The Novel Angiogenic Inhibitor, Angiocidin, Induces Differentiation of Monocytes to Macrophages

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Abstract
We previously showed that angiocidin, a tumor and vascular associated protein, is a potent inhibitor of angiogenesis and tumor growth. Angiocidin is a multidomain protein that exerts its antiangiogenic activity through multiple mechanisms, including effects on cell matrix interaction. Here, we describe another activity of angiocidin that may contribute to its antitumor activity. We show that angiocidin activates monocytes to secrete a mixture of proinflammatory cytokines and induces them to differentiate into macrophage-like cells. Using the monocytic cell line THP-1, we show that angiocidin induces the cells to become adherent and phagocytic, express macrophage markers, and secrete matrix metalloproteinase-9. Microarray analysis of control and angiocidin-treated THP-1 cells revealed that angiocidin up-regulated p105/p50, p100/p52, and rel B, components of the nuclear factor-kB (NF-kB) pathway. We confirmed the microarray data and showed that angiocidin induced phosphorylation of IKBa, p50, and p65 and translocation of p50 and p65 to the nucleus. We also showed that angiocidin activated up-stream mediators of NF-kB, such as the mitogen-activated protein kinase (MAPK) pathway and phosphoinositide-3 kinase (PI3K). Blockage of NF-kB and MAPK activation with small molecule inhibitors completely prevented angiocidin-mediated secretion of cytokines from THP-1 cells, but did not inhibit their adhesive phenotype. Blocking PI3K inhibited both secretion of cytokines, as well as the adhesive phenotype. These data suggest that angiocidin activates monocytes to secrete cytokines and differentiates them to a macrophage-like phenotype through at least two pathways mediated by MAPK and NF-kB, as well as PI3K.

Introduction
Solid human tumors are often infiltrated by host immune cells, which can have a diverse effect on tumor progression. Among other cell types, macrophages are a major component of the leukocyte infiltrate of tumors. These tumor-associated macrophages (TAM) have a complex dual function in their interactions with neoplastic cells (1). Cytotoxic T cells, when presented with the appropriate tumor antigen by macrophages, can kill tumor cells. In contrast, TAMs can also promote angiogenesis and metastasis. The contradictory reports that have surfaced can best be explained in terms of the "macrophage balance hypothesis," which asserts that the outcome of the interaction between macrophages and neoplastic cells depends on the number of macrophages recruited to the tumor microenvironment and their state of activation (2). The number and distribution of TAMs is linked to prognosis in different types of human malignancies (3, 4). Furthermore, ex vivo grown tumor cytotoxic macrophages are effective against murine models of metastasizing tumors (5). Immunomodulation, therefore, is a therapeutic strategy worthy of continued investigation.

Our laboratory has been studying the potential antitumor activity of a protein originally isolated from lung carcinoma (6). We cloned the protein and identified matrix protein binding domains within the molecule that were essential for its antitumor activity (7). The cloned recombinant protein, termed angiocidin, or its matrix-binding domain peptide, when injected systemically into tumor bearing mice, significantly inhibited tumor growth and angiogenesis (7–9). Because the matrix is important in binding cytokines that enable leukocytes to home to the tumor, and angiocidin binds important tumor matrix proteins, such as thrombospondin-1, we postulated that angiocidin might function to activate leukocytes.

In this report, we show that angiocidin potently stimulates monocytes and the monocytic cell line THP-1 to secrete a mixture of inflammatory cytokines. Angiocidin-treated THP-1 cells become adherent and differentiate into cells that morphologically resemble macrophages. We further show that this proinflammatory activity of angiocidin is mediated through a pathway involving the activation of nuclear factor-kB (NF-kB), mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase (PI3K). These macrophage-like cells are capable of phagocytosis and antigen presentation. These newly discovered activities of angiocidin likely contribute to its antitumor activity.

Materials and Methods

Chemicals and antibodies. RPMI 1640 was purchased from MediaTech. Trizol Reagent was purchased from Invitrogen. Ficoll Paque was purchased from Amersham Biosciences. Brefeldin A solution and the MCP-1 ELISA kit were purchased from eBioscience. The cytokine antibody array was obtained from RayBiotech, Inc. Fluorescent microparticles were purchased from Polysciences, Inc. The nuclear extraction kit was purchased from RayBiotech, Inc. The anti-p44/42 MAPK antibody was obtained from Cell Signaling. The anti–phosphorylated paxillin, anti–phosphorylated p44/42, and anti-p44/42 antibodies, as well as the NF-kB Pathway Sampler kit, were purchased from Cell Signaling. The anti–matrix metalloproteinase (MMP-9) antibody was purchased from R&D Systems. The tumor necrosis factor-a (TNF-a) antibody was obtained from BD Biosciences. The anti–phosphorylated sialyl adhesion kinase (FAK), anti–phosphorylated paxillin, anti–phosphorylated p44/42, and anti-p44/42 antibodies, as well as the NF-kB Pathway Sampler kit, were purchased from Cell Signaling. The anti–phosphorylated p50 and anti-p50 antibodies were obtained from Santa Cruz.

Expression and purification of his-tagged recombinant angiocidin. Expression and purification of his-tagged angiocidin was performed as previously described (7). Endotoxin was removed from the preparation by
Phosphorylation.

**Cell culture of THP-1 cells.** Human monomcytotic THP-1 cells were obtained from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2% glutamate, 0.5% penicillin/streptomycin, and 0.1% gentamicin. Cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO₂.

**Angiocidin treatment conditions.** Cells were treated for 24 h with or without 10 μg/mL angiocidin at a density of 2 × 10⁶ cells/mL in serum-free RPMI 1640 supplemented with 0.1% bovine serum albumin (BSA) for 24 h.

**Immunoblotting.** Lysate (25 μg) was run on a 10% SDS polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% milk/TBST, then probed with the appropriate antibody at a 1:1,000 dilution. The following day, after washing, membranes were incubated with a 1:10,000 dilution of goat anti-rabbit IgG/horseradish peroxidase and developed chemiluminescently with ECL Plus. To assess cytoplasmic to nuclear shuttling of the various NF-κB proteins, cytoplasmic and nuclear fractions were isolated using a nuclear extraction kit according to the manufacturer's protocol.

**Microarray analysis.** Total RNA was collected from cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Microarray analysis was performed by Fox Chase Cancer Center using an Agilent gene chip array.

**Flow cytometry.** Peripheral blood mononuclear cells (PBMC) were harvested from whole blood of a healthy human donor and isolated by density gradient centrifugation using Ficoll Paque according to the manufacturer's protocol. Peripheral blood monocytes were isolated from lymphocytes with anti-CD14–conjugated magnetic beads using a MidiMACS separator according to the manufacturer's instructions. After treatment with various concentrations of angiocidin for 6 h, purified monocytes were stained with a PE-conjugated anti–TNF-α antibody for 30 min in the dark before fixation with 4% paraformaldehyde. Cells were treated with brefeldin A solution to inhibit intracellular transport of the accumulated TNF-α protein according to the manufacturer's suggestions.

**Cytokine antibody array.** A cytokine antibody array was used according to the manufacturer's instructions.

**MCP-1 ELISA.** A capture ELISA was used according to the manufacturer's protocol.

**Phagocytosis assay.** Fluorescent green 0.5-μm microparticles diluted to a density of 5 × 10² particles/mL sterile PBS were added to cells at a concentration of 25 μL microparticles/mL media for 2 h at 37°C. Phagocytosis was stopped with the addition of 1 mL ice-cold sterile PBS. Cells were washed in ice-cold PBS five times to remove microparticles that remained in suspension or were loosely adherent to the tissue culture plastic or the surface of the cells. Cells were covered with a glass coverslip and observed and photographed using fluorescence microscopy.

**Gelatin zymography.** Gelatin zymography was performed, as previously described (11). Immunoblot confirmation of zymogram results was performed as described above using a 1 μg/mL solution of mouse monoclonal anti-MMP-9 antibody.

**Inhibitor studies.** THP-1 cells were pretreated with 1 μg/mL 6-amino-4-((4-phenoxyphenylethylamino)quinazoline, 10 μM/L U0126, or 50 μM/L LY294002 at 37°C for 60 min. Angiocidin was then added to the appropriate wells. The volume of DMSO (vehicle control) added to the cells was equivalent to the volume of inhibitor used.

**Results**

**Angiocidin induces THP-1 adhesion/differentiation.** Angiocidin-treated THP-1 cells, a suspension cell line, became more adherent to the plastic substrate in a dose-dependent manner, as shown in Fig. 1A. Whereas untreated cells remained spherical and completely in suspension, angiocidin-treated cells became flattened and elongated, extending numerous “processes.” The dose required to obtain optimum adhesion/differentiation was 10 μg/mL.

**Angiocidin induces focal adhesion kinase and paxillin phosphorylation.** Untreated cells showed barely detectable levels of phosphorylated FAK protein, consistent with their nonadherent morphology, whereas treatment of THP-1 cells with angiocidin resulted in a dramatic increase in levels of phosphorylated FAK protein, in accordance with the newly acquired adhesive phenotype of these cells (Fig. 1B). Angiocidin treatment also resulted in significant phosphorylation of paxillin protein, whereas untreated cells again contained barely detectable levels of the phosphorylated form of this molecule (Fig. 1B).

**Angiocidin-treated THP-1 cells express macrophage markers.** We next sought to analyze angiocidin-induced changes in gene expression in THP-1 cells. We hypothesized that various markers of monocyte activation and differentiation would become up-regulated. We found several important genes up-regulated at the mRNA level in angiocidin-treated cells, namely CD36, macrophage receptor with collagenous structure (MARCO), CD14, CD69, and α2-macroglobulin (A2M; Fig. 1C and D). There is evidence in the literature that up-regulation of each of these genes, discussed below, occurs upon differentiation of monocytes into macrophage-like cells.

**Angiocidin activates PBMCs.** To ascertain whether angiocidin is able to induce activation of freshly isolated peripheral blood monocytes, we used flow cytometric analysis to assay intracellular accumulation of TNF-α. Angiocidin-activated mononuclear cells in a dose-dependent manner at angiocidin levels as low as 1.0 μg/mL after only 6 h of treatment (Fig. 2A). This figure also shows a population of cells expressing low levels of CD14, which are not activated by angiocidin to produce TNF-α. This result is likely due to the presence of low levels of granulocytes present in our monocyte preparation because granulocytes are known to express low levels of this cell surface marker.

**Angiocidin induces secretion of a specific mixture of inflammatory mediators.** As monocyte activation is associated with the secretion of proinflammatory cytokines/chemokines, we sought to determine whether angiocidin-induced monocyte differentiation was concurrent with cytokine/chemokine secretion (12, 13). We used densitometry scanning of cytokine array membranes to evaluate changes in relative cytokine/chemokine secretion levels between angiocidin-treated and untreated cell supernatants. We found that 18 of the 42 cytokines/chemokines assayed by this array membrane were secreted at increased levels by angiocidin-treated THP-1 cells (Fig. 2B and C). The largest increase in secretion between angiocidin-treated and untreated cells occurred with MCP-1. We confirmed these results with our microarray analysis, which also showed that various inflammatory cytokines/chemokines, as well as their cognate receptors, became up-regulated at the mRNA level upon treatment with angiocidin (Supplementary data). For example, angiocidin treatment results in a nearly 800-fold increase in mRNA expression levels of MCP-1 (Supplementary data). We sought to confirm these results and quantitate the amount of MCP-1 secreted into culture supernatants upon treatment of THP-1 cells with angiocidin. Untreated cells secreted barely detectable levels of MCP-1, whereas treatment with angiocidin resulted in secretion of ~1.3 ng/mL MCP-1 (Fig. 2D). These results confirm the data obtained with our cytokine antibody array and microarray and show that angiocidin is indeed able to induce the secretion of important proinflammatory mediators.

**Angiocidin increases the phagocytic ability of THP-1 cells.** Monocyte to macrophage differentiation has been shown to be accompanied by an increase in the phagocytic activities of these cells (14). We therefore analyzed whether angiocidin-treated THP-1 cells acquired increased phagocytic activity toward fluorescent particles.
microparticles designed specifically for this purpose. Angiocidin-treated cells displayed an increased phagocytic activity, with >90% of the cells internalizing these fluorescent microparticles, whereas THP-1 cells not treated with angiocidin did not phagocytose microparticles as efficiently (Fig. 3A). These results indicate that, upon treatment with angiocidin, THP-1 cells acquire macrophage-like functions.

Angiocidin induces THP-1 cells to secrete MMP-9. Activated macrophages secrete several enzymes that cause changes in the molecular and mechanical structure of the extracellular matrix. They are a rich source of MMPs (15). MMP-9 expression typically falls into one of two distinct patterns. Some cell types, such as macrophages, only produce MMP-9 in response to prolonged exposure to an inflammatory stimulus. Other cell types constitutively produce MMP-9 and can rapidly release it from intracellular stores (16). Given the activating effect of angiocidin on both THP-1 and human peripheral blood monocytes, we next wanted to characterize the effect angiocidin treatment would have on the secretion of MMPs. Angiocidin stimulated THP-1 cells and PBMCs to secrete enzymatically active MMP-9 (Fig. 3B).

Angiocidin induces activation of MAPK and NF-κB pathways. We performed a series of immunoblots investigating key signaling molecules known to become activated by cytokine treatment (17). Angiocidin treatment resulted in activation of MAPK p44/42, as well as the upstream kinase c-Raf, as assessed by the phosphorylation state of these molecules (Fig. 4A).

Our microarray experiment showed that various members of the NF-κB signaling pathway became up-regulated at the mRNA level upon treatment with angiocidin (Supplementary data). We therefore decided to analyze the involvement of the NF-κB pathway in angiocidin-induced signaling. Both NF-κB p65 and p50 showed increased phosphorylation upon treatment with angiocidin, whereas total protein levels remained the same. Angiocidin-treated THP-1 cells also expressed much higher levels of phosphorylated IκB-α and IKK proteins (Fig. 4B). The IKK complex is responsible for phosphorylation of the inhibitory IκB-α protein, which results in polyubiquitination and degradation of this inhibitor, subsequently releasing the p65/p50 heterodimer to translocate into the nucleus and initiate DNA transcription.

We also performed immunoblots on isolated nuclear and cytoplasmic lysates to determine whether p65 and p50 showed increased nuclear localization in angiocidin-treated THP-1 cells. Levels of phosphorylated p65 are significantly increased in the nuclear fractions of angiocidin-treated THP-1 cells, whereas
untreated nuclear lysates contain barely detectable levels of these proteins (Fig. 4C). Figure 4C also shows a similar result was achieved when analyzing cytoplasmic and nuclear lysates for phosphorylated p50. These data indicate that, once the inhibitory IKB-α molecule is phosphorylated and degraded, the p50/p65 heterodimer is released and able to translocate into the nucleus and initiate transcription of its target genes.

**MAPK, NF-κB, and PI3K pathways are involved in angiocidin-induced cytokine and MMP-9 secretion.** It has been shown in the literature that various MAPK pathways are involved in the induction of cytokine secretion (18–20). Likewise, activation of the NF-κB pathway has been shown to initiate the secretion of a wide variety of cytokines and immunomodulatory molecules. Therefore, we wanted to determine whether inhibition of the MAPK and NF-κB pathways would minimize angiocidin-induced cytokine secretion. We chose to assay for secreted levels of MCP-1 due to the high abundance of this chemotactic cytokine in the conditioned media of angiocidin-treated THP-1 cells (Fig. 2D).

THP-1 cells treated with angiocidin alone secreted ~1.4 ng/mL MCP-1, whereas untreated cells secreted undetectable levels of this chemokine. Cells treated with both angiocidin and the U0126 MAPK MEK 1/2 inhibitor secreted only 77 pg/mL MCP-1, demonstrating that this MAPK pathway is involved in angiocidin-induced cytokine secretion (Fig. 5A). Although cells treated with both U0126 and angiocidin secreted extremely low levels of MCP-1, the cells were observed to undergo dramatic morphologic changes. These results suggest that the adhesive phenotype induced by angiocidin is separate from angiocidin-induced cytokine/chemokine secreting activity.

Similarly, angiocidin-treated THP-1 cells pretreated with an NF-κB inhibitor molecule known to abrogate NF-κB p65 transcriptional activity did not secrete any detectable levels of MCP-1, suggesting that the NF-κB pathway is also involved in angiocidin-induced cytokine/chemokine secretion (Fig. 5B). As with U0126, cells treated with the NF-κB inhibitor were also observed to adhere/differentiate to the same extent as cells treated with
Angiocidin alone. These results once again suggest that the processes of adhesion/differentiation and cytokine/chemokine secretion are initiated by separate pathways in our system.

Use of both the MAPK MEK 1/2 and NF-κB inhibitors indicated that an additional signaling pathway was responsible for angiocidin-induced cellular adhesion/differentiation. We therefore sought to determine whether PI3K was responsible for the observed adhesive effect. PI3K has been implicated in the control of cytokine release from a variety of cell types (21–24). In addition, various studies have shown crosstalk between PI3K and the MAPK and NF-κB pathways (25–27). Treatment of THP-1 cells with angiocidin and the PI3K inhibitor LY294002 reduced the levels of MCP-1 in the conditioned media to the level present in the untreated media of untreated cells (Fig. 5C). These results indicate that an additional signaling pathway was responsible for angiocidin-induced MMP-9 secretion (Fig. 5C). These data show that, in addition to the MAPK and NF-κB pathways, PI3K also plays a role in angiocidin-induced cytokine/chemokine secretion. Of note, cells treated with both angiocidin and the PI3K inhibitor did not become adherent to the tissue culture plastic. Therefore, PI3K may be playing a dual role in our system, controlling cytokine/chemokine release and adhesion/differentiation of the THP-1 cells.

We also sought to clarify whether inhibition of MAPK MEK 1/2, NF-κB, and PI3K would inhibit angiocidin-induced MMP-9 secretion. Treatment with angiocidin elicited secretion of this matrix metalloproteinase. However, pretreatment of cells with the MEK 1/2 inhibitor, the NF-κB inhibitor, or the PI3K inhibitor, before treatment with angiocidin, completely abrogated angiocidin-induced MMP-9 secretion (Fig. 5D). As expected, cells left untreated or those treated with the inhibitor alone or the DMSO vehicle control did not secrete any detectable levels of MMP-9.

**MAPK and PI3K function upstream of NF-κB.** We have shown that inhibition of MAPK MEK 1/2 or NF-κB p65 elicit similar effects in our system. These results indicated the possibility that MAPK p44/42 (whose phosphorylation is prevented by the MEK 1/2 inhibitor) is acting upstream of NF-κB p65. There is extensive literature citing a relationship between the MAPK and NF-κB pathways (28–30). We therefore hypothesized that inhibition of MEK 1/2 and therefore p44/42 would result in inhibition of the NF-κB pathway. THP-1 cells treated with angiocidin showed increased levels of phosphorylated p65 compared with untreated cells, as previously shown, whereas pretreatment of cells with the U0126 inhibitor reduced angiocidin-induced NF-κB p65 phosphorylation to control levels (Fig. 6A). These results indicate that MAPK p44/42 is indeed acting upstream of the NF-κB pathway.

As inhibition of PI3K elicited similar results in our system as inhibition of MAPK MEK 1/2 and NF-κB, we next hypothesized that perhaps PI3K was functioning upstream of the MAPK–NF-κB pathway. We again used the LY294002 PI3K inhibitor to reduce PI3K activity before angiocidin treatment. We then probed these cell lysates for phosphorylated p65 protein. In agreement with our hypothesis, treatment with angiocidin alone once again resulted in phosphorylation of the NF-κB molecule, whereas treatment of the cells with LY294002 before angiocidin treatment reduced the levels of phosphorylated p65 protein (Fig. 6B). These results therefore indicate that PI3K is indeed functioning upstream of our MAPK–NF-κB pathway.

As a final step to confirm this hypothesis, we then probed our LY294002-pretreated cell lysates for the phosphorylated form of the MAPK p44/42 protein. We hypothesized that levels of phosphorylated MAPK p44/42 would be significantly reduced when the PI3K pathway was inhibited. However, we obtained unexpected results. There was virtually no difference in phosphorylated p44/42 levels, whether or not the cells were pretreated with LY294002 (data not shown). These results therefore indicate that angiocidin is inducing...
the activation of at least two separate pathways in THP-1 cells. In the first pathway, MAPK p44/42 is functioning upstream of the NF-κB pathway. In the second scenario, NF-κB is also activated; however, it is functioning downstream of PI3K, without using MAPK p44/42 as an intermediary signaling molecule. A diagram describing these proposed angiocidin-induced signal transduction pathways is depicted in Fig. 6C.

Discussion

Angiocidin was first isolated from lung carcinoma by thrombospondin-1 peptide affinity chromatography (6). Angiocidin was shown to be overexpressed by invasive cancer cells and capillary endothelial cells (31–33). This protein was also found in the blood of patients with advanced melanoma, as well as cancers of the colon, prostate, and breast. Patients with more advanced cancers present with higher levels of angiocidin in their sera than healthy individuals or patients with less advanced disease (34). These results indicated that angiocidin may regulate tumor progression.

Consistent with its regulatory role in tumorigenesis, we found that angiocidin inhibited critical steps of tumor progression in vitro, including angiogenesis and invasion. Systemically injected angiocidin also exhibited potent antitumor activity in vivo (7).

A counterintuitive observation previously set forth is that removal of a primary tumor stimulates growth of secondary tumors, suggesting that the primary tumor produces factors that inhibit the development of these secondary metastases. Folkman and colleagues (3) have identified a number of these factors, including endostatin and angiostatin. Our data suggest that angiocidin belongs to this family of molecules that suppress tumor growth. By regulating the levels of secreted, antitumorigenic molecules, such as angiocidin, we can take advantage of the therapeutic potential of these molecules in the treatment of cancer.

Angiocidin binds the matrix proteins TSP-1 and collagen, as well as the collagen receptor α2β1 (7, 8). This integrin may be a potential cell surface receptor for angiocidin in THP-1 cells as well. A 20–amino acid domain in the NH2-terminal domain of angiocidin mediates its matrix and integrin-binding activities (7, 8). This matrix-binding and integrin-binding domain of angiocidin is required for its antitumor activity. A mutant angiocidin, missing the high-affinity matrix-binding site, lacked inhibitory activity in tumor cell invasion of collagen and angiogenesis. Furthermore, a 25–amino acid peptide containing the matrix integrin binding domain of angiocidin inhibited progression of Lewis lung and human colon carcinoma in vivo (8, 9). These data support the hypothesis that the antitumor activity of angiocidin depends strongly on its capacity to interact with matrix and integrin receptors.
Here, we show that angiocidin has the capacity to potently induce the monocytic cell line THP-1 to differentiate into adherent cells resembling tissue macrophages (Fig. 1A). We have shown this adherence phenotypically, as well as by probing angiocidin-treated and untreated lysates for the phosphorylated form of the focal adhesion proteins FAK and paxillin (Fig. 1B).

We have also shown genotypically that THP-1 cells become differentiated upon treatment with angiocidin. Our microarray data shows that angiocidin induces mRNA up-regulation of various molecules known to be "differentiation state dependent" (Fig. 1C and D and Supplementary data). As shown in these figures, CD36 expression increased nearly 16-fold. CD36 is an adhesive receptor for TSP-1 and collagen, as well as a scavenger receptor expressed during the late stages of monocyte differentiation. The steady-state mRNA levels of CD36 increase during monocyte-to-macrophage differentiation (35, 36). We also observed a nearly 16-fold increase in mRNA expression levels of A2M, the synthesis of which has been shown to be differentiation state-dependent in THP-1 cells (44). These results help support the hypothesis that angiocidin is activating THP-1 cells to become macrophage-like.

Angiocidin treatment also induces cellular activation. Flow cytometric analysis revealed a nearly 40% activation of monocytes with a dose of 1 μg/mL angiocidin (Fig. 2A). Additionally, our cytokine antibody array data show that, of the 42 inflammatory mediators assayed, 18 become increasingly secreted upon...
Angiocidin treatment (Fig. 2B and C). We show that angiocidin treatment results in secretion of ng/mL quantities of MCP-1 into culture supernatants (Fig. 2D).

Angiocidin treatment also results in secretion of MMP-9 (Fig. 3B). This protease may aid tumorigenic leukocytes in their extravasation from the blood compartment into the tumor microenvironment. Angiocidin-treated THP-1 cells also presented with phagocytic activity (Fig. 3A). Phagocytosis by macrophages aids these cells in ingesting debris shed by tumor cells, which is then presented on the macrophage cell surface to immune effector cells, particularly tumoricidal CD8+ T cells. Angiocidin-activated PBMCs are able to effectively present antigen to CTLs, and angiocidin-treated THP-1 cells up-regulate proteins important to the process of antigen presentation, such as various MHC class I and class II molecules.

Angiocidin treatment results in an increase in the phosphorylation of MAPK p44/42, as well as its upstream kinase c-Raf (Fig. 4A). In addition, various players in the NF-κB pathway become phosphorylated and translocate into the nucleus (Fig. 4B and C). As shown in Fig. 5A–D, these signaling molecules are responsible for angiocidin-induced cytokine secretion and MMP-9 secretion. We also show the involvement of PI3K in angiocidin-induced MCP-1 and MMP-9 secretion and cell adhesion.

MAPK p44/42 is likely functioning upstream of NF-κB in our system, as is PI3K (Fig. 6A and B). Interestingly, inhibition of PI3K had no effect on angiocidin-induced phosphorylation of MAPK.

Figure 6. In angiocidin-treated THP-1 cells, both MAPK p44/42 and PI3K function upstream of NF-κB p65. THP-1 cells were treated with the appropriate inhibitors, and cell extracts were analyzed for the activation of signaling factors as described in Materials and Methods. A, inhibition of MAPK MEK1/2 abrogates angiocidin-induced phosphorylation of NF-κB p65. B, inhibition of PI3K abrogates angiocidin-induced phosphorylation of NF-κB p65. Each experiment was repeated thrice, and the data represent a typical experiment. C, proposed signal transduction mechanisms induced by angiocidin in THP-1 cells.
p44/42 (data not shown), indicating that two separate pathways may be at work in our system. The first pathway places MAPK p44/42 upstream of NF-κB. The second places PI3K also upstream of NF-κB; however, MAPK p44/42 is bypassed. A diagram describing these proposed angiocidin-induced signal transduction pathways is depicted in Fig. 6C.

Given its matrix-binding ability, angiocidin becomes localized to the matrix of the tumor microenvironment, where it is easily accessible and able to exert its proinflammatory effects on the immune system. Matrix-bound angiocidin, behaving in a “cytokine-like manner,” would be readily available to induce differentiation and activation of monocytes into phagocytic, tumor antigen-presenting macrophages. The specific mixture of inflammatory cytokines and chemokines inducibly released from these activated macrophages would further this immune response and allow this matrix-bound angiocidin to serve as the soil to which other activated, potentially tumoricidal leukocytes would be recruited. Activated macrophages can be tumoricidal. Macrophages can present tumor antigens to T cells or directly kill tumor cells after activation with cytokines, such as interleukin 2 (IL-2), INF-γ, and IL-12. The so-called macrophage-mediated tumor cytotoxicity involves the secretion of lytic factors into neoplastic cells, resulting in tumor cell lysis. Thus, monocyte-derived macrophages have powerful constitutive antineoplastic properties even in the absence of specific immunity. Tumor formation has been shown to depend on the level of MCP-1 secretion and macrophage infiltration. Cells producing high levels of MCP-1 invoked a massive TAM infiltrate and subsequent tumor destruction within a few days after injection into mice (4). As angiocidin has been shown to produce MCP-1 up-regulation at both the mRNA and protein levels of expression, it is conceivable that systemically injected angiocidin could inhibit tumor growth through the up-regulation of this important chemotactic cytokine in the tumor microenvironment.

We believe that angiocidin and its 25 amino acid matrix-binding peptide present a therapeutic advantage in immunomodulatory cancer therapy. Not only does angiocidin exert potent antitumor effects on a variety of cell types, but it does so without any cytotoxicity. Tumor-bearing mice injected systemically with angiocidin present with no evidence of necrosis in any of the organs examined, including liver, kidney, intestine, lung, and brain (7). Angiocidin’s matrix-binding activity suggests an additional therapeutic advantage, that of being localized to the matrix surrounding the tumor where it is readily available and accessible to exert a direct antitumorigenic effect or additionally through elicitation of an immune response.

An additional significance of our results lies in the fact that the primary cell type we are using in our investigations is the monocytic leukemia cell line THP-1. Our results therefore suggest that angiocidin may have therapeutic potential in the treatment of this hematologic cancer. In recent years, the question has been raised as to whether the malignant phenotype of leukemia can be reverted to a nonmalignant phenotype without correcting any genetic abnormalities. This reversion can be achieved by reprogramming tumor cells by epigenetic changes that induce differentiation. If leukemic tumor cells can be forced to differentiate and to cease proliferation, then their malignant potential will be controlled. Thus the epigenetic suppression of malignancy by the induction of differentiation bypasses the genetic abnormalities present in tumor cells (46–49). Differentiation therapy has aroused great interest because its mechanism is different from the chemotherapy currently in use, which is based on the theory of total cell kill and because it is expected to be less toxic than such cytotoxic chemotherapies (50, 51). We believe that our angiocidin protein may have therapeutic potential in differentiation therapy.

Disclosure of Potential Conflicts of Interest

G.P. Tuszynski: patent pending, Drexel University. The other authors disclosed no potential conflicts of interest.

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References

21. Gibbs BF, Grabbe J. Inhibitors of PI 3-kinase and


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