Ethanol Stimulates Tumor Progression and Expression of Vascular Endothelial Growth Factor in Chick Embryos

Jian-Wei Gu, M.D.
Amelia Purser Bailey, B.S.
Amanda Sartin, B.S.
Ian Makey, B.S.
Ann L. Brady, M.D.

Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi.

Supported by an American Cancer Society Institutional Research Grant (IRG-98-275-01); a National Alcohol Abuse and Alcoholism Institute grant (NIH AA013821-01A2); and a National Heart, Lung, and Blood Institute grant (NIH HL-51971).

Address for reprints: Jian-Wei Gu, M.D., Department of Physiology and Biophysics, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216; Fax: (601) 984-1817; E-mail: jgu@physiology.umsmed.edu

Received July 27, 2004; revision received September 21, 2004; accepted September 28, 2004.

BACKGROUND. The mechanisms by which alcohol consumption causes cancer have not been established due to a lack of experimental studies.

METHODS. A chick embryo chorioallantoic membrane (CAM) model that bore human fibrosarcoma (HT1080) was used to determine whether the administration of physiologically relevant doses of ethanol could stimulate tumor growth, angiogenesis, metastasis, and vascular endothelial growth factor (VEGF) expression in tumors. HT1080 cells were inoculated onto the "upper CAM" on Day 8, saline or ethanol was administrated at a dose of 0.25g/kg per day on the CAM, and the tumors were harvested on Day 17. VEGF mRNA and protein were determined by Northern blot analysis and enzyme-linked immunosorbent assay. Intratumoral vascular volume density (IVVD) was determined by point counting on periodic acid–Schiff-stained sections. Intravasation of HT1080 cells was determined using human-Alu polymerase chain reaction analysis. The effects of ethanol on VEGF expression and cell proliferation were examined in cultured HT1080 cells.

RESULTS. Ethanol treatment for 9 days caused a 2.2-fold increase in tumor volume (867 ± 138 mm^3 vs. 402 ± 28 mm^3), a 2.1-fold increase in IVVD (0.021 ± 0.004 mm^3/mm^3 vs. 0.010 mm^3/mm^3 $\pm$ 0.002 mm^3/mm^3), and a significant increase in VEGF mRNA or protein expression in tumors compared with a group of control embryos ($n = 6$ embryos; $P < 0.01$). Ethanol treatment caused an increase $>$ 8-fold in the intravasated HT1080 cells in the CAM group compared with the control group ($n = 6$ embryos; $P < 0.01$). Physiologically relevant levels of ethanol (10 mM and 20 mM) caused a dose-related increase in VEGF mRNA and protein expression in cultured HT1080 cells. The ethanol–HT1080-conditioned media increased the proliferation of endothelial cells, but not of HT1080 cells.

CONCLUSIONS. The findings suggest that the induction of angiogenesis and VEGF expression by ethanol represents an important mechanism of cancer progression associated with alcoholic beverage consumption. ***Cancer*** **2005;103:422–31.**

KEYWORDS: alcohol, tumor, angiogenesis, metastasis, vascular endothelial growth factor, chick embryo.

An association between alcohol consumption and cancer has long been observed. In 1910, it was noted in Paris that 80% of patients with cancer of the esophagus or gastric tract were alcoholics.[1] The relation between alcohol and cancer has been studied in various epidemiologic investigations. Correlation studies (or ecologic studies) have examined the geographic, temporal, or population-based relations between per capita alcohol consumption and cancer rates. A strong geographic correlation consistently has been found in France between mortality from cirrhosis, alcoholism, and esophageal carci-

DOI 10.1002/cncr.20781
Published online 13 December 2004 in Wiley InterScience (www.interscience.wiley.com).

noma.[2] Alcoholism also has been associated with malignancies of the mouth, pharynx, and stomach.[2] Based on random effect models summarizing the results of the five largest studies,[3–7] epidemiologic evidence has demonstrated that the consumption of alcoholic beverages increases the risk of carcinoma of the oral cavity, pharynx, larynx, esophagus, liver, breast, and large bowel. Although this causal relation clearly applies for cancers of certain organs, the question remains unresolved for others. A change in alcohol consumption over time also correlates with subsequent changes in the occurrence of rectal carcinoma.[8] Better evidence is available from cohort and case–control studies in which information on alcohol consumption, other cancer risk factors, and cancer occurrence was obtained at an individual level. Smith-Warner and associates[9] evaluated a total of 322,647 women, including 4335 participants who had a diagnosis of incident invasive breast carcinoma, and found that alcohol consumption was associated with a linear increase in breast carcinoma incidence in women over the range of consumption reported by most women. In that study, the multivariate-adjusted relative risk for total alcohol intakes of from 30 g per day to $<$ 60 g per day (about 2–5 drinks per day) versus nondrinkers was 1.41. A strong, positive association between the level of alcohol consumption and the risk of cancer was observed in all four prospective cohort studies.[10–13] Mounting epidemiologic evidence has indicated that alcoholic beverage consumption is a well established risk factor for human malignancies. It is worth noting that human data also suggest that alcohol consumption accelerates tumor growth. For example, Matsuhashi et al. examined the tumor volume doubling times in 35 nodules from 35 patients with hepatocellular carcinoma calculated through utrasonographic imaging and found that tumor volume doubling time was reduced significantly by 50% in alcohol drinkers (80 g ethanol [EtOH] per day) compared with patients who did not drink.[14]

The mechanisms by which alcoholic beverage consumption causes cancer are not established. A dogma in EtOH carcinogenesis has been that EtOH is not a carcinogen,[15] although acetaldehyde, which is a metabolite of EtOH, is a carcinogen in animal models. However, not all of the carcinogenic effects of alcohol may be through this mechanism.[16] The experimental studies have provided less than convincing evidence to support the positive EtOH-cancer correlation. In addition, the studies lack information on the effects of EtOH on mammary carcinogenesis in spontaneous, chemically induced, and metastatic models. However, the proposed mechanisms may include: 1) carcinogenicity of acetaldehyde, 2) displacement of nutrients in the diet, 3) impaired nutrient metabolism, 4) inhibition of detoxification, 5) activation of enzymes, 6) altered hormonal status, 7) increased oxidant exposure, 8) suppressed immune function, 9) altered membrane fluidity, and 10) increased cellular proliferation.[17] We were interested in finding other possible mechanisms of EtOH-induced cancer.

Growth and expansion of tumor masses are dependent strictly on the sustained formation of new blood vessels, a process termed *angiogenesis*. Vascular endothelial growth factor (VEGF), which is one of the most important angiogenic factors, is up-regulated markedly in the vast majority of human tumors.[18] We recently reported that physiologically relevant levels of EtOH induced VEGF expression and angiogenesis both in models of cell culture and in the chick chorioallantoic membrane (CAM).[19] Therefore, we hypothesized that EtOH-induced angiogenesis and VEGF expression represent an important mechanism of cancer progression associated with alcoholic beverage consumption.

For the current study, we used a chick embryo CAM model in which inoculated human fibrosarcoma cells could form primary and secondary tumors. Using this model, we examined whether the administration of physiologically relevant doses of EtOH could stimulate tumor growth, tumor angiogenesis, the expression of VEGF in tumors, and intravasation by cancer cells. In addition, we determined the effect of physiologically relevant levels of EtOH on the expression of VEGF in vitro.

MATERIALS AND METHODS

Endothelial and Tumor Cell Lines

Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase digestion, as described previously.[20] Human fibrosarcoma cells (HT1080 cells) were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were maintained in standard medium (50% M199 medium plus 50% Dulbecco modified Eagle medium [DMEM]) containing 10% fetal bovine serum (FBS), 100 μg/mg gentamicin, and 2 mM L-glutamine. For tumor cell implantation, cells generally were used between passages 8 and 12; for the proliferation assays, cells were used between passages 4 and 8.

Chick Embryo CAM Assay

Chick embryo CAM assays were performed by modification of previously published methods.[19,21,22] Fresh fertile eggs from White Leghorn hens were incubated in forced-draft incubators at 37.8 °C and 53% relative humidity. On Day 8, HT1080 cells ($\approx 1 \times 10^6$ cells in 50 μL phosphate buffered saline [PBS] solution) were

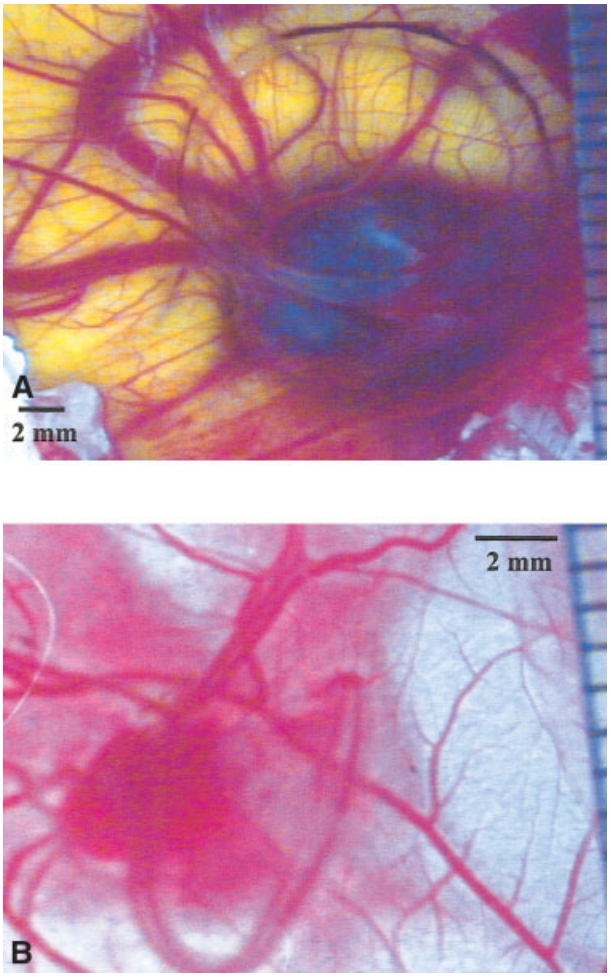

FIGURE 1. The digital images of primary (A) and secondary (B) human fibrosarcoma formed on chick embryo chorioallantoic membrane (CAM) 9 days after inoculating HT1080 cells. Many blood vessels, including the large vessels (0.5–1.0 mm in width) grew toward the tumors. Fertile eggs were incubated at 37.8 °C and 53% relative humidity. On Day 8, HT1080 cells ($\approx 1 \times 10^6$ cells in 50 μL phosphate buffered saline solution) were inoculated in a silastic ring on the "upper CAM" through an artificially generated air sac. On Day 17, the digital images of primary tumor formed on the "upper CAM" and secondary tumor formed on the "lower CAM" were acquired at $\times$ 15 magnification.

inoculated in a silastic ring on the "upper CAM" through an artificially generated air sac in the center of the large end of the eggs. The window was sealed with plastic tape of the same dimension, and the eggs were returned to the incubators. Figure 1 shows that the digital images of a primary tumor (Fig. 1A) formed on the "upper CAM" and a secondary tumor (Fig. 1B) formed on the "lower CAM" were acquired at $\times$ 15 magnification on Day 17. Because primary and secondary tumors could be formed on chick embryo CAM, we performed two series of experiments. One series of experiments studied the effects of EtOH on primary tumor growth. On Day 8, HT1080 cells ($\approx 1 \times 10^6$) were inoculated onto the "upper CAM," as described above. From Day 8 to Day 17, either normal saline (as control) or EtOH at 0.25 g/kg/day was administered into the CAM far away outside the silastic ring. EtOH was diluted to 15% with normal saline. The control and test embryos always came from the same batch of fertile eggs and were studied at the same time. On Day 17, 4 hours after administration of the test substances, the primary tumors were isolated and trimmed of CAM. After determination of tumor weight and volume, they were placed into either liquid nitrogen or 10% neutral formalin for later total RNA isolation, protein extraction, or morphologic study.

Intravasation Assay by Polymerase Chain Reaction

In a second series of experiments, we used a human Alu polymerase chain reaction (PCR) assay[22] to determine the intravasation of tumor cells in the chick embryo CAM model. The fertile eggs were incubated in forced-draft incubators at 37.8 °C and 53% relative humidity. On Day 8, a hole measuring 3–4 mm in diameter was made in the center of the large end of the eggs. The window was sealed with a plastic tape of the same dimension, and the eggs were returned to the incubators. On Day 9, HT1080 cells ($\approx 1 \times 10^6$) were inoculated onto the "upper CAM," as described above. From Day 8 to Day 11, either normal saline (as control) or EtOH at 0.25 g/kg per day was administered into the CAM through the artificially generated air sac, as described above. Forty-eight hours after inoculation of HT1080 cells, the shell was removed from the large end of each egg to make a big hole, and the embryo and yolk were removed carefully from the shell through the hole. After gently rinsing with PBS, the genomic DNA was extracted from the lower CAM tissues. The oligonucleotide primer pairs 5′-ACGCCTGTAATCCCAGCACTT-3′ (upstream) and 5′-TCGCCCAGGCTGGAGTGCA-3′ (downstream) for human Alu sequences were used to detect intravasated tumor cell DNA extracted from the lower CAM tissues. One microgram of genomic DNA was PCR-amplified under the following conditions: 95 °C for 10 minutes, 58 °C for 30 seconds, 72 °C 45 for seconds (1 cycle); and 95 °C for 30 seconds, 58 °C for 45 seconds, and 72 °C for 30 minutes (35 cycles). The PCR products were labeled with ^{32}P-deoxycytidine triphosphate (dCTP) and were electrophoresed on a 7% polyacrylamide gel, which produced a characteristic 220-base pair fragment. Quantification of the bands was analyzed with a Phospho-Imager System.

Northern Blot Analysis

Total RNA isolation and Northern blot analyses were performed as described previously.[19] Briefly, total RNA from tumor tissues or cultured cells was prepared using a total RNA isolation kit (Ambion, Austin, TX). Total RNA (20 μg per sample) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde in 1 $\times$ [3-(N-morpholino)-propanesulfonic acid] (MOPS) buffer, transferred to a nylon membrane (Bio-Rad Laboratories, Hercules, CA), and hybridized to a [α-^{32}P] dCTP-labeled VEGF cDNA probe (kindly provided by Dr. Werner Risau[22]; Max Planck Institute). Loading and integrity of each RNA sample were examined by observing the intensity of 18S and 28S in ethidium bromide-stained gels. Quantification of VEGF mRNA expression was determined on phosphor images of blots collected using a Phosphor-Imager (Molecular Dynamics) with ImageQuant software (Molecular Dynamics). To verify the relative amounts of total RNA, the membranes were hybridized with a [α-^{32}P] dCTP-labeled 28S rRNA antisense oligonucleotide probe (Ambion). The VEGF mRNA expression was normalized against 28S rRNA in each sample.

VEGF Protein

VEGF protein levels in the tumor tissues as well as in the media of cultured cells were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN), as described previously.[23] VEGF protein levels were normalized to the total amount of tissue or cellular protein and expressed as picograms per milligram (pg/mg) of the total protein. The total protein content was determined in duplicate with bovine serum albumin as the standard (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories, Hercules, CA).

Morphometric Analysis

Morphometric point counting in standard histologic sections of tumors was used to estimate the area occupied by vascular cells and intravascular space, as described previously.[23,24] Tumors were fixed in 10% formalin and then embedded in paraffin. Thin sections (5 μm) were mounted on glass slides and stained with periodic acid–Schiff reagent (1% periodic acid–Schiff reagent and Harris hematoxylin). Digital images of the tumors were captured at onscreen magnifications of $\times$ 830 and $\times$ 4333 and were overlaid with a 10 $\times$ 10 grid by image subtraction (Optimas version 5.3 software; Optimas Corp., Seattle, WA). Two observers evaluated the tissue sections without knowing the group from which they originated and simultaneously identified the microvascular vessels, and each identification was agreed upon in turn. Ten random fields (i.e., a total of 1000 points) were examined across each section. The area occupied by vascular cells and intravascular space that coincided with the points was counted, so that vascular volume density = number of points on component $\div$ number of points on containing tissue. Sampling was continued until the number of points was sufficient to put the relative standard error = $\sqrt{}$ [(1 $-$ volume density) $\div$ (total number of points $\times$ volume density)] $<$ 0.15. Between 8 and 16 sections were examined for each tumor.

Exposure of EtOH in Cell Cultures

We also tested the effect of physiologically relevant levels of EtOH on VEGF expression in cultured HT1080 cells. The cells were seeded into sterile culture flasks at $\approx$ 5 $\times$ 10^4 cells/cm^2 and incubated at 37 °C in a humidified atmosphere with 5% CO_2, 20% O_2, and 75% N_2. When the monolayers of cells reached $\approx$ 80% confluence, standard medium (50% M199 medium plus 50% DMEM) containing 10% FBS was replaced with media that had 4% heat-inactivated FBS to reduce the mitogenic influence of growth factors and hormones. The cells were then treated with EtOH (10 mmol/L or 20 mmol/L) for 18 hours. There were no changes in pH in the media between cells with EtOH and cells without EtOH. VEGF protein levels in the media were measured by ELISA, and VEGF mRNA expression in HT1080 cells was determined using Northern blot analysis.

Cell Proliferation

Cell proliferation was determined by [^{3}H]-thymidine incorporation. The uptake of [^{3}H]-thymidine by HUVECs or HT1080 cells was used as an indicator of DNA synthesis, as described previously.[20] Cells ($\approx$ 80% confluent) were cultured in standard media supplemented with 4% heat-inactivated FBS in the absence (control) and the presence of 20 mmol/L of EtOH for 8 hours. In a second series of experiments, we tested the effects of HT1080-conditioned media and EtOH-HT1080-conditioned media on proliferation of HUVECs or HT1080 cells. We harvested the conditioned media from cultured HT1080 cells 18 hours after incubation in standard media in both the absence (control) and the presence of 20 mmol/L of EtOH. The cells ($\approx$ 80% confluent) were cultured in the conditioned media (that had or had not been exposed to EtOH) for 24 hours. During the last 6 hours of incubation, the cells were pulsed with [^{3}H]-thymidine by adding 1 μCi per well. The cells were then washed, harvested, and processed for counting in a scintillation counter.

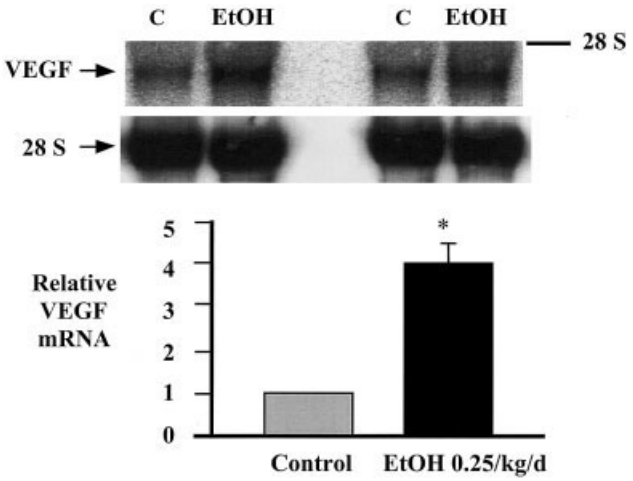

FIGURE 2. Ethanol (EtOH) treatment increased vascular endothelial growth factor (VEGF) mRNA expression in tumors formed on chick embryo chorioallantoic membrane (CAM). After inoculating HT1080 cells onto the CAM, the embryos were administered EtOH at a dose of 0.25 g/kg per day for 9 days. Northern blot analysis indicated that EtOH treatment caused a 3.9-fold increase in the expression of VEGF mRNA in the tumors compared with the saline-treated controls ($n = 6$ embryos; $P < 0.01$).

Statistical Analysis

All determinations were performed in duplicate, and each measurement was repeated at least three times. When indicated, data are presented as the mean ± standard error. Differences were considered statistically significant when two-sided P values were < 0.05 using a Student t test. All statistical calculations were performed with StatView software (BrainPower, Calabasa, CA).

RESULTS

Effects of EtOH on VEGF Expression

We used a model of chick embryo CAM bearing human fibrosarcoma formed by HT1080 cells and found that administration of a physiologically relevant dose of EtOH at 0.25 g/kg per day for 9 days caused a 3.9-fold increase in the expression of VEGF mRNA in the tumors compared with saline-treated controls ($n = 6$ embryos; $P < 0.01$), as shown in Figure 2. Moreover, we determined VEGF protein levels in the tumor tissue extract using an ELISA kit and demonstrated that EtOH treatment in chick embryos increased VEGF protein levels in tumors by 113% compared with control levels (736 ± 58 pg/mg vs. 345 ± 58 pg/mg protein; $n = 6$ embryos; $P < 0.01$).

In cell culture experiments, we found that the physiologically relevant levels of EtOH caused a dose-related increase in both VEGF mRNA and protein expression in cultured human fibrosarcoma (HT1080) cells. The Northern blot in Figure 3A shows that VEGF mRNA expression increased by 1.2-fold and 2.0-fold, respectively, compared with controls after HT1080 cells had been exposed to 10 mmol/L and 20 mmol/L of EtOH for 18 hours ($n = 6$ embryos; $P < 0.01$). We also found that 10 mmol/L and 20 mmol/L of EtOH increased VEGF protein levels in cultured HT1080 cells by 24% and 41%, respectively ($n = 6$ embryos; $P < 0.05$, respectively) compared with controls (basal VEGF level, 18.43 ng/mg ± 0.72 ng/mg total protein), as shown in Figure 3B.

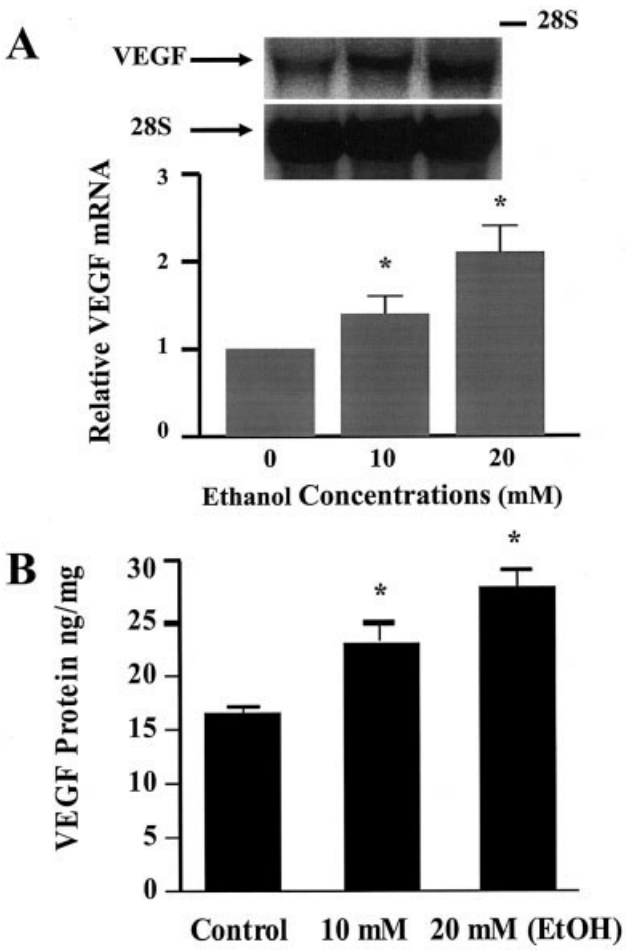

FIGURE 3. Exposure to physiologically relevant levels of ethanol (EtOH) caused a dose-related increase in both vascular endothelial growth factor (VEGF) mRNA (A) and VEGF protein (B) expression in cultured HT1080 cells. HT1080 cells were cultured in the absence or in the presence of EtOH (10 mM or 20 mM) for 18 hours. Northern blot analysis indicated that VEGF mRNA expression increased by 1.2-fold and 2.0-fold, respectively, compared with the controls after HT1080 cells had been exposed to 10 mmol/L and 20 mmol/L of EtOH for 18 hours ($n = 6$ embryos; $P < 0.01$). An enzyme-linked immunosorbent assay indicated that 10 mmol/L and 20 mmol/L of EtOH increased VEGF protein levels in cultured HT1080 cells by 24% and 41%, respectively ($n = 6$ embryos; $P < 0.05$) compared with the controls.

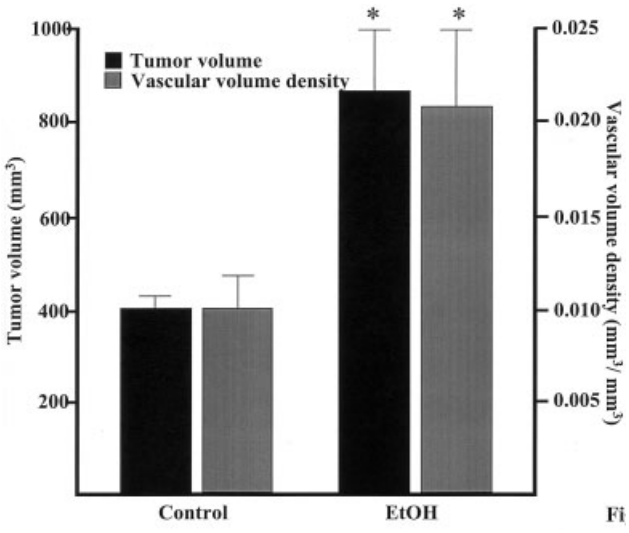

FIGURE 4. Effects of ethanol treatment on tumor growth and tumor angiogenesis. Administration of ethanol (EtOH) at a dose of 0.25 g/kg per day for 8 days in chick embryo caused a 2.2-fold increase in tumor volume compared with the control group (867 ± 138 mm^3 vs. 402 ± 28 mm^3; $n = 6$ embryos; $P < 0.01$), and a 2.1-fold increase in the intratumoral vascular volume density compared with the control group (0.021 ± 0.004 mm^3/mm^3 vs. 0.010 ± 0.002 mm^3/mm^3; $P < 0.01$). Digital images of the tumors were captured at onscreen magnifications of × 830 and × 4333 and were overlaid with a 10 × 10 grid by image subtraction (Optimas v5.3 software; Optimas Corp., Seattle, WA). Morphometric point counting in standard histologic sections from tumors was used to estimate the areas occupied by vascular cells and intravascular space that coincided with the points, so that vascular volume density = number of points on component ÷ number of points on containing tissue.

Growth Responses of Tumor to EtOH Administration

We established an in vivo model of chick embryo CAM bearing human fibrosarcoma formed by HT1080 cells. Using this model, we found direct evidence that the physiologically relevant dose of EtOH stimulated tumor growth and tumor angiogenesis, as shown in Figure 4. Administration of EtOH at 0.25 g/kg day for 9 days in chick embryos caused a 2.2-fold increase in tumor volume compared with control embryos (867 mm^3 ± 138 mm^3 vs. 402 mm^3 ± 28 mm^3; $n = 6$ embryos; $P < 0.01$) and caused a 2.1-fold increase in the intratumoral vascular volume density compared with control embryos (0.021 mm^3/mm^3 ± 0.004 mm^3/mm^3 vs. 0.010 mm^3/mm^3 ± 0.002 mm^3/mm^3; $P < 0.01$).

Using regression analysis, we observed that the growth responses of neoplasm and intratumoral vascular volume were different between the control group and the EtOH-treated group. In the control tumors (saline-treated), the intratumoral vascular volume relatively was as constant as the tumor volume ($n = 6$ tumors; $r = 0.472$). In EtOH-treated tumors, the increased intratumoral vascular volume was correlated strongly with the increased tumor volume ($n = 6$ tumors; $r = 0.962$; logarithmic curve fit). Biologically, the intratumoral vascular volume is a function of the growth of both the neovasculature and the neoplasm. These findings suggest that the physiologically relevant dose of EtOH modulated a positive feedback relation between the neoplasm and the neovasculature.

We also found that EtOH-treated tumors contained a larger fraction of the intratumoral connective tissue, whereas a smaller fraction of intratumoral connective tissue was observed in the control tumors, as shown in Figure 5. The regression analysis indicated that there was a good linear correlation between the increased intratumoral connective tissue volume density and the increased intratumoral vascular volume density ($n = 12$ tumors; $r = 0.714$). These findings may suggest that the intratumoral connective tissue likely serves as substratum for blood vessels and that EtOH-induced angiogenesis may be associated with the growth of intratumoral connective tissue. Indeed, the images under high magnification showed that the intratumoral connective tissue contained a lot of blood vessels, some of which were large.

Intravasation Response of HT1080 Cells to EtOH Treatment

Because we observed that secondary tumors could be formed on the chick embryo CAM (Fig. 1), we used a human Alu PCR assay[22] to determine the intravasation of tumor cells (HT1080) in the chick embryo CAM model under the influence of EtOH. The human Alu PCR in Figure 6 indicates that EtOH treatment significantly enhanced the intravasation of human fibrosarcoma cells in chick embryo CAM. Kim et al.[22] previously demonstrated that the intensity of the 220-base pair band of human Alu sequences increased with increased content of human tumor cells. We scanned the bands of human Alu sequences produced by PCR amplification and found that the intravasation of HT1080 cells under the influence of EtOH increased by > 8-fold compared with the control group ($n = 6$ embryos; $P < 0.01$).

Effect of EtOH on Cell Proliferation

We tested the effects of EtOH on cell proliferation in cultured HUVECs and HT1080 cells. Figure 7 indicates that 20 mmol/L of EtOH alone had no effect on cell proliferation in either HUVECs or HT1080 cells. However, exposure of HUVECs to EtOH-conditioned (20 mmol/L) media from HT1080 cell cultures increased cell proliferation by 26% ($n = 6$ embryos; $P < 0.05$) compared with the control group with HT1080-conditioned media. In contrast, the EtOH-conditioned me-

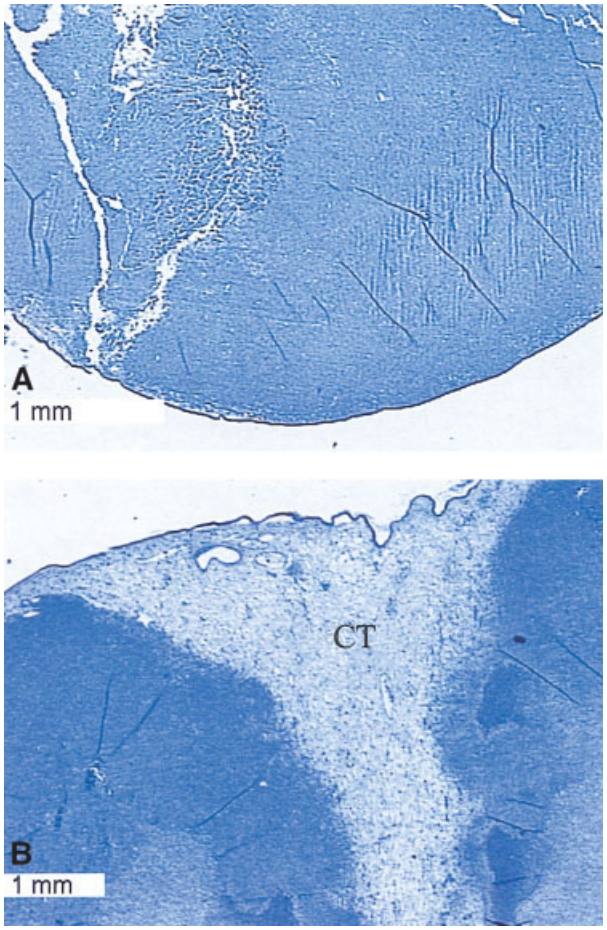

FIGURE 5. Periodic acid–Schiff-stained paraffin sections of tumors with or without ethanol (EtOH) treatment. EtOH-treated tumors (B) contained a larger fraction of the intratumoral connective tissue (CT), whereas a smaller fraction of intratumoral connective tissue was observed in the control tumors (A). The regression analysis indicated that there was a good linear correlation between the increased intratumoral CT volume density and the increased intratumoral vascular volume density ($n = 12$ embryos; $r = 0.714$).

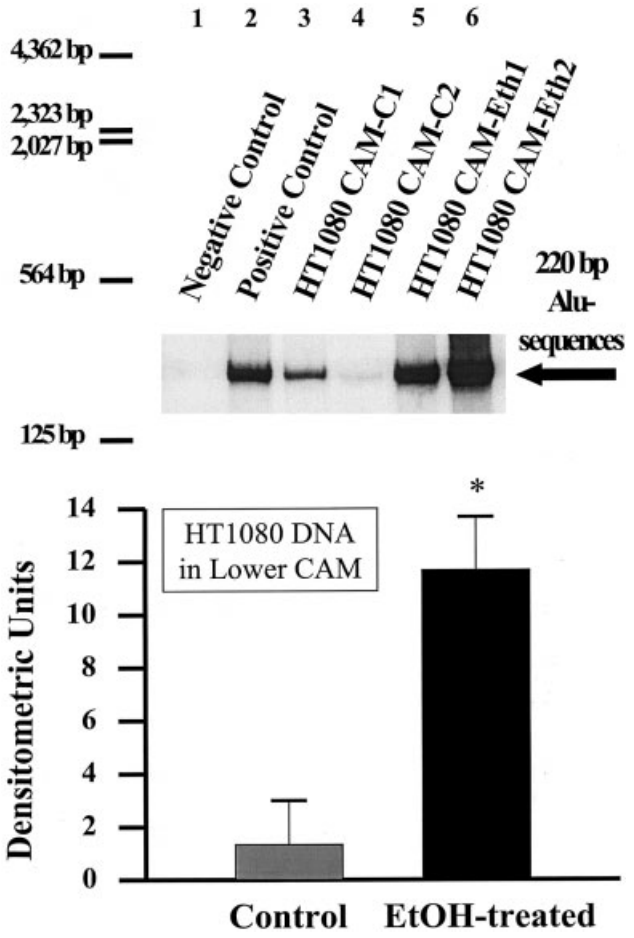

FIGURE 6. The effects of ethanol (EtOH) treatment on intravasation by HT1080 cells into the lower chorioallantoic membrane ("lower CAM"). The intensities of the 220-base pair (bp) band of human Alu polymerase chain reaction products indicate the degree of intravasation by HT1080 cells into the "lower CAM." Lane 1: This negative control shows the products of a reaction with template DNA extracted from the CAM without inoculating HT1080 cells. Lane 2: This positive control was a reaction with DNA from the HT1080 tumor. Lanes 3,4: Reactions with DNA from the "lower CAM" treated with normal saline (control) and from HT1080 cells that were inoculated onto the "upper CAM." Lanes 5 and 6: Reactions with DNA from the "lower CAM" treated with EtOH and from HT1080 cells that were inoculated onto the "upper CAM." The intravasation by HT1080 cells into the "lower CAM" under the influence of EtOH increased by $>$ 8-fold compared with the control group ($n = 6$ embryos; $P < 0.01$).

dia had no effect on HT1080 cell proliferation. Because HT1080 cells produce VEGF but lack VEGF receptors, these findings support the notion that increased HUVEC proliferation by EtOH-conditioned media may be due to increased VEGF protein levels in the conditioned media.

DISCUSSION

Although mounting epidemiologic evidence has indicated that alcoholic beverage consumption is a well established risk factor for human malignancies, experimental studies have provided less than convincing evidence to support the positive EtOH-cancer correlation. In the current study, we determined the effects of chronic administration of a physiologically relevant dose of EtOH on VEGF expression in tumor tissue or cells in relation to tumor growth, tumor angiogenesis, and intravasation of tumor cells in a chick embryo CAM model. To the best of our knowledge, this is the first report to show in vivo evidence that EtOH administration caused a significant increase in tumor size, intratumoral vascular volume density, and intravasation by tumor cells, which was associated with the up-regulation of VEGF expression.

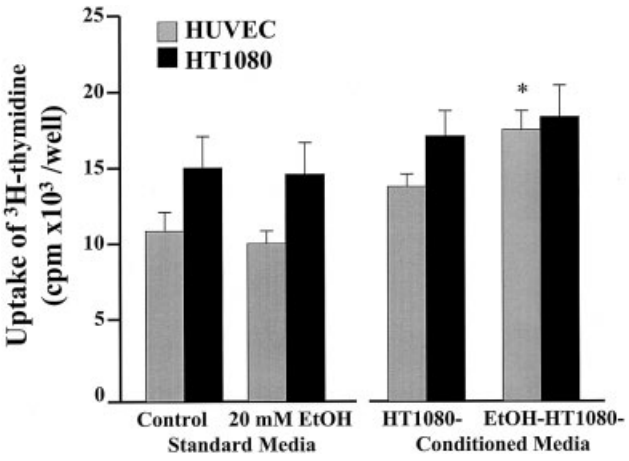

FIGURE 7. Effects of ethanol (EtOH) on cell proliferation in cultured human umbilical vein endothelial cells (HUVECs) and HT1080 cells. A DNA synthesis assay ([^{3}H]-thymidine incorporation) indicated that 20 mmol/L EtOH alone did not have an effect on proliferation of either HUVECs or HT1080 cells after 8 hours of incubation in standard media. Exposure of HUVECs to EtOH/HT1080-conditioned media increased cell proliferation by 26% ($n = 6$ embryos; asterisk indicates $P < 0.05$) compared with HT1080-conditioned media. Cell proliferation was unchanged in HT1080 cells that were exposed to EtOH-HT1080-conditioned media compared with HT1080-conditioned media.

We used a model of chick embryo CAM bearing human fibrosarcoma formed by HT1080 cells and found that the administration of a physiologically relevant dose of EtOH at 0.25 g/kg per day for 9 days caused a 2.2-fold increase in tumor volume compared with the control group. This finding was consistent with the study in humans reported by Matsuhashi et al.,[14] who examined the tumor volume doubling times of 35 nodules from 35 patients with hepatocellular carcinoma, as calculated through utrasonographic imaging, and found that tumor volume doubling times were reduced significantly by 50% in alcohol drinkers (80 g EtOH per day) compared with nonalcoholic patients.[14] Conversely, Blank and Meadows.[25] showed that very high EtOH consumption (10% or 20% weight/volume EtOH-drinking water) slightly reduced primary tumor size and significantly decreased body weight in melanoma-bearing mice compared with a water-drinking group. This discrepancy likely was due to the very high doses of EtOH used in the mice. Based on the study reported by Schmidt et al.,[26] 12% volume/volume alcohol consumption (11 g/kg per day) in mice is equivalent to 10–11 standard drinks per day in humans. A standard drink is the amount of EtOH in a 12-ounce bottle of 5% (alcohol) beer. The loss of body weight and slightly decreased primary tumor size in mice by the very high alcohol consumption at least suggested that high alcohol intake could not induce angiogenesis. Based on our previous report,[19] we believe that light-to-moderate alcohol consumption can induce angiogenesis. Judah Folkman[27] proposed that the growth of tumor was dependent on tumor angiogenesis, i.e., tumors could not grow beyond a maximum size of 1–2 mm^3. Therefore, the effect of EtOH on tumor growth may depend on the effect of EtOH on tumor angiogenesis. However, it is necessary to examine further the different effects of EtOH on tumor growth and tumor angiogenesis in mice using different alcohol consumption levels ranging from light to very heavy.

The results of the current study indicate that administration of a physiologically relevant dose of EtOH can increase VEGF expression in tumors and intratumoral vascular volume density (Figs. 2, 4). Our data do not directly prove that the up-regulation of VEGF expression causes tumor angiogenesis. However, we have examined the proliferative effect of EtOH and EtOH-conditioned media on endothelial cells (HUVECs) and human fibrosarcoma cells (HT1080). Figure 7 indicates that 20 mmol/L of EtOH alone has no effect on cell proliferation in either HUVECs or HT1080 cells. The EtOH-HT1080-conditioned media, in which VEGF levels were higher, increased the proliferation of HUVECs by 26% ($n = 6$ embryos; $P < 0.05$) and did not affect the proliferation of HT1080 cells. It is well known that HT1080 cells produce VEGF but lack VEGF receptors and that HUVECs have VEGF receptors but produce only small amounts of VEGF. Together, these results support the notion that EtOH-induced VEGF many play a role in the induction of angiogenesis. However, the EtOH-HT1080-conditioned media also may contain other factors that stimulate angiogenesis. Therefore, further studies to investigate the time course of VEGF up-regulation and vascular morphometric changes in the tumor tissue during exposure to physiologically relevant levels of EtOH and to apply a VEGF receptor blockade, such as soluble Flt-1,[28] will be necessary to confirm or refute this possibility.

A question that must be asked is what are possible mechanisms of EtOH-induced VEGF expression. Many of the pathophysiologic effects of alcohol ingestion relate to the metabolic consequences of EtOH in tissues or cells. We previously reported the evidence of metabolic response to EtOH in cell cultures.[19] We observed a significant dose-dependent increase in lactate production in coronary artery vascular smooth muscle cells incubated with EtOH.[19] More recently, Burns and Wilson[29] reported that the angiogenic potential of lactate was dependent upon increased expression of VEGF using an in vitro assay (AngioKit). Moreover, we also reported that physiologically rele-

vant levels of EtOH induced the mRNA expression of hypoxia-inducible factor 1 α (HIF-1α) in cultured vascular smooth muscle cells.[19] The classic pathway for EtOH metabolism is oxidation of EtOH to form acetaldehyde, a process that is catalyzed by alcohol dehydrogenase.[30] An important consequence of EtOH and acetaldehyde oxidation is an increase in both cytosolic and mitochondrial reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide (NADH/NAD). The increase in NADH/NAD ratios can increase the activity of xanthine oxidase, which is a free radical-generating enzyme. Many studies support the hypothesis that the pathophysiologic effects of EtOH ingestion are mediated primarily by the generation of free radicals.[31] It is well known that HIF-1 is necessary for a hypoxia-induced increase in VEGF expression. Semenze[32] suggested that some free radical molecules, such as hydroxyl radical, may be responsible for the hypoxia signal that leads to HIF-1 expression. It will be interesting to determine whether EtOH can cause a positive correlation between the expression of HIF-1 and NADH/NAD ratios. Additional studies will be required to investigate whether free radical molecules related to EtOH metabolism may be associated with induction of HIF-1 and VEGF expression.

We have observed that there is a more significant increase in the up-regulation of VEGF by EtOH in an in vivo human fibrosarcoma tumor than the in vitro cultured human fibrosarcoma cells (HT1080). We speculate that cell-cell communication may enhance the effect of EtOH on VEGF expression in tumors. It is believed that a tumor is an ecosystem for both tumor cells and endothelial cells. Judah Folkman[33] proposed that tumor and vascular endothelial cells existed as a highly integrated ecosystem within a neoplasm and that endothelial cells could be switched from a resting state to a rapid growth phase by diffusible chemical signals from tumor cells. Neovascularization permitted rapid tumor growth by providing an exchange of nutrients and oxygen to the tumor. The vascularized tumor also received paracrine stimuli from endothelial cells that stimulated growth.[34–36] Several studies[37–39] demonstrated that tumor angiogenesis mediated down-regulation of endothelial adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1). The speculation was that the reduction of VCAM-1 may promote tumor growth by avoiding the immune response. The neovasculatization will promote cell-cell communication further in tumors. Therefore, both tumor growth and tumor vessel growth are perpetuated continuously.[40] The findings of the more significant influence of EtOH on VEGF expression in tumor tissue compared with the tumor cell alone lead us to assume that EtOH and, perhaps, EtOH-mediated angiogenic factors drive a system of positive feedback between the expanding neoplasm and vasculature wherein each promotes the growth of the other. It will be interesting to test this hypothesis using a model of cocultures between HUVECs and HT1080 cells or an in vivo model of cell-cell communication.

In conclusion, in the current study, a chick embryo CAM model bearing human fibrosarcoma was used to demonstrate that the administration of a physiologically relevant dose of EtOH caused a significant increase in both tumor size and intratumoral vascular volume density, and those increases were accompanied by the up-regulation of VEGF expression in tumors. We found that the physiologically relevant levels of EtOH caused a dose-related increase in both VEGF mRNA and protein expression in cultured human fibrosarcoma (HT1080) cells. The EtOH/HT1080-conditioned media, in which VEGF levels were higher, increased the proliferation of HUVECs, but not HT1080 cells. The secondary tumors were observed on chick embryo CAM, in which EtOH treatment stimulated the intravasation of tumor cells (HT1080). These findings support the hypothesis that the induction of angiogenesis and VEGF expression by EtOH represent an important mechanism of cancer progression associated with alcoholic beverage consumption.

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