic potential of angiogenic factors, with particular emphasis on the role and hypothesized mechanism of ANG in a non-solid tumor setting.

Keywords

angiogenesis; angiogenin; bone marrow diseases; leukemia; malignant hematopoiesis; cancer; cell proliferation; RNA processing

Growth control is a fundamental property throughout the body and is regulated at molecular, cellular, and tissue levels. Perturbations at each of these levels lead to uncontrolled growth, a defining feature of cancer[1, 2]. At the tissue level, the formation of blood vessels, or angiogenesis, is critical for growth and metastasis of solid tumors[3–9], and inhibitors against this process are current standard of care approaches for “starving” tumors of nutrients and oxygen required for continued growth and subsequent metastasis[10–13]. It is therefore not surprising that considerable effort has been concentrated on this aspect of tumor biology. Indeed, decades of work resulted in the identification of a panoply of intracellular or soluble regulators, including cytokines and growth factors, that either promote or inhibit angiogenic capabilities locally or systemically: among these, vascular endothelial growth factor or VEGF[14–16], fibroblast growth factor or FGF[17, 18], platelet-derived growth factor or PDGF[19], hypoxia-inducible factor-α or Hif1-α[20], matrix metallopeptidase-9 or MMP-9[21], angiopoietin-1 and 2[22–24], and angiogenin (ANG)[25], were found to be among the most well-characterized and most potent pro-angiogenic factors. Anti-angiogenic factors such as endostatin[26] and thrombospondin[27, 28], were also identified. Indeed, much of this work culminated in 2004 with the first US Food and Drug Administration approval of an

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anti-VEGF agent, bevacuzimab, for treatment of colorectal cancer and, since then, for other solid tumors, including kidney, lung, and glioblastoma multiforme, all to varying degrees of success[9–13, 29–31]. However, at the time of initial discovery and characterization of angiogenesis, the prevailing theory was that its role was largely limited to solid tumors, and liquid "tumors", such as leukemias and other hematological malignancies, were considered angiogenesis-independent.

1. Recognition of hematological malignancies as angiogenic diseases

In 1993, the hypothesis that hematological malignancies could also be "angiogenic" was proposed by Judah Folkman based on initial evidence from his group, which demonstrated elevated levels of basic FGF in patients with leukemia, and E. Lynette Wilson's laboratory, which showed the presence of basic FGF in human bone marrow and peripheral blood[8, 32, 33]. A series of subsequent imaging studies showed that angiogenesis was increased in multiple myeloma[34–36] and B-cell leukemia[37]. Of these early studies, the most convincing evidence arose from confocal imaging performed by the Folkman laboratory, which showed that bone marrow from patients with acute lymphoblastic leukemia (ALL) have similar neovascularization patterns as in solid tumors[38]. Increased bone marrow vascularity was subsequently found in a variety of other hematologic disorders, including myelodysplastic syndromes[39], acute myeloid leukemia or AML[40], chronic myelogenous leukemia or CML[41], polycythemia vera[41], and idiopathic myelofibrosis[42], as well as in various lymphoid-based disorders, such as B-cell chronic lymphocytic leukemia or CLL[43], and B-cell non-Hodgkin's lymphoma[44].

Simultaneously, attention was focused on identifying the factors responsible for increased vascularity in hematopoietic tissues in these diseases. As VEGF was originally cloned from HL-60 leukemic cells[15], early efforts focused on the role of VEGF in hematologic disorders, and led to the discoveries that VEGF levels were significantly higher in patients with AML[45] and that such high levels were a predictor of patient outcome[46]. As with solid tumors, other proangiogenic factors, including FGF-2[47, 48], PDGF[49], MMP-9[50, 51], HIF1-α[52], and angiopoietin-1 and -2[53, 54], were identified as mediators of bone marrow angiogenesis in the context of hematologic disorders. Several soluble factors with considerable anti-angiogenic properties, including endostatin[55] and thrombospondin-1[56, 57] were also identified. Together, these initial studies ultimately gave rise to hundreds of subsequent reports, which demonstrated the presence of angiogenesis in a variety of hematological disorders[58–65], and suggest that anti-angiogenesis approaches may also have significant therapeutic potential in the treatment of hematologic disorders.

2. Anti-angiogenic therapy in hematologic diseases

The findings described until now simply reflect correlation between leukemic and angiogenic states; however, direct support that leukemia is angiogenesis-dependent quickly followed with experimental evidence showing that combinatorial treatment with direct angiogenesis inhibitors, angiostatin and endostatin, could inhibit leukemia progression in animal models[66]. In 1999, a clinical study assessing the role of another anti-angiogenesis therapy, thalidoamide, demonstrated complete remission in two of 76 patients with multiple

myeloma who were refractory to other frontline therapies[67]. Stunningly, one third of these patients also showed a reduction of myeloma protein for at least 6 weeks, demonstrating the potential for anti-angiogenesis therapy in treating hematological malignancies. Therefore, these earliest reports, together, are indicative of angiogenesis as a powerful therapeutic target in hematological disorders such as leukemias and multiple myeloma.

Further, it was shown that a VEGF receptor blockade could slow down progression of a variety of hematological malignances, including T-leukemia/lymphoma[68], promyelomonocytic leukemia[69, 70], and subcutaneous implanted erythroleukemia[71]. Together, these preclinical data indicate that anti-angiogenic approaches have significant capabilities in murine xenograft models. Nonetheless, the translational capabilities of these agents and others were found to be less promising in initial human studies[72], therefore necessitating additional mechanistic studies of other angiogenic factors in the context of blood disorders. In this review, we discuss one of these impactful mediators of angiogenesis, ANG, in the context of hematological malignancies.

3. ANG is overexpressed in leukemia patients: insights into its function?

ANG is a 14 kD secreted ribonuclease with considerable angiogenic potential; the precise biochemical mechanisms of ANG function are described below. Originally identified from conditioned medium of HT-29 colon adenocarcinoma cells[25], ANG transcripts were detected in a number of tumor cell lines and tumor tissues[73, 74]. Indeed, secretion of ANG could be observed in virtually all cell types, including endothelial cells, fibroblasts, tumor cells, and hematopoietic cells[75]. Importantly, some of these cell types are present in the leukemic bone marrow niche, the main site of malignant hematopoiesis[76]. While ANG can be detected in plasma and serum of healthy individuals, elevated ANG levels were observed in patients with solid tumors, and such high levels are associated with poor prognosis and/or an advanced cancer stage in a variety of cancers, including colorectal[77], pancreatic[78], gastric[79], urothelial[80], prostate[81], and breast[82] cancer. Further, anti-ANG therapies showed significant prevention of human xenograft tumor growth[83]. These data point to ANG as a contributor to cancer development, particularly in the context of solid tumors. Significantly, two studies in 1987 and 1994 detected ANG in leukemia cell lines[75, 84]. Therefore, a number of laboratories set forth to characterize ANG levels in patients with various hematological disorders on the basis of three correlative findings described above: (1) ANG is a prognostic factor in solid tumors, (2) ANG is expressed in hematopoietic and leukemic cells, and (3) increased bone marrow vascularity is observed in patients with hematopoietic disorders and can be targeted by anti-angiogenic therapies.

4. ANG levels are elevated in patients with myelodysplastic syndromes and myeloid malignancies

The first of these studies, from Maher Abitar’s group in 2001, examined ANG levels in the plasma of patients with AML and advanced MDS, and found elevated levels of ANG in the plasma of these patients[85]. Of these patient samples, ANG levels were lower in AML patients than in advanced MDS patients. Significantly, high plasma levels of ANG were associated with prolonged survival in both AML and advanced MDS patient cohorts. This

contrasts reports in solid tumors, which showed that high ANG levels are associated with cancer progression and/or poor prognosis in solid cancers[77–82]. No significant correlation between ANG levels and remission was observed in both patient cohorts in the Abitar study. Nonetheless, multivariate analyses revealed a weak correlation between ANG, survival, and prognostic characteristics of AML patients, including age and hemoglobin levels; however, no correlation was observed in MDS patients, which may be attributed to the patient treatment options pursued in this study, as well as a small cohort size.

A subsequent study from Reinhard Stauder's group in 2002 showed elevated serum levels of ANG in MDS and AML patients, including across various disease stages[86]. However, ANG levels did not correlate with IPSS scoring or complete blood cell count parameters. Interestingly, serum ANG levels were higher in RAEB/RAEB-t patients with increased bone marrow blast counts than in RA/RAS low-risk MDS patients, which have lower bone marrow blast counts. The authors of this study therefore proposed that ANG is perhaps secreted by myeloid blasts themselves.

A report in 2005 from Despina Kyriakou's laboratory confirmed elevated levels of serum ANG in MDS patients, observing significantly higher levels of ANG in high-risk (RAEB, RAEB-t, and CMML) compared to low-risk (RA and RARS) patients[87]. In this study, ANG levels correlated with IPSS groupings, and patients with a score of 3 showed significantly higher levels of ANG than patients with a score of 0 or 1. Interestingly, ANG levels also significantly correlated with the cytokine and acute phase regulator, interleukin-6 (IL6)[88]. Given that ANG is excreted in a similar manner to type 2 acute phase proteins[89], and IL-6 stimulates excretion of ANG from human hepatoma cells[90], the authors proposed a mechanism by which IL-6 excretion from leukemic blasts triggers ANG excretion from the liver; however, further work beyond expression and correlation studies are necessary to either support or refute this hypothesis.

In 2002, Oystein Bruserud's group examined the effect of chemotherapy on level of ANG in AML patients[91]. Consistent with the previously described findings from the Abitar and Stauder groups[85, 86], this study found elevated serum levels of ANG in patients with untreated AML; however, treatment with chemotherapy resulted in a significant decrease in serum ANG levels. As ANG levels are altered during an acute phase reaction[89, 90], ANG levels were examined in cytopenic patients before, during, and after bacterial infection, and no alterations of serum ANG levels during febrile neutropenia were observed, despite evidence of an acute phase reaction. Further, undetectable levels of secreted ANG was observed in cultured patient-derived blasts in 56 of 59 patient samples examined; a maximum of 175 pg/mL ANG in the three detectable samples. Together, these data indicate that the elevated levels of ANG observed in the serum of AML patients are neither due to an acute phase reaction nor due to secretion of endogenously produced ANG from AML blasts. Further, ANG levels were also significantly increased in patients with PBSC autografts before and after apheresis. These data suggest that high ANG levels following mobilization of PBSC with granulocyte-colony stimulating factor (GCSF) may be relevant for autologous transplantation therapies in treatment of various hematological malignancies, including AML.

Further evidence came from Caterina Musolino's laboratory in 2004, which reported elevated levels of ANG in serum from patients with chronic myeloproliferative disorders, including CML and essential thrombocythemia, with highest levels in serum from CML patients[92]. These data, however, did not correlate with standard clinical parameters, including blast count, tumor burden, spleen size, and complete cell counts. Moreover, circulating levels of ANG in a subset of patients with CML were measured after they had achieved hematological remission following treatment with interferon (IFN)-α. Interestingly, these patients displayed reduced levels of circulating ANG compared to their pre-treatment levels at time of diagnosis. This is consistent with the findings from the Bruserud group that ANG levels decrease following chemotherapy[91], as described above, and is also consistent with other findings that show reduced bone marrow microvessel density following IFN-α treatment of patients with CML[93].

The finding that ANG levels are higher in serum from patients with CML compared to healthy was confirmed by Sang Kyun Sohn's group in 2005; however, in these 14 patient samples, the levels of ANG significantly correlated with WBC and platelet counts, and LDH levels[94]. No differences in ANG levels, however, were observed in the serum of patients diagnosed with AML, in contrast to the findings from the Bruserud group[91]. These conflicting findings, therefore, warrant future studies with larger patient cohorts with a variety of molecular and cytogenetic characteristics.

5. The role of ANG in lymphoid-based malignancies is not quite as clear

The role of ANG, however, is not likely limited to hematological disorders of the myeloid lineage. Nonetheless, studies assessing levels of ANG in patients with hematological disorders of the lymphoid lineage are more limited.

To explore whether ANG levels are also elevated in patients with lymphoid malignancies, Stefan Molica's group assessed serum ANG levels in patients with early B-cell CLL[95]. In this report, the authors found no difference in levels of ANG, and no association with microvessel density, levels of other angiogenic factors except soluble VCAM-1, patient karyotype, or clinical-hematological patient features, except for a positive correlation with lymphocyte doubling time and β2-microglobulin. Interestingly, however, patients with ANG levels higher than the cohort median showed a dramatically elevated five-year progression-free survival (85% for patients higher than the median, versus 51. 5% for patients lower than the median). Importantly, this report suggests that ANG levels may be incorporated into Rai substages to identify risk categories with well-defined differences in 40-month progression-free survival. This positive correlation between high ANG levels and increased survival is consistent with the study by the Albitar group in AML and advanced MDS patients[85]. Together, these data poignantly contrast correlation studies in solid tumors, which show reduced survival and/or increased disease progression in patients with high ANG levels[77–82]. In the same report from the Bruserud group[91], described above, a reduction in serum ANG levels was observed following chemotherapy in ALL patients, although another study using a larger cohort is necessary confirmation. In contrast to these two studies, however, the report from Sang Kyun Sohn's group, described above, did not observe any significant differences in serum ANG levels in patients with ALL[94]. Given the number of

inconsistencies in levels of ANG among different patient cohorts, it is therefore necessary to pinpoint the exact function of ANG, rather than solely examine levels of ANG in patient serum and/or plasma samples.

6. Insights into the mechanism of ANG in non-solid tumors

6.1. The precise biochemical and cellular functions of ANG in hematological disorders are still unknown

While the aforementioned studies indicate potential prognostic and translational capabilities of ANG in hematological malignancies, functional studies directly assessing the role of ANG in various hematological diseases are limited. To our knowledge, the only functional study of ANG in leukemia cells came from Steven Benner's lab in 1995, where the authors demonstrated that culture with recombinant ANG protein had no effect on ^{3}H-thymidine incorporation in K562 CML cells and HL-60 promyelocytic leukemia cells[96]. While this study examined a number of doses of ANG, the only functional readout was DNA synthesis. Therefore, further validation with additional time points of treatment with ANG and other functional assays, are necessary.

This important functional study, coupled with analyses of ANG levels in patients with hematological disorders, raise a number of basic, but significant, questions that require experimental validation: (1) is ANG secreted from leukemic blasts, (2) does ANG affect the proliferation, survival, and/or growth of leukemic blasts, (3) can this be recapitulated in a mouse model of hematological disease, (4) do ANG inhibitors show equal or greater success in pre-clinical mouse models of hematological disorders compared to other anti-angiogenesis therapies, and if so, are these therapies synergistic, and (5) does this translate into humans?

It would be amiss, however, to imply that ANG might act solely through angiogenic pathways in promoting the progression of cancer. Indeed, work from our laboratory and others have shown that ANG function in tumor progression is actually two-fold: while its angiogenic potential promotes tumor formation in later steps of cancer development, its ribonucleolytic activity promotes the survival and/or proliferation of cancer cells in early stages. These functions are described in great detail in other review articles by our laboratory[97, 98]. Indeed, ANG has been shown to promote the growth of a variety of cell types, include cancer cells, largely through ribosomal RNA (rRNA) transcription, the rate-limiting step for ribosomal biogenesis, and an important facet of cell growth[99]. Under growth conditions, ANG translocates to the nucleus, where it stimulates rRNA transcription[100]. Under stress, ANG localizes to the cytoplasmic foci in cells, termed stress granules; this localization is required for tRNA cleavage into tRNA fragments termed tRNA-derived stress-induced small RNA (tiRNA)[101–104]. These RNA fragments are directly responsible for reprogramming global protein synthesis as a mechanism for enhancing cellular survival: tiRNA production leads to suppression of global protein translation which saves anabolic energy, while simultaneously permitting translation of anti-apoptotic genes by internal ribosomal entry sequence (IRES) - mediated protein translation[101–104].

Therefore, ANG has a powerful role as a regulator of RNA processing, with considerable, cell state-dependent effects on protein translation. Future studies assessing the role of ANG in RNA processing in leukemic blasts are necessary. These studies are especially warranted, given the recent plethora of studies assessing the drastic number of ribosomopathies resulting from defects in ribosome function or protein synthesis; while a number of regulators of these biological processes have been identified, only a handful have been implicated in disease, or in normal or malignant hematopoiesis[105, 106]. Indeed, hematopoietic stem cells were recently shown to have a tightly regulated rate of protein synthesis[107], suggesting that this housekeeping function must be maintained for proper and continuous cell output of viable blood and immune cells. It is therefore conceivable that such regulation extends to leukemic stem cells, a functionally-related stem cell population[108]. Given the wide range of experimental options available for study of protein synthesis, including in vivo study of protein synthesis in bone marrow[107], these biochemical studies are especially critical.

6.2. The physiologic function of ANG is also unknown

A considerable amount of evidence supports the working model that signals from leukemic blasts as well as from the bone marrow microenvironment in which leukemic blasts reside, orchestrate the development and/or progression of hematological cancer[76, 108]. While it is likely that some or all of the aforementioned biochemical functions of ANG may, at least in part, contribute to the disease state, it is equally as likely that ANG derived from stromal cells present in the bone marrow niche also contribute to malignant hematopoiesis. As ANG is a secreted factor, there is a considerable possibility that ANG participates in a crosstalk between leukemic cells and the tumor microenvironment, and may be a factor involved in the ‘self-reinforcing’ leukemic niche[76]. Indeed, many of the cell types that compose the bone marrow niche have been shown to express and/or secrete ANG, as discussed above[75, 76].

Work from the Burserud group showed that leukemic blasts, themselves, likely produce minimal ANG, suggesting that a non-cell autonomous source may contribute to cancer development. It is therefore necessary to pinpoint the precise cell type(s) involved in secretion of ANG secretion; future work is required to determine whether bone marrow stromal cells known to regulate leukemic stem cell and/or blast function secrete high levels of ANG, and also determine the functional consequence of such high levels. Whether ANG also affects development of leukemia due to ineffective hematopoiesis as a result of hypercellular bone marrow remains an unaddressed question. A number of mouse models to faithfully recapitulate leukemias have been developed over the last several decades, including MLL-AF9 oncogene-induced AML[109] and BCR-ABL1 oncogene-induced CML-like myeloproliferative neoplasia[110], and are the best tools available for modeling hematopoietic and leukemic cell function in a complex milieu of regulatory stromal cells[111]. Quite broadly, it is imperative to assess whether ANG contributes to and/or is regulated by the hostile, hypoxic bone marrow niche.

6.3. Broad questions that still exist

A number of clinically-based studies have assessed the levels of ANG in patients with a variety of myeloid and lymphoid-based disorders; however, comprehensive analyses are still lacking, and there are some contrasting findings, particular with respect to the levels of ANG in patients with lymphoid leukemias. Indeed, the expression studies described above are largely limited to myelodysplastic syndromes and leukemias[91, 94, 95]. Further expression and functional studies in other non-solid tumors, such as lymphoma, are warranted. Nonetheless, the most important unaddressed question remains: does ANG have a similar function in non-solid tumors as in solid-tumors and, if so, how does it work? These questions, as outlined above, can be addressed by *in vitro* proliferation and functional assays as well as *in vivo* mouse models of defined leukemias. Given that ANG is a secreted factor, it can be neutralized by monoclonal antibodies, which have shown incredible promise in solid tumor studies in mice[83]. Testing the potential therapeutic use in hematological disorders must necessarily follow early functional studies in this non-solid tumor setting.

While a number of anti-angiogenic therapies have been developed in solid tumors, more work is required to understand specific functional characteristics of various angiogenic factors, including ANG, in hematological malignancies. In light of a number of instances of resistance against anti-angiogenic therapies[10, 29], understanding the precise mechanisms behind such therapies will be vital to better development of therapeutic approaches, particularly in unique tumor types such as the leukemias.

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