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Inhibition of Angiogenesis by Humulone, a Bitter Acid from Beer Hop

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On the basis of our previous finding that humulone, a bitter acid from beer hop extract, was a potent inhibitor of bone resorption and inhibited the catalytic activity of cyclooxygenase-2 (COX-2) and more potently the transcription of the COX-2 gene, we examined the effect of humulone on angiogenesis, using chick embryo chorioallantoic membranes (CAMs) and vascular endothelial and tumor cells. Humulone significantly prevented *in vivo* angiogenesis in CAM in a dose-dependent manner with an ED_{50} of 1.5 μg/CAM. Humulone also inhibited *in vitro* tube formation of vascular endothelial cells. Moreover, it suppressed the proliferation of endothelial cells and the production of vascular endothelial growth factor (VEGF), an angiogenic growth factor, in endothelial and tumor cells. Thus, humulone is a potent angiogenic inhibitor, and may be a novel powerful tool for the therapy of various angiogenic diseases involving solid tumor growth and metastasis. © 2001 Academic Press

Key Words: **angiogenesis; humulone; bitter acid; beer hop; cyclooxygenase; COX-2; VEGF; endothelial cells; tube formation; cancer.**

Angiogenesis, the formation of new capillary blood vessels for supplying oxygen and nutrients, plays a key role in the development of malignant tumors (1). The inhibition of angiogenesis prevents tumor growth and metastasis (2, 3). Recently, prostagrandin-forming cyclooxygenases (COXes) were postulated to be involved in tumor growth, metastasis, and angiogenesis. It is well known that two COX isozymes (COX-1 and -2) are involved in PG synthesis. COX-2 is an inducible enzyme with various pathological roles (4). It was reported that the microvessel density within tumors grown in animals correlated with the intensity of COX-2 expression as was observed in different types of human cancers and the COX-2 inhibitor exhibited potent antiangiogenic activity (5). On the other hand, COX-1 as well as COX-2 was reported to play an important role in vascular endothelial cells during the modulation of angiogenesis (6).

In the screening test for the inhibitor of bone resorption, we found humulone (Fig. 1) that was isolated from beer hop (*Humulus lupulus* L.) extract, to be a strong inhibitor of bone resorption. The value of IC_{50} (50% inhibition of bone resorption) was 5.9 nM in the pit formation assay (7). In order to study the mechanism of action of humulone, we noted the similarity in structures between humulone and prostaglandin (PG) molecules. The two molecules have a 5- or 6-membered ring structure, unsaturated side chains, and carbonyl and hydroxyl groups. PGE_2 is a well known bone resorbing-compound and produced in murine osteoblastic cells (8). Therefore, we studied the interaction between humulone and PG biosynthesis. Previously, it was reported that tumor necrosis factor (TNF) α induced the expression of the COX-2 enzyme markedly in the murine osteoblastic cell line, MC3T3-E1, releasing PGE_2 into the culture medium (9). Recently, we found that humulone potently blocked the transcription of COX-2 gene of MC3T3-EI cells but inhibited the catalytic activities of cyclooxygenase-1 and -2 much less potently (10).

In view of the involvement of COX-2 in angiogenesis, we investigated the possible effect of humulone on angiogenesis, using chick embryo chorioallantoic mem-

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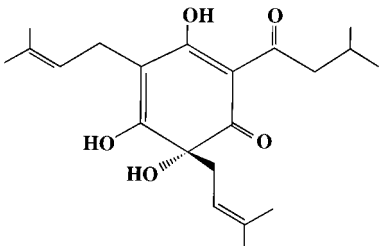

FIG. 1. Structure of humulone.

branes (CAMs), vascular endothelial cells and tumor cells.

MATERIALS AND METHODS

Reagents and cells. Humulone was isolated and purified from hop paste as described previously (7). NS-398 was obtained from Biomol Research Lab., Inc. The ELISA kit for mouse VEGF was purchased from R&D Systems (Minneapolis, MN). The fertilized eggs were obtained from Omiya Kakin (Omiya, Japan). Murine endothelial (KOP2.16) cells were a kind gift from Dr. N. Toyama-Sorimachi (Rinshoken) and maintained in PRMI 1640 containing 20% fetal calf serum (FCS), 2-mercaptoethanol, MEM nonessential amino acids, and MEM sodium pyruvate. Rat lung endothelial (RLE) cells were a gift from Dr. T. Aoyagi (Showa Pharmaceutical University) and maintained in MEM containing 10% FCS. Mouse colon carcinoma (Co26) cells were a gift from Dr. M. Iigo (National Cancer Center Research Institute) and maintained in RPMI 1640 medium supplemented with 10% FCS.

Chick embryo chorioallantoic membrane assay. In vivo angiogenic activity was assayed using CAMs as described previously (11). Humulone was mixed in 1% methyl cellulose/0.9% NaCl. On day 4 of the fertilized chick embryo in a shell, 10 μl of humulone preparation was applied to the silicon ring that was placed on the surface of the CAM. After 48-h exposure at 37°C, the fat emulsion was injected into CAM to visualize the blood vessels. Angiogenic inhibition was indicated by the formation of an avascular zone around the ring of 3 mm diameter. The results were expressed as the percentage of embryos showing inhibition.

Tube formation assay of endothelial cells. RLE cells (5×10^4 cells/ml) were suspended with humulone in serum-free MEM medium at room temperature for 30 min. Separately, the wells of 24-well tissue culture plates were coated with 150 μl/well of Matrigel and 150 μl/well of MEM, which were incubated at 37°C for 24 h for solidification. The cell suspension was then plated onto the surface of the Matrigel and incubated at 37°C. After 8- and 16-h incubation, respectively, the cells were photographed and analyzed for the extent of tube formation.

Proliferation assay of endothelial cells. KOP2.16 cells (3×10^4 cells per well) were seeded onto 24-well tissue culture plates and incubated for 24 h. After the samples were dissolved in DMSO, the medium in each well was replaced with fresh medium containing 1% FCS, and the samples in triplicate were added to each well. After 30 min of incubation, bFGF was added to a final concentration of 10 ng/ml. The cells were cultured for 48 h, trypsinized, and counted with a Coulter counter.

Analysis of VEGF production. The expression of VEGF in KOP2.16 cells and Co26 cells was evaluated using ELISA. KOP2.16 cells or Co26 cells (3×10^5 cells per well) were cultured in 24-well tissue culture plates with medium containing 10% FCS. After 24-h incubation, the medium was changed to serum-free one containing 10 ng/ml of bFGF for KOP2.16 cells or to a serum-free one for Co26 cells, and the samples in triplicate were added to each well. The 24-h cultured medium was collected for ELISA.

RESULTS

Inhibition of angiogenesis in chick embryo CAM. The *in vivo* antiangiogenic activity of humulone was tested with CAM in Fig. 2. Humulone strongly inhibited angiogenesis, clearly producing an avascular zone in CAM (Fig. 2B). In contrast, no avascular zones were observed in any of the control embryos treated with 0.9% NaCl alone (Fig. 2A). Figure 2C shows the dose-dependent inhibition of *in vivo* angiogenesis in CAM by humulone and a specific COX-2 inhibitor, NS-398. Humulone at concentrations of 0.1–100 μg/CAM substantially inhibited new blood vessel growth of chick embryos. The ED_{50} of humulone was 1.5 μg/CAM. NS-398 inhibited the angiogenesis in CAM in a dose-dependent manner with an ED_{50} of 65 μg/CAM. The antiangiogenic activity of humulone was 40 times more potent than that of NS-398 in CAM assay.

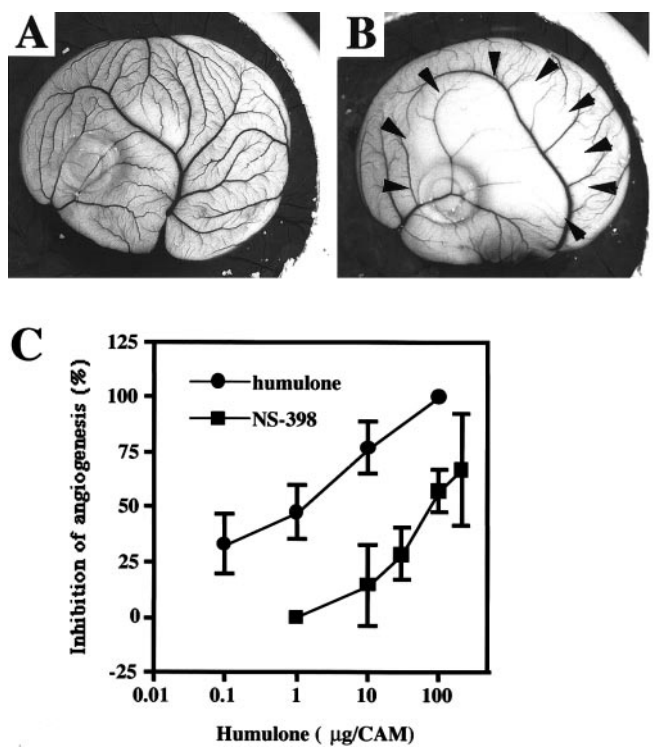

FIG. 2. Inhibitory effect of humulone on *in vivo* angiogenesis in CAM. No (A) or 10 μg/ml (B) humulone was added to the CAM surfaces of 4-day-old fertilized eggs, and then the eggs were incubated for 46–48 h as described under Materials and Methods. Humulone produced an avascular zone (surrounded by arrows), indicating its antiangiogenic activity. (C) Dose-dependent inhibition of *in vivo* angiogenesis in CAM by humulone and NS-398. The antiangiogenic activity was assessed as described under Materials and Methods. Values are the means of five experiments. Humulone potently inhibited angiogenesis in CAM in a dose-dependent manner.

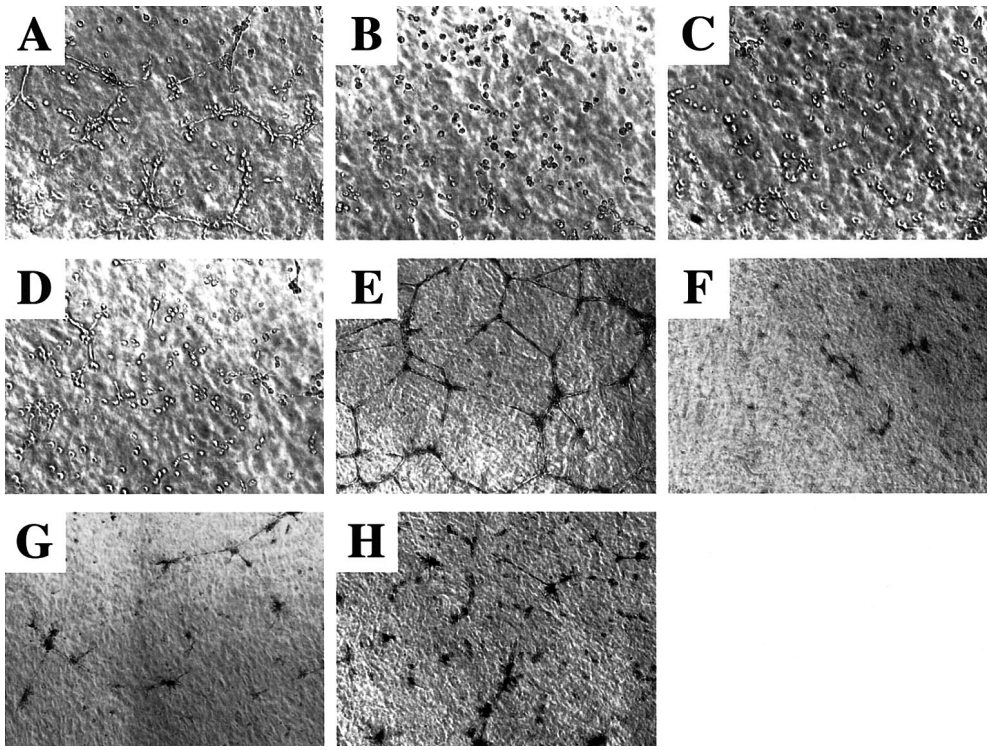

FIG. 3. Effect of humulone on *in vitro* vascular endothelial cell tube formation. (A) Serum-starved RLE cells were plated on Matrigel and incubated for 6 h. Cells migrated and started to form capillary-like structures, reflecting *in vitro* angiogenesis. (B–D) After 6 h of incubation on Matrigel, serum-starved RLE cells failed to migrate and form capillary-like structures in the presence of 30 μM (B), 10 μM (C), or 3 μM (D). (E) Serum-starved RLE cells were plated on Matrigel and incubated for 16 h. All cells formed capillary-like structures. (F–H) Serum-starved RLE cells were plated on Matrigel and incubated for 16 h in the presence of humulone. 30 μM (F), 10 μM (G), and 3 μM (H) of humulone inhibited the ability of RLE cells to form capillary-like structures.

Inhibition of vascular endothelial cell tube formation by humulone. Tube formation by vascular endothelial cells was investigated in the presence of humulone. RLE cells were preincubated with humulone (0–30 μM) and put on Matrigel. Figure 3 shows the inhibition of tube formation of RLE cells by humulone. After 6 h of incubation on Matrigel, nontreated RLE cells migrated and then started to form capillary-like structures (Fig. 3A). In contrast, all RLE cells treated with humulone exhibited delayed migration and tube formation (Figs. 3B–3D). After 16 h of incubation, the tube formation of nontreated RLE cells was completed (Fig. 3E). However, the tube formation was significantly inhibited by humulone, at concentrations of 3 to 30 μM (Figs. 3F–3H).

Inhibition of endothelial cell proliferation by humulone. The effect of humulone on the proliferation of endothelial cells was investigated using KOP2.16 cells stimulated by 10 ng/ml bFGF (Fig. 4). Humulone at 10 μM inhibited endothelial cell proliferation by 80%. Humulone had a strong inhibitory effect on the growth of endothelial cells. In contrast, NS-398 showed a moderate inhibitory effect on cell growth in the concentration range of 3 to 100 μM.

Suppression of VEGF expression in tumor cells and endothelial cells. Tumor cells are known to produce a variety of factors, such as VEGF, bFGF, and PDGF-B which contribute to angiogenesis. To evaluate if humulone suppresses the production of VEGF by KOP2.16 endothelial cells or Co26 colon cancer cells which constitutively express COX-2 protein (12) we measured VEGF in the culture medium of these cells that were

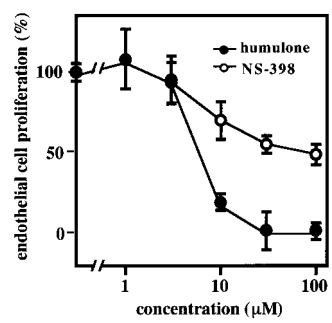

FIG. 4. Effect of humulone on proliferation of endothelial cells. Kop2.16 cells were treated for 48 h with humulone or NS-398 at various concentrations indicated. Data are means ± SD from five separate wells. Humulone potently inhibited the proliferation of endothelial cells.

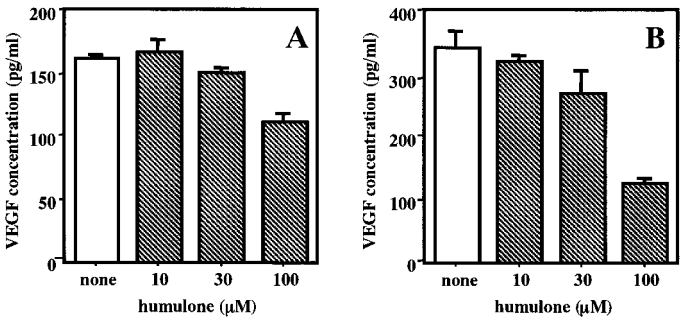

FIG. 5. Effect of humulone on production of VEGF by endothelial cells and tumor cells. Kop2.16 cells (A) and Co26 cells (B) were treated for 24 h with humulone at various concentrations indicated. VEGF in conditioned medium was assayed by ELISA. Humulone suppressed the production of VEGF by endothelial cells and tumor cells.

treated or not treated with humulone (Fig. 5). Humulone at 100 μM substantially inhibited the production of VEGF by KOP2.16 cells (Fig. 5A) and Co26 cells (Fig. 5B). The inhibition was more significant in tumor cells than in endothelial cells at 10–100 μM of humulone.

DISCUSSION

In this study, humulone was demonstrated to be a novel angiogenesis inhibitor that was sufficiently potent to suppress angiogenesis *in vivo* (Fig. 2). *In vitro,* this compound inhibited vascular endothelial cell tube formation (Fig. 3), endothelial cell proliferation (Fig. 4), and VEGF production in endothelial cells and tumor cells (Fig. 5).

Previously, we reported that humulone inhibited COX-2 enzyme activity more potently than COX-1 enzyme activity, with IC_{50} values of 1.6 and 45 μM, respectively. Furthermore, humulone suppressed TNFα-induced COX-2 gene transcription with an IC_{50} of as low as approximately 30 nM. Its signal transduction may not involve a glucocorticoid receptor but may be mediated by NFκB and NF-IL6. Thus, humulone inhibited COX enzyme activity and more potently the transcription of the COX-2 gene (10).

Nonsteroidal antiinflammatory drugs (NSAIDs) as COX inhibitors are known to prevent gastrointestinal tumor and polyp, and reduce mortality from cancers of the stomach (13) and the colon (14). One of the mechanisms of the anticancer activity by COX is the suppression of angiogenesis. Many reports have described the direct relationship between COX and angiogenesis, and the suppression of angiogenesis by COX inhibitor has been demonstrated (15–19). However, the question as to which of the two isozymes contributes to angiogenesis, remains to be answered.

Recently, the role of COX in tumor-induced angiogenesis was estimated, using an endothelial cell/colon carcinoma coculture model system incubated with NS-398 and aspirin, and it was demonstrated that both COX isozymes regulated colon-carcinoma-induced angiogenesis caused by two mechanisms: COX-2 could modulate the production of angiogenic factors by colon cancer cells, while COX-1 regulated angiogenesis in endothelial cells (6). A study to evaluate the role of COX, using a nonselective inhibitor and a selective COX-2 inhibitor, demonstrated that both COX isozymes were important for the regulation of angiogenesis (20). However, COX-2 was clearly detected not only in most of colon, lung, breast, and prostate tumors in humans, but also in the angiogenic vasculature in their tumors. It was also reported that cornea blood vessel formation was suppressed by a selective COX-2 inhibitor but not by a COX-1 inhibitor in a rat model of angiogenesis, suggesting that COX-2 plays a critical role in tumor angiogenesis (5). In our study, humulone inhibited angiogenesis in both *in vivo* and *in vitro* assays more potently than the specific COX-2 inhibitor, NS-398. The IC_{50} of NS-398 against COX-2 catalytic activity was reported to be 3.8 μM (21). This value is almost the same as the IC_{50} of humulone against COX-2 catalytic activity. On the other hand, humulone exhibited the potent inhibition of the transcription of COX-2 gene with an IC_{50} of 30 nM (10). Therefore, the potent antiangiogenic activity of humulone may depend on the inhibition of COX-2 on catalytic activity and gene transcription. Furthermore, humulone, was reported to have radical scavenging activity and lipid peroxidation inhibitory activity (22) which induce angiogenic inhibition (23, 24). The antiangiogenesis of humulone may also involve these biological activities. In view of the importance of COX-2 in angiogenesis, humulone may be useful as an angiogenic inhibitor, because it can significantly inhibit both enzyme activity and the transcription of the COX-2 gene.

However, compounds that suppress pathological angiogenesis are almost certain to suppress the physiological angiogenesis required for wound and ulcer healing. NSAIDs produce gastroduodenal ulcers in about 25% of users and delay ulcer healing, presumably by blocking PG synthesis by COX-1 and -2. These harmful side effects by NASIDs on the gastric mucosa were presumed to result from the inhibition of gastric COX-1. Thus, a selective COX-2 inhibitor was expected to serve as an effective anti-inflammatory and analgesic agents with less harmful side effects (25). However, it was reported that both indomethacin and a selective COX-2 inhibitor delayed healing of experimental gastric ulcers because of their inhibition of angiogenesis, and that COX-2 as well as COX-1 were required for unimpeded healing of the gastric mucosa as well as for gastric mucosal defense (20). Therefore, this finding indicates the advantage of humulone as an antiangiogenic and anti-inflammatory agent, since humulone has the ability to block COX-1 activity to a lesser extent.

In conclusion, humulone, a bitter acid from hops, potently inhibits *in vivo* and *in vitro* angiogenesis presumably through the regulation of COX, and may be applied clinically as an antiangiogenic agent in the treatment of cancer, rheumatoid arthritis, and diabetic retinopathy.

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