Mainstream and Sidestream Cigarette Smoke Inhibit Growth and Angiogenesis in the Day 5 Chick Chorioallantoic Membrane

Goar Melkonian, Lucia Cheung, Rebecca Marr, Cathy Tong, and P. Talbot

Department of Cell Biology and Neuroscience, University of California, Riverside, California 92521

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The purpose of this study was to test the hypothesis that components in mainstream (MS) and sidestream (SS) cigarette smoke inhibit growth and angiogenesis using the chick chorioallantoic membrane (CAM). Varying doses of whole or gas-phase MS and SS smoke solutions were placed on day 5 CAMs, and their effects on angiogenesis were evaluated on day 6. All parameters evaluated (CAM area, major blood vessel area, major blood vessel diameter, blood vessel pattern formation, and capillary plexus formation) were inhibited to different degrees in a dose-dependent manner by both MS and SS smoke treatment. Inhibition of growth and vessel development was correlated with inhibition of cell proliferation. Inhibition of capillary plexus formation was caused by failure of mesodermal blood vessels to migrate to the ectoderm. SS smoke solution was more inhibitory than MS smoke solution in all assays, except for capillary plexus formation. In all assays, the toxicants in SS smoke partitioned mainly with the gas phase, whereas those in MS smoke were deduced to be mainly in the particulate phase. Some of the inhibitory doses of MS and SS smoke solutions had nicotine concentrations within the range found in human smokers. Taken together, these data demonstrate that exposure to complex mixtures of chemicals in MS and SS cigarette smoke adversely affect growth, vessel development, vessel migration, and cell proliferation.

Key Words: cigarette smoke; angiogenesis; chick chorioallantoic membrane; CAM; vessel migration; cell proliferation; BrdU labeling.

Angiogenesis, the formation of blood vessels from preexisting vessels, is a vital process in both normal and diseased states (Hanahan, 1997; Hanahan and Folkman, 1996; Nicosia and Villaschi, 1999; Zachary, 1998). Angiogenesis occurs normally during embryonic development (Flamme et al., 1997; Wilting et al., 1995), cyclically in some reproductive tissues of adult females (Augustin et al., 1995; Findlay, 1986; Goorder and Rogers, 1995; Redmer and Reynolds, 1996; Reynolds and Redmer, 1995), and during wound healing (Arbiser, 1996; Martins-Green and Hanafusa, 1997). It also occurs in certain disease states such as inflammation, psoriasis, and diabetic retinopathy (Folkman and Shing, 1992), and tumors require angiogenesis to sustain growth (Gastl et al., 1997; Hanahan and Folkman, 1996; Uhr et al., 1997). Because of its important role in both normal and pathological processes, there is current interest in identifying the factors that regulate or affect angiogenesis.

The influences of cigarette smoke on angiogenesis are just beginning to be investigated. In vivo studies on mammals have shown that smoke inhalation inhibits angiogenesis in gastric ulcers of rodents, and this inhibition is accompanied by dose-dependent decreases in nitric oxide synthase (Ma et al., 1999a,b) and epidermal growth factor biosynthesis (Ma et al., 2000). Reduced expression of vascular endothelial growth factor (VEGF) and lower vessels counts have been observed in squamous cell lung carcinomas (Volm et al., 1999), and a similar downregulation of VEGF has been reported in head and neck tumors (Tae et al., 2000). Smoking has also been reported to cause adaptive angiogenesis in placentas (Pfarrer et al., 1999) and impair development of chorionic villi (Genbacev et al., 2000).

Additional evidence that smoke impairs angiogenesis has come from in vivo studies showing that exposure of hamsters to either mainstream (MS) smoke (the smoke inhaled by active smokers) or sidestream (SS) smoke (the major component of environmental tobacco smoke) at doses equivalent to those received by human smokers for 30 days prior to and during pregnancy resulted in corpora lutea with diminished vascular area (Magers et al., 1995). The decreased vascularization in corpora lutea of smokers could influence steroid hormone production and distribution and may contribute to the increased incidence of spontaneous abortions observed in human smokers (Atrash et al., 1986; Saraiya et al., 1998; Stillman et al., 1986).

To investigate the effect of smoke on angiogenesis more directly, we subsequently exposed day 7-day 11 chick choioallantoic membranes (CAM) to MS and SS cigarette smoke solutions that altered normal pattern formation of the major blood vessels (Melkonian et al., 2000). In these experiments, alterations in blood vessel pattern formation were correlated with increases in collagen I and III, decreases in extracellular...
hyaluronan in the mesodermal layer, and in the case of SS treatment, with increases in extracellular fibronectin.

The purpose of the current study was to test the hypothesis that MS and SS cigarette smoke inhibit angiogenesis, specifically formation of the major vessels and capillary plexus in day 5/day 6 CAMs. We were able to address this hypothesis by using day 5/day 6 CAMs, which provide advantages over older CAMs for studying development of both the major vessels and the capillary plexus that forms beneath the ectoderm (Melkonian et al., 1999b). In the day 5/day 6 CAM assay, test compounds are placed directly on the surface of day 5 CAMs, and their effects on vessel development are evaluated 24 h later on day 6. Solutions of whole MS and SS smoke and their gas phases were compared in dose-response experiments, and their effects on various aspects of blood vessel development were evaluated. MS and SS cigarette smoke solutions caused abnormalities in the development of both the major vessels and the capillary plexus of CAMs. To test the hypothesis that these effects were due to inhibition of cell proliferation, BrdU-labeled nuclei were compared in control and treated CAMs.

MATERIALS AND METHODS

Media and Reagents

All media and solutions were made in deionized water that was further purified in a Barnstead/Thermolyne nanopure water system (Fisher Scientific, Tustin, CA). EBSS (Earle’s balanced salt solution) was made from reagent-grade chemicals as described in detail previously (Talbot et al., 1998). Prior to use, sodium bicarbonate (26.2 mM) and HEPES (25 mM) were added to EBSS, and the pH was adjusted to 7.4 to create EBSS-H, which was used as a control medium and to make all smoke solutions.

Whole MS (MSW) and SS (SSW) smoke solutions were made fresh in EBSS-H using 2R1 research-grade cigarettes (University of Kentucky, Louisville, KY) that were smoked on a puffer box built at the University of Kentucky (Knoll and Talbot, 1998). Diagrams showing the arrangement of the puffer box and vacuum pumps have been published previously (Knoll and Talbot, 1998). MSW smoke solutions were made from 60 puffs of MS smoke pushed through 10 ml of EBSS-H. SSW smoke solutions were made by collecting the smoke that was produced at the burning end of a cigarette during 30 puffs of MS smoke and pushing it through 10 ml of EBSS-H. The gas phases of MS (MSG) and SS (SSG) smoke were made by passing whole smoke through a Cambridge filter prior to bubbling it into EBSS-H (Knoll and Talbot, 1998). The Cambridge filter removes the particulate phase while allowing the gas phase to pass into the EBSS-H. Only a small fraction of the total smoke actually dissolves in the EBSS-H. All smoke solutions and control medium (EBSS-H) applied to CAMs were passed through a 0.22-m Acrodisk filter (Fisher Scientific, Tustin, CA) to remove bacteria and handled using sterile technique. In experiments in which the filter was not used, similar results were obtained. Smoke solutions were checked spectrophotometrically and adjusted if necessary to the same reading before use. This was done to assure that different batches of smoke solution were similar in concentration. The pH of each solution was adjusted to 7.4, and solutions were added to CAMs immediately after preparation. Prior studies had shown that smoke solutions remain stable at room temperature for several hours. Doses were defined as puff equivalents (PE) or the number of puffs/ml, and varying doses were made by serially diluting the master solution with EBSS-H.

Glutaraldehyde, cacodylate, and Spurr’s plastic kits were purchased from Electron Microscopy Supplies (Fort Washington, PA). Paraformaldehyde was purchased from Sigma (St. Louis, MO). BrdU evaluation was done using a kit from Boehringer Mannheim (Indianapolis, IN) and BrdU antibody was purchased from Sigma (St. Louis, MO). Antimouse IgG conjugated to Alexa-488 was purchased from Molecular Bioprobes (Eugene, OR). Hemo-De and para-plast plus were purchased from Fisher Scientific (Tustin, CA).

CAM Assay

Angiogenesis was evaluated in day 5 CAMs. Fertilized chicken eggs were obtained from Hi-Vac International (Lakeview, CA) and incubated at 37°C, 85–90% relative humidity throughout the experiment. Albumin was removed on the fourth day after fertilization to drop the embryo away from the shell and to allow the CAM to develop in a way accessible to treatment. A window was opened in each egg to allow subsequent access to the CAM and then sealed with transparent tape. At 11 A.M.–12 P.M. on day 5, 200 ml of EBSS-H or serial dilutions of MSW, MSG, SSW, or SSG smoke solution were placed on the surface of each CAM. Untreated control CAMs had windows made in their shells, but received no solution. At 11 A.M.–12 P.M. on day 6, CAMs were fixed and prepared for evaluation by video microscopy and light microscopy as described below.

Fixation and processing of CAMs. To terminate experiments, CAMs were fixed in place at room temperature by depositing 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) both on top and underneath the CAM. After 2 h, CAMs were dissected out of the eggs and fixation was continued overnight at 4°C. After fixation, CAMs were rinsed thoroughly and stored in phosphate-buffered saline (PBS). Each CAM was first measured with a ruler, then imaged with a video camera as described below. Small pieces containing one tertiary vessel and the adjacent area containing capillary plexus were dissected from CAMs and postfixed for 30 min in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. Following postfixation, tissue was rinsed in deionized water, dehydrated in a graded ethanol series, and infiltrated and embedded in Spurr’s plastic. Plastic was polymerized at 75°C for 12 h. Sections (1–2 μm) were cut on a Sorval MT2B ultramicrotome using glass knives, and sections were stained using methylene blue.

BrdU labeling. EBSS-H or MSW or SSW smoke solution were placed on CAMs between 11 AM and 12 PM on day 5 after fertilization. At 8 h after the start of an experiment, control and treated CAMs were exposed to 100 μl of BrdU (10 mM) for 55 min, after which they were transferred to a –20°C freezer for 2–3 min, then fixed at –20°C in 70% ethanol containing 50 mM glycine buffer, pH 2.0, for 1.5 h. CAMs were cut out of eggs and washed in PBS, dehydrated in an ethanol series, and further processed in a Hypercenter II Tissue Processing System (Shandon Inc., Pittsburgh, PA), then embedded in paraffin using a Sakura Tissue-TE (Shandon Inc., Pittsburgh, PA). Sections (5 μm) were cut on an 820 Spencer microtome (Labequip, American Optical Corp., Ontario, Canada) and placed on Superfrost/plus glass slides (Fisher Scientific, Tustin, CA). Sections were deparaffinized with three changes of Hemo-De for 15 min each, washed in 100% ethanol two times for 5 min each, then rehydrated to PBS. Sections were incubated in 1N HCl for 1 h at 40°C, washed in PBS for 10–15 min, incubated in 1% sodium borohydride (Sigma, St. Louis, MO) for 10 min, and washed again in PBS. Sections were incubated in anti-BrdU IgG (1/100 dilution in PBS containing 1% BSA) for 2 h at room temperature. Slides were washed in PBS containing 0.1% BSA for 45–60 min, then incubated in secondary antibody (Alexa antimouse IgG, 1/100 dilution) for 1 h at room temperature. Slides were washed with PBS plus 0.1% BSA for 45 min, and cover slips were mounted with Vectashield. BrdU and antibody controls were performed on both control and treated CAMs. Controls consisted of CAMs that were not exposed to BrdU and CAMs exposed to secondary antibody only. Slides were examined using a Zeiss photomicroscope with epifluorescence. Images were recorded using a Spot digital camera-SP401-115 (Diagnostic Instruments, Sterling Heights, MI) and processed and montaged using Adobe Photoshop (San Jose, CA).

Quantification of CAM assay results. To determine CAM area, each CAM was placed in PBS in a Petri dish, then the longest and shortest lengths were measured with a ruler to a precision of 0.5 mm using a Wild-M5A dissecting microscope (Max ERB instrument Co., Burbank, CA). Data were recorded in Excel spreadsheets, and CAM areas were calculated using the formula:

\[
\text{CAM area} = \frac{1}{2} \times (\text{longest length} + \text{shortest length}) \times \text{CAM thickness}
\]
area = \( (1/2 \times A) \times (1/2 \times B) \times \pi \)

where \( A \) = longest length, \( B \) = longest width, \( \pi = 3.14 \).

Means and standard deviations were then calculated for each control and treatment group. The means of the treated and control groups were compared to determine the effect of each chemical on CAM growth.

To evaluate blood vessel pattern formation, fixed CAMs were further dissected to remove edge tissue and mounted in tissue culture dishes under cover slips to flatten the CAMs. Video images (640 x 480 TIF) were captured at a magnification of approximately 3 x using a Hitachi KP-D50U camera (Hitachi Inc., Torrance, CA). To evaluate the degree of the effect of MS and SS smoke solutions on pattern formation, a template was made containing four images with varying degrees of disruption of pattern formation. The images were ranked from 0 to +++ with 0 representing the branching pattern observed in controls and +++ representing the most severe disruption of pattern. The images were printed on a sheet of paper, then control and test CAMs were compared blindly with the template, and each CAM was assigned a ranking from 0 to +++ based on its closest match to the template images.

Video images were also used to analyze: (1) the area occupied by primary, secondary and tertiary vessels, and (2) the diameter of secondary and tertiary vessels. These parameters were quantified using NIH Image (Scion, Frederick, MD) running on Windows 98. To quantify the area occupied by the major blood vessels, a micrometer was used to calibrate the number of pixels/mm\(^2\) at the magnification used. The area occupied by the primary, secondary, and tertiary vessels was quantified by placing a 220 x 180 pixel selection box on each CAM at random. The chosen area was then pasted into a new blank image and processed using the threshold tool to maximize contrast and present each pixel as either black or white. Image brightness was then adjusted so that the primary, secondary, and tertiary blood vessels appeared black and other pixels became white. The measurement tool was then used to determine the area occupied by the blood vessels/4.5 mm\(^2\) in each CAM.

The diameters of secondary and tertiary vessels were measured in each CAM using the perimeter/length tool in NIH Image. For each vessel analyzed, both the thickest and thinnest regions were measured. Two CAMs were evaluated for each group (control or treated), and two secondary and two tertiary vessels were evaluated in each CAM.

To evaluate capillary plexus formation, histological sections were digitized with a Spot camera using a 16 x objective. To determine the percentage of ectoderm subtended by capillary plexus (this is a measure of how much plexus has formed), an 18-cm length of each CAM was marked off on the computer monitor, and the length of the plexus that had formed immediately beneath the ectoderm was measured and presented as a percentage of the total projected length (18 cm).

**Estimation of Nicotine Concentration in Smoke Solutions**

We have previously determined nicotine concentrations in smoke solutions produced in the same manner as those used in this study (Knoll et al., 1995). The nicotine concentrations in 6-PE solutions of MSW and MSG and in 3-PE solutions of SSW and SSG smoke in our previous study were divided by the dilution that gave the lowest observed adverse effect level (LOAEL) in the current study to obtain an estimate of the nicotine concentration at the LOAEL dose. LOAEL in this study is defined as the lowest exposure level at which there were statistically significant increases in the frequency or severity of adverse effects between the exposed group and its control group. NOAEL in this study is defined as the dose at which there was no statistically significant increase in the frequency or severity of an adverse effect between the exposed group and its control.

**Statistics**

For each experiment, data were analyzed using a one-way analysis of variance (ANOVA). When significance was found \( p < 0.05 \), post hoc comparisons were made using the Student-Newman-Keuls test. Control groups were compared with each other to determine if the vehicle (EBSS-H) alone produced an effect, and each smoke treatment group was then compared with the EBSS-H control.

**RESULTS**

**Mainstream and Sidestream Smoke Solutions Inhibit CAM Growth**

MSW-, MSG-, SSW-, and SSG-phase smoke solutions were tested at varying concentrations to determine their effect on CAM growth. Smoke solutions were applied to day 5 CAMs, and CAM area (mm\(^2\)) was evaluated 24 h later (Fig. 1). CAMs treated with culture medium only (EBSS-H) were not significantly different in size from untreated controls in either the MS or SS experiments (Figs. 1A and 1B; \( p > 0.05 \)). However, all smoke solutions, except MSG, significantly inhibited CAM growth in a dose-dependent manner. SSW and SSG solutions were more inhibitory than the MSW solution. These data show that growth-inhibitory chemicals in SSW smoke partition, at least in part, with the gas phase, whereas in MS smoke they are not detected in the gas phase and can therefore be deduced to be in the particulate phase.

**Smoke Solutions Decrease Vascular Area in CAMs**

The preceding measurements show that smoke solutions inhibited the overall growth of CAMs. To determine if blood vessel growth was specifically inhibited, the area occupied by the major blood vessels was determined in control and smoke-treated CAMs using NIH Image as described in Materials and Methods (Fig. 2). There was no significant difference in vascular area in the EBSS-H and untreated controls in either the MS or SS experiments (Figs. 2A and 2B). However, MSW (but not MSG) smoke solutions (Fig. 2A) and both SSW and SSG smoke solutions (Fig. 2B) significantly decreased vascular area in a dose-dependent manner when compared with the EBSS-H controls. The effect was stronger in the SS-treated groups than in the MSW group. SSW solutions were slightly more effective than SSG-phase solutions. These data show that the chemicals that affect blood vessel area partition mainly with the gas phase of SS smoke solution. Because MSG-phase smoke solutions were not effective, the active components in MS smoke can be deduced to reside in its particulate phase.

**Smoke Solutions Decrease Vessel Diameter and the Length of Secondary and Tertiary Branches**

To determine if vessel diameter had been affected by smoke solutions, the diameters of the secondary and tertiary vessels were measured. The effect of smoke solutions on vessel diameter was quantified by measuring the thickest and thinnest diameters of secondary and tertiary vessels in images projected on a computer screen (Fig. 3). In the MS experiment, EBSS-H treatment did not significantly affect the diameters at the thickest or thinnest regions in either the secondary or tertiary vessels when compared with the untreated control groups (Figs. 3A and 3B). However, treatment of CAMs with MSW smoke
solutions produced a significant dose-dependent decrease in the diameter of both secondary and tertiary blood vessels when compared with the EBSS-H control (Figs. 3A and 3B). MSG-phase smoke did not significantly decrease vessel diameter at any concentration, suggesting activity resides mainly in the particulate phase.

FIG. 1. MS and SS smoke solutions inhibit CAM growth. Day 5 CAMs were treated with nothing (control), EBSS-H culture medium (EBSS), mainstream whole smoke (MSW), mainstream gas-phase smoke (MSG), sidestream whole smoke (SSW), or sidestream gas-phase smoke (SSG), and CAM area (mm²) was evaluated on day 6; doses given in puff equivalents (PE)/ml as defined in Materials and Methods. Each group is the mean ± SD of five CAMs. Control groups (control vs. EBSS) did not differ significantly. Each smoke-treated group was compared with the EBSS control; *p < 0.05; **p < 0.01; ***p < 0.001.

FIG. 2. MS (A) and SS (B) smoke solutions decreased vascular area of the major vessels. Day 5 CAMs were treated with nothing (control), EBSS-H culture medium (EBSS), mainstream whole smoke (MSW), mainstream gas-phase smoke (MSG), sidestream whole smoke (SSW), or sidestream gas-phase smoke (SSG), and major blood vessel area/4.75 mm² of CAM was evaluated on day 6; doses given in puff equivalents (PE)/ml. Control groups (control vs. EBSS) did not differ significantly. Each smoke-treated group was compared with the EBSS control. (A), each group is the mean ± SD of 11–13 measurements. (B), each group is the mean ± SD of 5–7 measurements; *p < 0.05; **p < 0.01; ***p < 0.001.
In the SS experiment, secondary vessel thickness did not differ significantly in the control and EBSS-H–treated groups (Figs. 3C and 3D). However, SSW- and SSG-phase smoke solutions significantly decreased the diameter of both secondary and tertiary vessels in a dose-dependent manner when compared with the EBSS-H–treated controls (Figs. 3C and 3D). SSW smoke solutions were more effective at lower doses than SSG solutions, indicating some, but not all, of the activity is in the SSG phase.

**Smoke Treatment Alters Pattern Formation of Secondary and Tertiary Vessels in Day 5 CAMs**

The branching pattern formed by major vessels was adversely affected by smoke solutions placed on older CAMs (Melkonian et al., 2000). To determine if a similar effect occurred in day 5 CAMs, video images of control and treated CAMs were compared (Figs. 4A–4D). In control CAMs, major blood vessels established a dendritic pattern in which primary vessels gave off secondary vessels, which in turn produced tertiary vessels (Fig. 4A). In contrast, treated CAMs showed varying degrees of disruption of this normal pattern (Figs. 4B–4D). The video images further revealed that differentiation of the vasculature in the CAM is altered by smoke treatment. Networks of vessels that were not characteristic of normal development appeared in many treated CAMs (e.g., Figs. 4B and 4C near arrowheads). In these networks, vessels were about equal in diameter and interconnected with each other, but did not form the dendritic branching pattern characteristic of normal development.

To compare the effect of various doses of MSW, MSG, SSW, and SSG smoke solutions on the branching pattern formed by major vessels, patterns for each control and treated CAM were accessed using a scale of 0 (similar to controls) to 111 (unlike controls) as described in Materials and Methods. All CAMs in the control- and EBSS-H–treated groups were ranked as 0, except for one EBSS-H–treated CAM that was ranked 1 (Fig. 5). In contrast, all treatment groups, except the 0.06-PE dose of MSW, produced some level of adverse effect on blood vessel pattern formation (Figs. 5A and 5B). The most severe disturbances in pattern were caused by 6 PE of MSW, 3 PE of SSW, and 3 PE of SSG smoke solutions, which in most cases caused CAMs to be ranked +++. CAMs with +++ ranking would be similar in appearance to Figure 4D. Because 6 PE of MSW produced a stronger effect than MSG in the pattern formation assay, it can be deduced that both the gas and the particulate phase of MS smoke have activity in this assay. In contrast, because the SSW and SSG solutions were about equally effective at disrupting pattern, it is likely that most of the components in SS smoke solutions that disturb pattern formation reside in the gas phase.

**Smoke Solutions Inhibit Formation of the Capillary Plexus**

The capillary plexus undergoes very rapid growth between days 5 and 6 of development (Melkonian et al., 1999a). To determine if capillary plexus formation was affected by exposure to MS or SS smoke solutions, histological sections of control and treated CAMs were examined (Fig. 6). Both untreated (Fig. 6A) and EBSS-H–treated (not shown) controls showed normal plexus formation beneath the ectoderm. In contrast, MS- and SS-treated CAMs had little or no capillary plexus beneath the ectoderm (Figs. 6B and 6C). Unlike controls, the smoke-treated CAMs had numerous mesodermal blood vessels (arrows, Fig. 6C) that apparently failed to migrate to the ectoderm to form plexus. The percentage of ecto-
derm subtended by capillary plexus was quantified as described in Materials and Methods (Fig. 7). The untreated controls and EBSS-H–treated controls did not differ significantly from each other in any experiment. However, all four types of smoke solution significantly inhibited formation of the capillary plexus when compared with the EBSS-H controls (Figs. 7A–7D). MSG-phase smoke solutions had less activity than MSW solutions, indicating that activity partitioned between the particulate and gas phase of MS smoke. Because SSW and SSG smoke solutions were about equally effective, the active components can be deduced to have partitioned mainly with the SSG phase.

Mainstream and Sidestream Smoke Solutions Inhibit Cell Proliferation

To test the hypothesis that smoke solutions were impairing CAM growth and angiogenesis by inhibiting cell proliferation, CAMs were exposed to EBSS-H or smoke solutions on day 5 and then labeled with BrdU 8 h after the start of exposure. Histological sections revealed that CAMs treated with MSW (Fig. 8B) or SSW (Fig. 8C) smoke solutions had fewer labeled nuclei than CAMs treated with EBSS-H (Fig. 8A). Cell division appeared to be inhibited in all layers of the smoke-treated CAMs. In addition, blood vessels from control CAMs had numerous labeled endothelial cells (Fig. 8A, small arrowheads), whereas endothelial cells from both treatment groups were less frequently labeled (Figs. 8B and 8C, small arrowheads). The number of labeled cells/45 \( \mu \text{m}^2 \) was counted in CAMs exposed to EBSS-H or to smoke solutions and was significantly less in both smoke-treated groups than in the EBSS-H control (Fig. 8D).

DISCUSSION

We have examined the effects of a one-time application of MS and SS cigarette smoke solutions at varying doses on CAM growth and angiogenesis in day 5 CAMs. All of the parameters examined (CAM area, area occupied by major vessels, diameters of secondary and tertiary vessels, pattern formation of major vessels, and capillary plexus formation) were adversely affected, to different degrees, in a dose-dependent manner by
both MS and SS smoke solutions. The effects of smoke solutions on CAM growth and vasculature development were correlated with significant inhibition of cell proliferation in both vascular and nonvascular cells. The inhibition of cell proliferation by MS and SS smoke solutions suggests that smoke may adversely affect other processes, such as wound healing, that depend on cell division.

The NOAELs and LOAELs for each type of smoke solution in each assay are shown in Table 1. LOAELs for the three growth parameters (CAM area, vascular area, and vessel diameter) indicate that SS smoke solutions are more potent inhibitors of growth than MS solutions. In addition, the active factors in each of these assays appeared in the gas phase of SS but not of MSG smoke. In contrast, pattern formation and capillary plexus formation were affected by the gas phases of both MS and SS smoke, which were about equally effective at disrupting pattern formation. Plexus formation was the only parameter that was more sensitive to MS smoke than SS smoke. These data indicate that the various aspects of angiogenesis studied in these assays show different sensitivities to MS and SS smoke and suggest that more than one chemical is likely involved in affecting these processes. Moreover, the active factors in some assays (growth parameters, SS smoke) partitioned mainly with the gas phase, whereas in other assays (growth parameters, MS smoke), they partitioned with the particulate phase.

The estimated nicotine concentration in each type of smoke solution for each LOAEL is also shown in Table 1. Nicotine concentrations were estimated from values obtained previously (Knoll et al., 1995) for smoke solutions produced in the same manner as those used in this study. Although nicotine is probably not the active factor in these assays (Melkonian et al., 2000; also see later section of the Discussion), nicotine is a useful biomarker and can be used to interpret PE relative to doses of smoke received by human smokers. Active human smokers typically have serum concentrations of nicotine ranging from 4 to 72 ng/ml (average = 33 ng/ml) (Russel et al., 1980), whereas tissue levels may be 11 times higher than serum levels (McCann et al., 1992; McLachlan et al., 1976; Weiss and Eckert, 1989). The data in Table 1 show that several LOAELs (pattern formation, MSG and SSG; plexus formation, MSG) had concentrations of the biomarker nicotine that were within serum and tissue levels in human smokers. Additionally, several LOAELs (CAM area, SSW; vascular area, MSG; diameter, SSW; pattern formation, SSW; plexus formation, MSW) had nicotine concentrations that were within a factor of 2–3 of levels in human smokers. These data suggest that many of the doses used in our study were similar to doses received by
human smokers. Our data are based on single exposures. It will be important in future work to determine if the smoke inhaled by humans produces similar effects in angiogenic tissues and if chronic exposure in a smoker decreases the LOAEL.

The CAM has been used twice previously to study smoke solutions. The initial study was not directed at angiogenesis, but did show that smoke condensates could cause hemorrhage, necrosis, and hyperplasia in day 10 CAMs (Comber and Grasso, 1975). These changes, which probably occurred because high doses of condensate were applied directly to CAMs, were not observed in our studies. In our previous study with day 7–day 11 CAMs, MS and SS smoke solutions produced profound effects on the pattern of major vessels that correlated with alteration in the composition of the extracellular matrix (Melkonian et al., 2000). The extracellular matrix often plays roles in establishing branching patterns in developing tissues (Hisaoka et al., 1993; Matsui et al., 1996). Significant increases in interstitial collagen (type I and III), combined with decreases in hyaluronic acid in the mesoderm of MSW- and SSW-treated CAMs, could have altered normal pattern formation of the major vessels. Both MS and SS smoke solutions disrupted normal pattern formation of the major vessels in 5 day CAMs, in agreement with our previous study of older CAMs (Melkonian et al., 2000). Moreover, pattern formation was particularly sensitive to smoke solutions, as both MSG and SSG had LOAELs of 0.06 and 0.03 PE, respectively. However, the most characteristic abnormal pattern produced by smoke on the day 7–day 11 CAMs (long thin vessels that ran parallel to each other and lacked many branches) was rarely observed in the younger CAMs in the current study. Thus, although pattern was abnormal in both the young and older CAMs, the specific pattern produced in response to smoke components differed at different stages of CAM development.

In our previous study on day 7–day 11 CAMs, we found preliminary evidence that vascular area was affected by smoke solutions, but were unable to get reliable quantitative data to substantiate this observation using older CAMs. To directly test the hypothesis that smoke solutions decrease vascular area quantitatively, we used day 5 CAMs and found that MS and SS smoke solutions decreased both overall CAM growth (CAM area) and the percentage of the CAM area occupied by major blood vessels. Thus, not only was the total amount of vasculature decreased (CAM area), but the density of vasculature within treated CAMs was also decreased. This decrease in vascular area correlated with decreased diameter of secondary and tertiary vessels. These observations with CAMs are in agreement with in vivo studies showing that vascular area decreased in the corpora lutea of smokers exposed to both MS and SS (Magers et al., 1995), that the number of microvessels decreased in areas of ulcer healing in smokers (Ma et al., 1999a), and that tumors of smokers had lower vessel counts than those from nonsmokers (Volm et al., 1999).
By using aqueous extracts of smoke, the composite effect of the numerous chemicals contained in smoke can be evaluated simultaneously and their net effect on angiogenesis and cell proliferation determined. The specific chemicals in these solutions that inhibited angiogenesis and cell division are not yet known; however, their aggregate effect on growth and angiogenesis was inhibitory in the CAM assays. Nicotine by itself can inhibit cell division in endothelial cells and cytotrophoblast cells at high doses (Genbacev et al., 2000; Villablanca, 1998), but may actually stimulate cell division at lower doses (Villablanca, 1998). Low doses of nicotine alone have also been reported to stimulate angiogenesis in several pathological states (Heeschen et al., 2001). We observed inhibitory effects of smoke solutions on CAM growth, blood vessel area, and vessel diameter, suggesting that chemicals other than nicotine play a role in inhibiting cell division, and that in complex mixtures of chemicals containing nicotine, other chemicals dominate over nicotine and inhibit vessel growth. The chemicals in MS and SS smoke solutions that inhibit angiogenesis and cell proliferation are currently being identified (Melkonian et al., 1999a).

The effect of smoke solutions on CAM capillary plexus formation has not been studied previously. The plexus begins its development between days 5 and 5.3, making CAMs of this age ideal for studying capillary formation (Melkonian et al., 2001). During this interval, the plexus forms mainly by cell proliferation, migration of mesodermal vessels toward the ectoderm, and vascularization (formation of vessels from angioblasts) beneath the ectoderm (Melkonian et al., 2001). Our study shows that both MS and SS treatment significantly inhibited plexus formation. Interestingly, plexus formation was the only parameter that was more sensitive to MS than SS smoke solutions. Histological sections showed that plexus formation was inhibited by failure of mesodermal vessels to migrate to the ectoderm, an important process in angiogenesis. Plexus formation may also have been slowed by inhibition of cell division in the mesodermal vasculature.

BrdU-labeling data show that both MS and SS smoke solutions have a general inhibitory effect on cell division in the CAM. Mesodermal (including endothelial cells), ectodermal, and endodermal cells were less frequently labeled by BrdU in smoke-treated CAMs than in controls. This inhibition of cell proliferation by smoke solutions could account for their effects on CAM and blood vessel area. Several other processes in smokers are linked to inhibition of cell proliferation. Inhalation of smoke by rats decreased cell proliferation in vivo in gastric ulcers and could explain their delayed healing in smokers (Ma et al., 2000). Slower healing of wounds has also been clinically

**FIG. 7.** Smoke solutions inhibit formation of the capillary plexus. (A–D) Day 5 CAMs were treated with nothing (control), EBSS-H culture medium (EBSS), mainstream whole smoke (MSW), mainstream gas-phase smoke (MSG), sidestream whole smoke (SSW), or sidestream gas-phase smoke (SSG), and the percentage of ectoderm that had plexus beneath it in histological sections was determined on day 6; doses given in puff equivalents (PE/ml). Control groups (control vs. EBSS) did not differ significantly. Each smoke-treated group was compared with the EBSS control. All smoke solutions inhibited plexus formation. Each group is the mean ± SD of measurements made on 5 CAMs for the MS groups and on 10 CAMs for the SS groups; *p < 0.05; **p < 0.01; ***p < 0.001.
observed in smokers (Silverstein, 1992) and could be due to an inhibition of cell proliferation by chemicals in smoke. In addition, incomplete development in the placentas of human smokers has been correlated with a decrease in the number of cytotrophoblasts in the S phase of the cell cycle (Genbacev et al., 2000).

Although MS and SS are chemically similar, the relative amounts of many chemicals vary in MS and SS smoke (U.S. EPA, 1992). For example, N-nitrosodimethylamine, analine, 4-aminobiphenyl, and nickel are at least 20 times more abundant in SS smoke (U.S. EPA, 1992). The distribution of chemicals in the particulate and gas phases also varies between MS and SS smoke. For example, nicotine is more abundant in the particulate than in the gas phase of MS smoke (U.S. EPA, 1992). Thus, it is probable that SSG-phase smoke has higher concentrations of chemicals that affect cell proliferation and major vessel development than its MSG counterpart. However, it is also possible that the gas phase of SS smoke has additional inhibitory chemicals that are not present in MSG-phase smoke.

In conclusion, our study shows that day 5 CAMs provide a useful assay for evaluating the effects of chemicals on CAM

![FIG. 8. MSW and SSW smoke solutions inhibited cell proliferation. Day 5 CAMs were treated with 6 PE of MSW or 3 PE of SSW smoke solution, then labeled 8 h after the start of treatment with BrdU to detect cell proliferation. Sections of control-treated (A), MSW-treated (B), and SSW-treated (C) CAMs were labeled with BrdU to detect cell proliferation. The ectoderm is toward the top. The control had more labeled nuclei than either treatment group. Small arrowheads in (A) point to labeled endothelial cells of a mesodermal blood vessel. In (B) and (C), small arrowheads point to blood vessels with unlabeled endothelial cells. The large arrowhead in (B) points to a group of autofluorescent red blood cells. (D) Eight hours after smoke solution treatment, the number of labeled nuclei was significantly lower in the MSW and SSW groups than in the control. Each group is the mean ± SD of 5–7 measurements; **p < 0.01.]

### TABLE 1

Summary of Dose Response Data for All Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>NOAEL (PE)</th>
<th>LOAEL (PE)</th>
<th>Estimated nicotine in LOAEL dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAM area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSW</td>
<td>0.06</td>
<td>0.6</td>
<td>12.9 (µg/ml)</td>
</tr>
<tr>
<td>MSG</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SSW</td>
<td>ND</td>
<td>0.03</td>
<td>2.7 (µg/ml)*</td>
</tr>
<tr>
<td>SSG</td>
<td>0.3</td>
<td>3.0</td>
<td>54 (µg/ml)</td>
</tr>
<tr>
<td>Vascular area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSW</td>
<td>0.6</td>
<td>6.0</td>
<td>129 (µg/ml)</td>
</tr>
<tr>
<td>MSG</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SSW</td>
<td>ND</td>
<td>0.03</td>
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<tr>
<td>SSG</td>
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<td>5.4 (µg/ml)</td>
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<tr>
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<td>6.0</td>
<td>129 (µg/ml)</td>
</tr>
<tr>
<td>MSG</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>SSW</td>
<td>ND</td>
<td>0.03</td>
<td>2.7 (µg/ml)*</td>
</tr>
<tr>
<td>SSG</td>
<td>0.3</td>
<td>3.0</td>
<td>54 (µg/ml)</td>
</tr>
<tr>
<td>Pattern*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MSW</td>
<td>0.06</td>
<td>0.6</td>
<td>12.9 (µg/ml)</td>
</tr>
<tr>
<td>MSG</td>
<td>ND</td>
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<td>7 (ng/ml)*</td>
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<tr>
<td>SSW</td>
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<tr>
<td>SSG</td>
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<tr>
<td>Plexus</td>
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<tr>
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<tr>
<td>SSG</td>
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<td>3.0</td>
<td>54 (µg/ml)</td>
</tr>
</tbody>
</table>

Note. ND, could not be determined from our data.

*Concentrations are within a factor of 2–3 of serum/tissue levels of smokers (Knoll et al., 1995).

*LOAEL for MSG, SSW, and SSG may be lower than indicated.

*Concentrations are within serum/tissue levels of human smokers (Knoll et al., 1995).
growth, angiogenesis, and cell proliferation. CAM growth, development of the major vessels, and formation of the capillary plexus were adversely affected in a dose-dependent manner by MS and SS smoke treatment. In all assays except capillary plexus formation, SS smoke solutions were more inhibitory at lower doses than MS solutions. The inhibitory chemicals in SS smoke solutions partitioned with the gas phase in all assays, whereas the MS toxicants partitioned with the particulate phase in the growth assays and with the gas phase in the pattern formation and capillary plexus assays. The effects of smoke solutions on CAM and major blood vessel growth can be explained by inhibition of cell proliferation, although other modes of action are also possible. Moreover, both MS and SS smoke solutions inhibited vessel migration, an important aspect of angiogenesis during plexus formation. Together, these data demonstrate that both MS and SS cigarette smoke contain chemicals that inhibit growth, major vessel development, capillary plexus formation, and cell proliferation in CAMs and could contribute to problems in vascular development in human smokers.

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REFERENCES


SMOKE INHIBITS GROWTH AND ANGIOGENESIS


