Lipoxin A<sub>4</sub> and Its Analogue Suppress the Tumor Growth of Transplanted H22 in Mice: The Role of Antiangiogenesis

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Abstract

Tumor angiogenesis plays an essential role in carcinogenesis, cancer progression, and metastasis. Some studies indicate that lipoxins, endogenous anti-inflammatory lipid mediators, might be involved in tumor angiogenesis; however, the governing mechanisms are still unknown. In the present study, we examined the effects of exogenous lipoxin A<sub>4</sub> (LXA<sub>4</sub>) in mouse hepatocarcinoma cell line (H22) and H22-bearing mice model. It was found that in H22 cells, LXA<sub>4</sub> inhibited the production of vascular endothelial growth factor and reduced hypoxia-inducible factor-1α level. In addition, its analogue, BML-111, blocked the expression of vascular endothelial growth factor in serum and tumor sections from H22-bearing mice. H&E staining and immunostaining with antibodies against CD34 revealed that BML-111 suppressed tumor-related angiogenesis in vivo, but LXA<sub>4</sub> could not influence the proliferation of primary cultured human umbilical vein endothelial cells. The tumor growth was also inhibited by BML-111. We also found that BML-111 enhanced the in situ apoptosis while inhibiting macrophage infiltration in tumor tissue. The results provide new evidence that LXA<sub>4</sub> suppresses the growth of transplanted H22 tumor in mice through inhibiting tumor-related angiogenesis. Mol Cancer Ther; 9(8): 2164–74. ©2010 AACR.

Introduction

Tumor angiogenesis, the proliferation of blood vessel networks that penetrate into cancerous tissue, plays an essential role in carcinogenesis, cancer progression, and metastasis (1). The proangiogenic signals within tumors have been a focus of cancer research for several years, and targeting tumor vasculature represents a promising tool for cancer therapy (2).

In 1863, Rudolf Virchow described that cancer and inflammation were connected on the basis that tumors often arose at sites of chronic inflammation and inflammatory cells were present in biopsied samples from tumor (3). Today, inflammation is believed to be a key feature of the environment within and around tumors.

Lipoxins (LX) are trihydroxytetraene-containing eicosanoids typically generated through transcellular biosynthetic pathways involving either 5- and 15-lipoxygenases or 5- and 12-lipoxygenases (4). As the first family of endogenous “braking signals” in inflammation identified in vivo (5), LXs have been extensively studied preclinically in a growing list of inflammation-related disease models (6–8). Our group has described important properties of LXs, including their ability to attenuate reactive oxygen species generation in lipopolysaccharide-stimulated macrophages, their influence on stored-operated channels in erythroleukemia cells, and their capability to attenuate acute lung injury in lipopolysaccharide-treated mice (9–11). Some evidence also indicated the inhibitory effects of LXs and aspirin-triggered 15-epi-lipoxins on angiogenesis induced by vascular endothelial growth factor (VEGF; refs. 12, 13).

Note, microbial pathogens such as Toxoplasma gondii express 15-lipoxygenase and can trigger high levels of LXs in the host serum and then cause immune escape (14, 15). Importantly, it was recently identified that tumor growth was inhibited concomitantly with suppressed angiogenesis during acute Toxoplasma gondii infection in mice (16, 17). These observations suggest that LXs might be involved in tumor angiogenesis, but the governing mechanisms are unknown.

LX is rapidly converted to biologically inactive lipid with lower potency as an anti-inflammatory agent. To circumvent such degradation, a panel of synthetic, stable lipoxin A<sub>4</sub> (LXA<sub>4</sub>) analogues has been designed (18). These longer acting analogues represent useful...
tools to evaluate the potential of pharmacologic manipulation of LXs, which is not suitable for long-term in vitro experiments. Among the various LXs analogues studied, 5(S), 6(R)-7-trihydroxyxymethyl heptanoate (BML-111) is a C-7–truncated analogue that was originally proved to be equiactive with LXA4 in the inhibition of leukotriene B4–induced polymorphonuclear chemotaxis (19). Our group previously showed that, similar to LXA4, BML-111 protected from carbon tetrachloride–induced liver injury in mice (20) and regulated the cytosolic calcium concentration (11).

Thus, in the following in vitro experiments on mouse hepatocarcinoma cell line (H22) and primary human umbilical vein endothelial cells (HUVEC), LXA4 was applied, whereas in in vivo experiments involving H22-bearing mice, BML-111 was used to mimic the effect of LXA4. Their effects on tumor-related angiogenic factors, angiogenesis, and tumor growth were determined. The results, coupled with previous findings, suggest that LXs and their analogue are potential angiogenesis inhibitors and antitumor agents.

Materials and Methods

Materials

Fetal bovine serum and RPMI 1640 were obtained from Life Technologies. Endothelial cell growth medium (ECGM) was purchased from Promocell. LXA4, from Cayman Chemical Company, was stored at −80°C until being diluted in serum-free culture medium immediately before use. BML-111, purchased from Biomol, was stored in ethanol at −20°C at a concentration of 1 mmol/L. On the day of administration, it was diluted in PBS. The daily working stocks were routinely discarded after use. Vehicle control comprised PBS (maximal ethanol concentration, <0.5%). Anti–hypoxia-inducible factor-1α (HIF-1α) antibody (for immunofluorescence assay) and Hoechst 33258 were obtained from Novus. Anti-CD34 was purchased from Bioss. Anti–HIF-1α (for Western blot) and anti-VEGF antibodies were purchased from Santa Cruz. Anti-CD45, anti-CD68, VEGF ELISA kit, and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay kit were from Boster. Penicillin and streptomycin in saline citrate buffer were from Invitrogen. CoCl2 and RNase were from Sigma Aldich. Triton X-100 and BCA protein assay kit were purchased from Pierce. Propidium iodide (PI) was the product of KPL. Cell Counting Assay Kit-8 was from Dojindo Molecular Technologies.

In vitro studies

Cell culture. H22 cells were tested by isoenzyme analysis and α-fetoprotein in the China Center Type Culture Collection from June 1 to June 21, 2009. Cells were resuscitated and obtained on September 12, 2009 from the China Center Type Culture Collection. In our laboratory, from that day to January 20, 2010, H22 cells were cultured in a humidified incubator at 37°C under 5% CO2 in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Primary HUVECs were isolated and grown as previously reported (21). Cells from passage 3 to 8 were used and cultivated in endothelial cell growth medium and 10% FCS. All cells were refed every 1 to 2 days and subcultured approximately once every 4 days. CoCl2 (200 μmol/L) was applied in vitro to mimic hypoxia.

ELISA. H22 cells were plated in 24-well plates (2 × 10^4/well), grown until 80% confluence, and then starved in serum-free RPMI 1640 overnight. After the cells were incubated with 0 to 200 nmol/L LXA4 for 24 hours, supernatants were collected and analyzed for VEGF protein levels using mouse ELISA kit.

Immunofluorescence assay. H22 cells were grown to ~80% confluence on poly-d-lysine-coated glass cover-slips in 24-well plates and serum starved overnight. After cells were treated with 100 nmol/L LXA4 for 30 minutes, 200 μmol/L CoCl2 was then added to each well (except control). After 12 hours of incubation, HIF-1α protein level and location were studied.

Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, rinsed three times with PBS, permeabilized by 0.2% Triton X-100/PBS for 2 minutes, and then with 0.5% Triton X-100/PBS for 10 minutes. Nonspecific binding was prevented by the addition of 5% bovine serum albumin/PBS for 30 minutes at 37°C. The samples were then incubated overnight at 4°C with rabbit anti–HIF-1α (1:200) in 2% bovine serum albumin/PBS. Following three PBS rinses, the cells were incubated for 2 hours with fluorescein-conjugated ImmunoPure goat anti-rabbit IgG (H+L; 1:200) in 5% bovine serum albumin/PBS at room temperature. After washing three times with PBS, cell nuclei were counterstained with Hoechst/PBS (1:1,000, v/v) for 15 minutes, followed by additional three rinses with PBS. Cells were then mounted by inversion onto glass slides dotted with Gel/Mount. Images were taken by an inverted microscope (IX70, Olympus America, Inc.) with a 1.40NA ×60 objective and FITC, rhodamine, and Cy5 filter sets. Image size was 2,560 × 1,920, and further adjustments to images were done using Adobe Photoshop.

Western blot analysis. H22 cells were cultured to ~80% confluence in six-well plates and starved overnight. Cells were then incubated with 0, 50, 100, or 200 nmol/L LXA4 30 minutes before CoCl2 (200 μmol/L) application. After a 12-hour incubation, protein was isolated with 200 μL of cold lysis buffer [150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.2), 0.2 mmol/L sodium vanadate, 1% phenylmethylsulfonyl fluoride, and 0.2% aprotinin]. Samples were kept on ice for 20 minutes and then spun at 12,000 × g for 10 minutes. Protein concentrations of the supernatants were determined using a BCA protein assay kit. The protein extracts were then subjected to 10% SDS-PAGE and subsequent nitrocellulose membrane transfer. HIF-1α protein was...
determined using primary rabbit antibody (1:1,000) and secondary horseradish peroxidase–conjugated goat anti-rabbit IgG. β-Actin served as an internal control. The bound antibody was detected by enhanced chemiluminescence (ECL) on an X-ray film.

**Cell cycle analysis.** Cells were treated with 0 to 200 nmol/L LXA₄ for 24 hours and then suspended in PBS. After fixed in cold 75% ethanol overnight at −20°C, cell pellets were washed twice with ice-cold PBS, resuspended in PBS, and stained with PI solution that contained 50 μg/mL PI and 25 μg/mL RNase. Stained cells were maintained on ice and protected from light. They were then analyzed on the FACScan flow cytometer. Data were analyzed using CellQuest Software.

**Cell proliferation analysis.** Proliferation of H22 cells and HUVEC was measured by the Cell Counting Assay Kit-8 according to the manufacturer’s protocol. Cells were plated in 96-well plates at a density of 4 × 10⁴ per well, grown until 75% confluence, and then starved in serum-free RPMI 1640 overnight. After cells were incubated with 0 to 200 nmol/L LXA₄ for 24 hours, 10 μL Cell Counting Kit-8 solution was added to each well, the cells were incubated for another 2 hours, and the absorbance at 450 nm was measured by using a microplate reader. The amount of the formazan dye, generated by the activities of dehydrogenases in cells, was directly proportional to the number of living cells. RPMI 1640 was used as black control group. All experiments were done three times independently.

**In vivo studies**

**Animals.** All studies involving mice were approved by Animal Care and Use Committee of Huazhong University of Science and Technology. Five- to 6-week-old, 18 to 22 g male Imprinting Control Region mice were purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. The animals were maintained under pathogen-free conditions, in a controlled environment of temperature at 20°C to 25°C and 12-hour cycles of light and dark. Mice were fed a standard laboratory diet and water ad libitum for at least 1 week before study.

**Establishment of H22-bearing mice model.** According to the method published (22), before implantation, H22 cells were injected and grown i.p. for 7 days in mice. After the mice were killed by cervical dislocation, H22 cells in ascites were collected in the super clean bench, washed with PBS, and resuspended in PBS. Cell viability was determined by trypsin blue exclusion. Only preparations with >90% viability of the cells were used. Then, to establish a solid tumor model, each Imprinting Control Region mouse was inoculated with 0.2 mL H22 tumor cell suspension (1 × 10⁶/mL PBS) by s.c. injection to the right flank.

**Groups and observations.** Twenty-four mice were weighed and randomly divided into a negative control group (s.c. injected with 0.2 mL PBS only), a positive control group (s.c. injected with 0.2 mL H22 cell suspension in PBS), and BML-111 group (s.c. injected with 0.2 mL H22 cell suspension in PBS followed by BML-111). BML-111 (0.1 mL PBS/mouse, final dosage of 1 mg/kg) was i.p. injected 5 minutes before and 4 hours after H22 cell inoculation, then every 12 hours for 2 consecutive days, then daily in an additional 3 days and every other day for the last 10 days. The general conditions of mice including activity, fur growth, and weight were observed daily. The size of the tumors was determined by caliper, measuring length (L) and width (W) of tumors every other day. Tumor volumes (V) were calculated by the formula: $V = L \times W^2 \times 0.5$. The tumor growth inhibition rate was calculated by the formula: inhibition (%) = (1 − V-volume of BML-111 group/volume of positive control group) × 100%.

**ELISA**

Blood was drawn from the orbital venous plexus on day 21 after inoculation as previously described (23). Samples were incubated in an upright position at room temperature for 30 to 45 minutes to allow clotting. Serum was separated by centrifuge at 2,000 g for 15 minutes at 4°C. VEGF protein levels in serum were measured through ELISA kits according to the manufacturer’s instruction.

**Morphologic analysis.** On day 21 postinoculation, mice were weighed and sacrificed. The transplanted tumor tissues were surgically excised, weighed, and then fixed in 10% neutral-buffered formalin, and frozen at −80°C until processing for histochemical and microscopic examination.

For histologic assessment, specimens in 4-μm frozen sections were stained with H&E for conventional morphologic evaluation under light microscope (Nikon eclipse 90i). For immunohistologic assay, VEGF, CD34, CD45, or CD68 expression was detected by anti-VEGF (1:100), anti-CD34 (1:350), anti-CD45 (1:100), or anti-CD68 (1:100) primary antibodies, respectively. Secondary antibodies were all conjugated to horseradish peroxidase.

CD34 was used as marker of endothelial cells. All brown endothelial cells, or endothelial cell clusters with dyed cytoplasm that were separated from adjacent tumor cells, microvessels, or connective tissues, were counted as independent microvessels. Vessels with thick muscle layers or with lumen diameters larger than the width of eight RBC were excluded (24). Microvessel density was determined by the mean number of CD34-positive vessels counted in 10 random fields.

CD68 was chosen as the specific marker of macrophages (25). CD45, also called leukocyte common antigen, was chosen as the specific marker of leukocyte (26).

**In situ apoptosis detection.** Slices were examined by TUNEL method to show apoptosis in tissue sections. The TUNEL assay kit was applied according to the instructions in the user manual.

**Statistical analysis.** All statistical analyses were done using the SPSS software. Numerical results were expressed as mean ± SEM of multiple experiments. ANOVA was
used to determine the level of difference between groups. Pairs of groups were compared by unpaired two-tailed Student’s t test. Significance was determined with P values of <0.05.

Results

Inhibitory effect of LXA₄ on VEGF production in H22 cells

Because VEGF is a well-known potent inducer of angiogenesis (12, 13), in this study, after confirming the expression of LXA₄ receptor in H22 cells by Western blotting (seen in Supplementary Fig. S1), we first tested the effect of exogenous LXA₄ on VEGF secretion in H22 cells. After cells were incubated with 0 to 200 nmol/L of LXA₄ for 24 hours, VEGF protein levels in the supernatant were measured by ELISA. As shown in Fig. 1A, VEGF secretion was inhibited by LXA₄ in a dose-dependent manner. It decreased from 120.80 ± 17.00 pg/mL in control group to 56.84 ± 5.38 pg/mL in 100 nmol/L LXA₄-treated group and 34.92 ± 3.58 pg/mL in 200 nmol/L LXA₄-treated group (P < 0.05).

Inhibitory effect of BML-111 on VEGF production in vivo

We further evaluated the influence of BML-111 on serum VEGF concentrations in H22-bearing mouse through ELISA. As seen in Fig. 1B, serum VEGF was nearly undetectable in control mice, but reached 2,218.9 ± 53.7 pg/mL on day 21 after H22 inoculation (P < 0.05). Importantly,
VEGF concentrations substantially decreased to 1,364.5 ± 62.6 pg/mL when mice were coadministrated with BML-111 (\(P < 0.05\)).

VEGF expression in tumor tissue was also studied by immunohistochemical staining. As seen in Fig. 1C, brown cytoplasmic staining mainly appeared in tumor cells. Positive control group had obvious stronger brown cytoplasmic staining than that in BML-111 group.

**Inhibitory effect of LXA4 on HIF-1\(\alpha\) in H22 cells**

In most tumors, hypoxia promotes angiogenesis, metastasis, and resistance to therapy (27). As a crucial cellular

Figure 2. LXA4 and its analogue BML-111 reduced HIF-1\(\alpha\) protein level in H22 cells or H22-bearing mice. A, LXA4 reduced HIF-1\(\alpha\) protein level in cytoplasm and nuclear accumulation. H22 cells were immunostained with anti-HIF-1\(\alpha\) (green fluorescence) and counterstained with Hoechst (blue fluorescence) to visualize the nuclei. Cells exposed to 200 \(\mu\)mol/L CoCl2 for 12 h were applied as positive control of HIF-1\(\alpha\) protein stabilization. LXA4 (100 nmol/L) was administrated simultaneously in the CoCl2+LXA4 group. Images were captured using the Olympus IX 70 Fluorescent microscope and adjusted using the Adobe Photoshop 7.0 software (\(\times 200, \times 400\)). B, Western blot analysis of HIF-1\(\alpha\) protein content in whole-cell lysates. Cells were cultured under indicated concentrations for 12 h. \(\beta\)-Actin served as a loading control. The representative photomicrographs are from one of three separate experiments that yielded similar results.
response to hypoxia, enhanced stabilization of HIF-1α promotes transcriptional activation of many target genes, including VEGF. Thus, we next investigated the effect of LXA₄ on HIF-1α protein level and location in H22 cells, which were first exposed to 200 μmol/L CoCl₂ for 12 hours to mimic hypoxic condition. It was confirmed that, similar to data from other laboratories (28), HIF-1α protein level and accumulation inside nucleus were both strongly enhanced (Fig. 2). When cotreated with 100 to 400 nmol/L LXA₄, these were markedly suppressed.

Suppression of tumor-related angiogenesis by BML-111

Because both VEGF and HIF-1α are important angiogenic factors, we next evaluated the influence of BML-111 on tumor-induced neovascularization. Tumor tissue sections were collected at the time of autopsy on day 21 after H22 inoculation. Figure 3A showed H&E staining results. Immunostaining with antibodies against CD34, special marker for endothelial cells, also visualized the vascular reticulum of blood vessels (Fig. 3B). Figure 3C presented the mean number of CD34-positive vessels from 10 randomly selected high-power fields, which was substantially decreased in mice treated with BML-111 (P < 0.05).

We also chose vascular endothelial cells as our target to explore whether the antiangiogenic effect of LXA₄ is directly on the proliferation of endothelial cells or indirectly through the regulation of VEGF or HIF-1α. Then, cell counting assay and flow cytometric assay were carried on primary HUVEC. It was shown as in Fig. 4A and B that the cells in each phase of cell cycle did not change obviously after different concentration of LXA₄ administration for 24 hours. Cell counting assay also showed no difference between groups treated with different concentrations of LXA₄ for 24 or 72 hours (P > 0.05). Data indicated that LXA₄ applied in our experiment had no direct influence on endothelial cell proliferation.
Suppression of primary tumor growth by BML-111

Given the observed antiangiogenic effect of BML-111 in H22-bearing mice, we next determined whether BML-111 could inhibit tumor growth *in vivo*. First, mice were weighed on day 0 and day 21 after H22 inoculation. As seen in Fig. 5A, body weight of all the mice increased over 21 days. No obvious differences were observed between BML-111 group and negative control group, indicating that the dose of BML-111 applied in our *in vivo* study was not toxic to mice. On the contrary, mean body weight in positive control group was relatively lower ($P < 0.01$). Next, the volume of peripheral tumors was measured every other day. At all times, tumors of BML-111 group were significantly smaller compared with that of positive group (Fig. 5C). On day 21 postinoculation, its inhibition rate was as high as 80.78%.

At the time of autopsy (day 21 after inoculation), tumors were dissected and weighed. At surgery, tumors in the positive control group appeared less circumscribed, more extensively invading adjacent tissue, and more difficult to be dissected (Fig. 5B). The tumor weight and its ratio to body weight of BML-111 group were <50% of that in positive control ($P < 0.01$ (Fig. 5D)).

The direct effect of LXA₄ on H22 proliferation was also studied by cell counting assay combined with flow cytometric assay. It was shown as in Fig. 4A and B that the cells in each phase of cell cycle did not change obviously after different concentration of LXA₄ administration for 24 hours. Cell counting assay also showed no difference between groups treated with different concentrations of LXA₄ for 24 or 72 hours ($P > 0.05$). Data indicated that LXA₄ applied in our experiment had no direct influence on H22 cell proliferation.
Effect of BML-111 on in situ apoptosis of tumor tissue

To further explore the mechanism of H22-bearing tumor growth in mice, TUNEL assay on tumor section at day 21 after inoculation was done to detect the morphologic changes. As shown in Fig. 6A, apoptosis was more obvious after BML-111 treatment.

Effect of BML-111 on inflammatory cell infiltration in tumor tissue

Because LX is an important factor to trigger inflammatory resolution, we next investigated the influence of BML-111 on the inflammatory cell infiltration in tumor tissue. CD68 and CD45 were chosen as the markers for macrophages or leukocyte, respectively. Results on day 21 after inoculation were shown in Fig. 6B and C. First, it could be seen that infiltrations of macrophages were more serious than leukocytes in both groups. In addition, BML-111 could obviously inhibit macrophage infiltration, whereas its effect on leukocytes was not significant.

Discussion

A link between inflammation and liver cancer has been suspected for many years. However, most previous work focused on the imbalance between cell apoptosis and proliferation (29). For example, nonsteroidal anti-inflammatory drugs reduce the incidence and mortality of certain malignancies by targeting the cyclooxygenase enzymes, of which the overexpression increases hepatocellular carcinoma cell proliferation and survival through the Akt pathway, whereas inhibition leads to marked induction of apoptosis (30, 31).

Antiangiogenic approaches have become important therapeutic anticancer strategies (2). Important clues indicate that LXs, the first family of endogenous “braking signals” in inflammation identified in vivo (5), might interfere with tumor angiogenesis (16, 17). HCC is a highly vascular tumor, and angiogenesis is believed to play a crucial role in its development (32). At the same time, the transcellular route of LX biosynthesis has been recognized in the liver (33). To further explore the effect and mechanism of LXs on HCC growth, in the present investigation, H22 cell line and H22-bearing mice model were applied. To avoid the challenges of using LXA4 in vivo, owing to its short lifetime (18), BML-111, a C-7–truncated analogue of LXA4 (19), was applied in mice experiments.

VEGF, identified as a primary and potent angiogenic factor to promote tumor growth and metastasis, was
chosen as our initial research target. Our results showed that VEGF secretion by H22 cells was significantly inhibited by LXA4 in a dose-dependent manner. At the same time, in H22-bearing mice, both at serum and tumor tissue, BML-111 group showed lower VEGF levels than positive control group. It was shown in vivo that VEGF was mainly expressed in tumor cells, which was similar with results from other groups (34, 35). This suggested that LXA4 might diminish the ability of H22 cells to trigger new vessels formation.

Hypoxia is well accepted as one of the main characteristics of the tumor microenvironment and has been widely implicated in the progression of tumor angiogenesis (36, 37). HIF-1α, a heterodimeric transcription factor, serves as a master switch to regulate cell adaptation to hypoxic stress by modulating several genes involved in vascular growth and cellular metabolism, including VEGF (37). Therefore, we investigated the influence of LXA4 on HIF-1α protein content in H22 cells. Like in some other tumor cells (38, 39), it was also found that HIF-1α was undetectable in control H22 cells but strongly enhanced in cytoplasm and accumulated in nuclei under CoCl2-mimiced hypoxic condition. Unlike other antiangiogenic agents that only blocked VEGF but not HIF-1α (40), we found that CoCl2 stabilization of HIF-1α was markedly countered by LXA4.

This antiangiogenic effect was finally confirmed in vivo. Tumor tissues in mice of BML-111 group exhibited strikingly less CD34-positive vessels, indicating that tumor-related angiogenesis was suppressed by BML-111. Interestingly, LXA4 showed no direct influence on HUVEC proliferation. This discrepancy between in vivo and in vitro studies might be caused by enhanced VEGF level in H22-bearing mice, which would in turn evoke endothelial cell proliferation. A lot of articles confirmed that LXA4 could inhibit VEGF-stimulated proliferation of HUVEC (12, 13, 41-43).

In tumor tissue, one would expect a feedback cycle between VEGF and HIF-1α whereby decreased VEGF would lead to reduced angiogenesis, the later subsequently increasing hypoxia in the tumor, activating HIF-1α, and consequently increasing the expression of angiogenic genes such as VEGF (37). This negative feedback may lessen, at least partly, the effectiveness of antiangiogenic molecules targeting VEGF. But here, we proved that LXA4 blocked both HIF-1α and VEGF. This could avoid the flaw of traditional antiangiogenic agents.

As we know, LXA4 is first recognized as important anti-inflammatory and proresolving lipid-based autacoids in inflammation (44). To further explore whether its effect on angiogenesis is dependent on inflammation resolution, we next detected the infiltration of two main inflammatory cells, leukocyte and macrophage, in tumor tissue. We found that on day 21 postinoculation, more macrophages appeared in tumor tissue than leukocytes, and BML-111 mainly interfered with the infiltration of macrophages. This might be because macrophages are often prominent. For example, they compromise up to 80% of the cell mass in breast carcinoma (45), and they can live longer than leukocytes.

Like other solid tumors, HCC also includes a diverse array of host cells that are either recruited into the tumor microenvironment or reside inside tumor tissue, resulting in interactions between malignant cells, epithelial cells, inflammatory cells, and even other stoma cells (29). It is now becoming clear that the inflammatory cells, especially tumor-associated macrophages, play an indispensable role in cancer progression by producing a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators (45). Endogenous VEGF could also be generated by nontumor tissues, such as macrophages and even endothelial cells (46, 47). Thus, in our study, inflammatory cell infiltration blocking by BML-111 might contribute to its antiangiogenic effect in H22-bearing mice.

Finally, all of the tumor growth indices, including tumor size, weight, and morphologic features, revealed that the primary tumor growths were inhibited by BML-111. It was encouraging to observe that on day 21

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**Figure 6.** Effect of BML-111 on tumor cell apoptosis and inflammatory cell infiltration of tumor tissue. A, TUNEL assay was conducted on tumor frozen sections for neovascularization analyses at the time of autopsy on day 21 postinoculation. Yellow arrow, apoptotic cells. Representative images of multiple microscopic fields observed in six mice per group (×200). B, tumor sections were resected at the time of autopsy and stained with anti-CD68 on day 21 postinoculation by H22. White arrows, CD68+ macrophages. Representative images of multiple microscopic fields observed in six mice per group (×200). C, tumor sections were resected at the time of autopsy and stained with anti-CD45 on day 21 postinoculation by H22. White arrows, CD45+ leukocytes. Representative images of multiple microscopic fields observed in six mice per group (×200).
postinoculation, the tumor growth inhibition rate was as high as 80.78% by tumor size. In addition, in situ, apoptosis was elevated by BML-111. On the contrary, as studied by flow cytometry and CCK-8 kit, LXA₄ did not exert any direct antiproliferative effect on H22 cells. Although Claria et al. (48) found that LX could inhibit alveolar type II epithelial cell proliferation in vitro, it might be because of the organism—as well as cell—specificity. The results of the current investigation showed that LXA₄, an important proresolution lipid, can suppress HCC tumor growth by inhibiting tumor-related angiogenesis.

Compared with other conventional antitumor therapies, antiangiogenic treatment has clear merits, such as higher genomic stability of vascular endothelial cells than tumor cells, greater access, and lack of restriction to only specific tumor types (49). However, to date, anti-VEGF tumor cells, greater access, and lack of restriction to only higher genomic stability of vascular endothelial cells than tumor cells, greater access, and lack of restriction to only specific tumor types (49). However, to date, anti-VEGF therapy alone has shown little antitumor or survival benefit in humans (50). LXA₄ itself is still not suitable for clinical use because of its short life, whereas BML-111 contains just part but not the complete molecule of LXA₄. This structural difference might cause loss of some agonist actions of LXA₄ when BML-111 was applied in vivo. At the same time, without blocking the LXA₄ pathway by either receptor antagonist or lipoxigenase gene knockout, results in this study cannot reveal the full story of endogenous LXs in HCC progress. Although there are still substantial gaps to address, our findings may pave the way for development of new antiangiogenic therapeutic strategies that target both tumor-related inflammation and angiogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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