Biochemical and Biophysical Research Communications **276,** 371–378 (2000)
doi:10.1006/bbrc.2000.3474, available online at http://www.idealibrary.com on IDEAL®

Anti-Angiogenic Potential of a Cancer Chemopreventive Flavonoid Antioxidant, Silymarin: Inhibition of Key Attributes of Vascular Endothelial Cells and Angiogenic Cytokine Secretion by Cancer Epithelial Cells

Cheng Jiang, Rajesh Agarwal, and Junxuan Lü[1]
Center for Cancer Causation and Prevention, AMC Cancer Research Center, Denver, Colorado 80214

Received August 9, 2000

In recent studies, we have shown that silymarin, a naturally occurring flavonoid antioxidant, exhibits anti-cancer effects against several epithelial cancers. Here, we assessed its potential as an anti-angiogenic agent employing human umbilical vein endothelial cells (HUVEC) and human prostate and breast cancer epithelial cells. When sub-confluent HUVEC were treated for 48 h, adherent cell number decreased by 50 and 90% at 50 and 100 μg/ml doses, respectively. Apoptotic cell death principally accounted for cell loss at >50 μg/ml doses. In biochemical analysis, silymarin treatment of HUVEC for 6 h resulted in a concentration-dependent decrease in the secretion and cellular content of matrix metalloproteinase (MMP)-2/gelatinase A. Silymarin also inhibited HUVEC tube formation (*in vitro* capillary differentiation) on a reconstituted extracellular matrix, Matrigel. In other studies, 5 to 6 h exposure of DU145 prostate, and MCF-7 and MDA-MB-468 breast cancer cells to silymarin resulted in a dose-dependent decrease in the secreted vascular endothelial growth factor (VEGF) level in conditioned media without any visible change in cell morphology. The inhibitory effect of silymarin on VEGF secretion occurred as early as 1 h. These observations indicate a rapid inhibitory action of silymarin on the secretion of this primary angiogenic cytokine by cancer epithelial cells. Taken together, the results of this study support the hypothesis that silymarin possesses an anti-angiogenic potential that may critically contribute to its cancer chemopreventive efficacy.

Key Words: **silymarin; HUVEC; MMP-2/gelatinase A; *in vitro* capillary differentiation; vascular endothelial growth factor; angiogenesis switch.**

It is now well established that angiogenesis, that is, the growth of capillary vessels from existing blood vessels, is obligatory for the growth and progression of solid cancers (1–3). During solid cancer genesis, initiated cells undergo clonal expansion in an avascular state when the expanding lesions are small enough to take in nutrients and to expel metabolic wastes by diffusion. However diffusion is not sufficient to support continued growth of the lesion beyond a certain physical size (estimated ~2 mm diameter) because the expanding lesions consume nutrients at a rate proportional to their volume whereas the supply of nutrients is delivered at a rate proportional to their surface area (4, 5). In order for avascular lesions to progress beyond the size limit imposed by simple diffusion, they must turn on their angiogenic switch to form a neovasculature. Angiogenesis critically depends on several conditions such as the endothelial cells must proliferate to provide the necessary number of cells for the growing vessels, the activated endothelial cells must secrete matrix metalloproteinases (MMP) required to break down surrounding tissue matrix and the endothelial cells must be capable of movement/migration. In addition, the angiogenic stimuli (for example, hypoxia and production of angiogenic cytokines such as vascular endothelial growth factor [VEGF]) must be sustained. Because of the critical dependence of tumor growth and metastasis on angiogenesis, therapeutic strategies have been developed targeting various aspects of the angiogenic processes, many with promising results. Cancer chemoprevention aims to block or reduce the occurrence or progression of human malignancies by the chronic administration of naturally occurring or synthetic chemical agents. Chemoprevention can be most effective on early lesions, the fate and growth of which are likely to be more critically dependent on angiogenesis. Since the vascular endothelial cells constitute the first line of exposure to blood-borne agents, it is plausible that cancer chemopreventive

[1] To whom correspondence should be addressed at AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Fax: 303-239-3560. E-mail: luj@amc.org.

0006-291X/00 $35.00

activity of many agents may be attributable, at least in part, to anti-angiogenic properties through an inhibition of one or more of the angiogenic responses of the endothelial cells.

Fruits, vegetables, tea as well as many medicinal herbs and plants have been shown to be rich sources of phytochemicals with chemoprevention potential for some kinds of human cancer (6–9). Naturally occurring polyphenolic antioxidants are among these phytochemicals that have received increasing attention in recent years (6–9). Silymarin is a polyphenolic flavonoid antioxidant isolated from milk thistle (*Silybum marianum (L.) Gaertn*) and is used clinically as a liver detoxicant for almost three decades (10, 11). Several studies in recent past have shown anti-carcinogenic effects of silymarin in short-term bioassays (12–14). More recently, we have shown the cancer preventive efficacy of silymarin in several mouse skin tumorigenesis models (15–19), and its anti-cancer potential for human breast, prostate and cervical cancers (20–24).

Whereas all the mechanistic studies done with silymarin in recent years have focused on the cancer epithelial cells as the targets, the present study was conducted to explore potential inhibitory effects of silymarin on key parameters critical for tumor angiogenesis. In this paper, we report that silymarin treatment of human umbilical vein endothelial cells (HUVEC) inhibits their growth and survival, the secretion and expression of matrix metalloproteinases (MMPs) and capillary tube formation (*in vitro* angiogenesis). In addition, we report a rapid inhibitory action of silymarin on the secretion of a primary angiogenic cytokine VEGF by human prostate and breast cancer epithelial cells. Together, these results support an anti-angiogenic activity of silymarin that may contribute critically to its cancer chemopreventive potential.

MATERIALS AND METHODS

Chemicals and reagents. Silymarin, bovine endothelial cell growth supplement (ECGS) and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was purchased from Becton-Dickinson Labware (Bedford, MA). VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN).

Cell lines and cell culture. HUVEC cells, DU145 prostate cancer cells, and MCF-7 and MDA-MB-468 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). HUVEC were propagated in F12K medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml of heparin (Sigma Chemical Co., St. Louis, MO) and 30 μg/ml of bovine endothelial cell growth supplement (ECGS) (Sigma Chemical Co.) as described previously (25). DU145 cells were cultured in RPMI1640 medium supplemented with 10% FBS. MCF-7 and MDA-MB-468 breast cancer cells were cultured in DMEM medium supplemented with 10% FBS and 2 mM L-glutamine.

HUVEC growth/survival. Cells were seeded into 6-well plates for 24–48 h to reach ~50% confluence. Fresh medium was replaced and silymarin was added from 100× stock solutions prepared in DMSO/ethanol (20:80). In all the studies, the selection of silymarin doses was based on our earlier studies showing anti-proliferative and differentiation-inducing effects in several human epithelial carcinoma cells (20–24). Morphological responses were monitored over time under a phase contrast microscope. Adherent cells after 48 h of treatment were fixed in 1% glutaraldehyde and stained. The cell number was counted under 100× magnification for 5 random fields for each condition. The experiment was repeated at least once.

Zymogram analysis for MMP-2. HUVEC were grown in 6-well plates in complete medium for 24–48 h to near confluence. The cells were washed two times with PBS to remove spent medium and fed serum-free medium supplemented with 100 μg ECGS/ml and treated with silymarin for 6 h (a time frame that did not result in any visible morphological changes). Conditioned medium and cell lysate (prepared in 1% Triton X-100, 0.5 M Tris-HCl, pH 7.6, 200 mM NaCl) were analyzed for gelatinolytic activities on substrate gels as we previously described (25). The gels were digitized with a transmission scanner and band intensity (on inverted images) was quantified using the UN-SCAN-IT gel scanner software (Silk Scientific, Inc. Orem, UT). As a comparison for the efficacy of silymarin to inhibit HUVEC MMP-2, curcumin, a polyphenolic component of the food flavor turmeric, was included in some experiments. Curcumin has been reported to potently inhibit MMP-2 expression and tube formation in this model (26).

Capillary tube formation on Matrigel (in vitro angiogenesis). The method of Kubota *et al.* was used (27). When seeded on Matrigel, a reconstituted extracellular matrix preparation of EHS mouse sarcoma, vascular endothelial cells undergo rapid *in vitro* differentiation into capillary like structures (27), providing a simple assay for assessing impact of agents on endothelial differentiation process which requires cell-matrix interaction, intercellular communication as well as cell motility. To examine the effect of silymarin on this process, HUVEC were treated in two ways in relationship to the time frame of cell seeding onto the Matrigel. (A) Silymarin simultaneous with cell seeding: Twenty-four-well cell culture plates were coated with 0.3 ml of Matrigel and allowed to solidify at 37°C for 1 h. Then 0.5 ml medium was added to each well and silymarin was added at 2 times of the desired concentrations. HUVEC were trypsinized and 20,000 or 40,000 cells were added per well in 0.5 ml medium. Tube formation was observed periodically over time under a phase contrast microscope. Representative Polaroid pictures were taken at 6 or 17 h. (B) Treatment of preformed tubes: HUVEC were seeded onto Matrigel for 6 h to form rudimentary tubes, then the medium was replaced and silymarin was added. Tube morphology was observed over time and representative Polaroid pictures were taken at 20 h after the initiation of silymarin treatment. Curcumin was included in some experiments as a comparison for the efficacy of silymarin to inhibit tube formation. The experiments were repeated twice.

VEGF secretion and expression in cancer epithelial cells. In dose-response experiments, DU145 prostate cancer cells and MCF-7 (estrogen dependent) and MDA-MB-468 (estrogen independent) breast cancer cells were grown in T25 flasks in complete medium until confluence (~48 h). The spent medium was removed, and cells were washed 3× with PBS. Cells were treated in serum-free medium with increasing concentrations of silymarin. Conditioned media and cell lysates were analyzed for VEGF protein content by an ELISA kit as per manufacturer's instructions (R&D Systems, Minneapolis, MN). In time course experiments, confluent DU145 or MDA-MB-468 cells were treated in serum-free media with solvent vehicle (DMSO/ethanol), 50 or 100 μg/ml silymarin. Serial 1-ml aliquots were taken of the culture media for VEGF ELISA. Each sample was measured in triplicate. Experiments were repeated at least once.

RESULTS

HUVEC growth and survival. As shown in Fig. 1 (A–D), treatment with silymarin for 48 h led to a

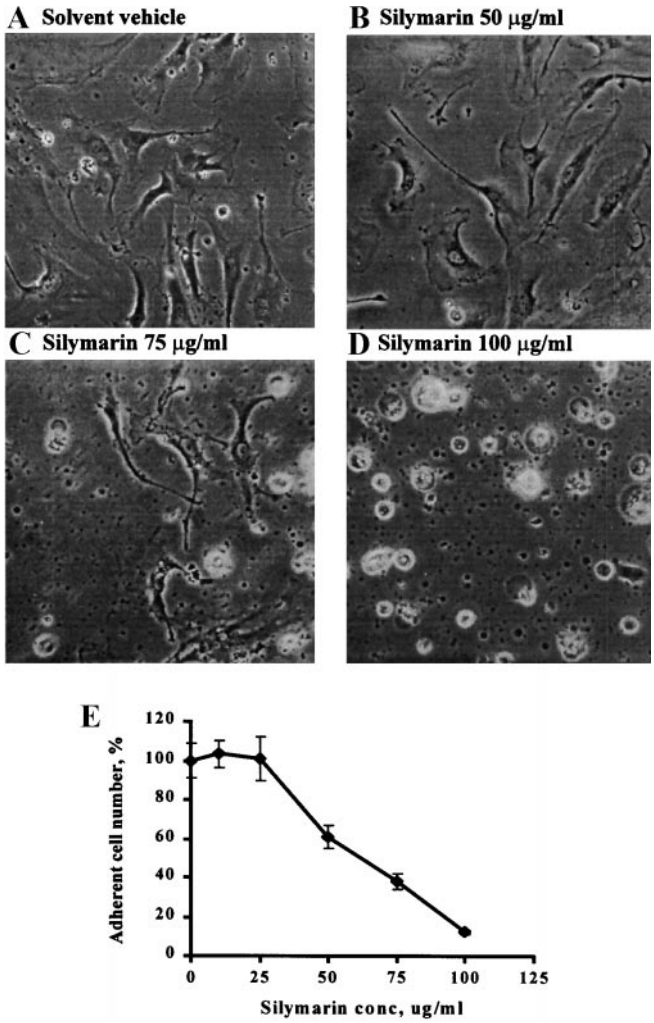

FIG. 1. Effect of silymarin treatment on HUVEC growth and survival. (A–D) Representative phase-contrast photomicrographs of HUVEC at 48 h after treatment was initiated with DMSO/ethanol vehicle (A), 50 (B), 75 (C), and 100 (D) μg/ml of silymarin. Most floaters showed typical apoptotic morphology such as cell retraction, condensation, and fragmentation into apoptotic bodies. (E) Adherent cell number as a function of initial silymarin treatment concentration. Each data point represented the mean ± SEM of the adherent cells in 5 randomly chosen fields.

concentration-dependent decrease of cells remaining adherent to the culture vessel and an increase of detached floaters. The adherent cell number was inhibited by 50 and 90% at 50 and 100 μg/ml doses of silymarin, respectively (Fig. 1E). The floaters displayed typical apoptotic morphology as indicated by cell condensation and fragmentation into apoptotic bodies. Such floaters did not re-attach or grow upon reseeding into silymarin-free fresh medium (data not shown).

HUVEC MMP-2 expression. Treatment with silymarin for 6 h, an exposure time that did not result in any visible morphological changes, led to a concentration-dependent decrease of MMP-2 (72 kD gelatinase A) in the conditioned media (i.e., secreted MMP) as detected by gelatin zymogram analyses (Fig. 2A). The extent of inhibition of the secreted MMP-2 by 100 μg/ml of silymarin was comparable to that induced by 25 μM curcumin, which has been shown to inhibit HUVEC MMP-2 and *in vitro* angiogenesis (26). In the cell lysate (Fig. 2B), 100 μg/ml silymarin inhibited MMP-2 by 67% and this effect was greater than that exerted by 25 μM curcumin, even though the secreted MMP-2 was decreased to the same extent by both compounds at the respective concentrations. At 50 μg/ml dose, silymarin did not decrease MMP-2 in the cell lysate even though it decreased the secreted MMP-2 by as much as 63%, indicating that at this level, silymarin might only inhibit the secretion of MMP-2 from the cells but not the cellular level. Incubation of the control medium (MMP-2 containing) with silymarin directly in the test tube did not inhibit its zymographic activity (data not shown), indicating a cellular dependent process for the inhibitory action on MMP-2 secretion and expression by silymarin.

In vitro angiogenesis on Matrigel by HUVEC. In experiments assessing the inhibitory effects on capillary tube formation, silymarin exposure, commenced at the time of seeding HUVEC onto Matrigel,

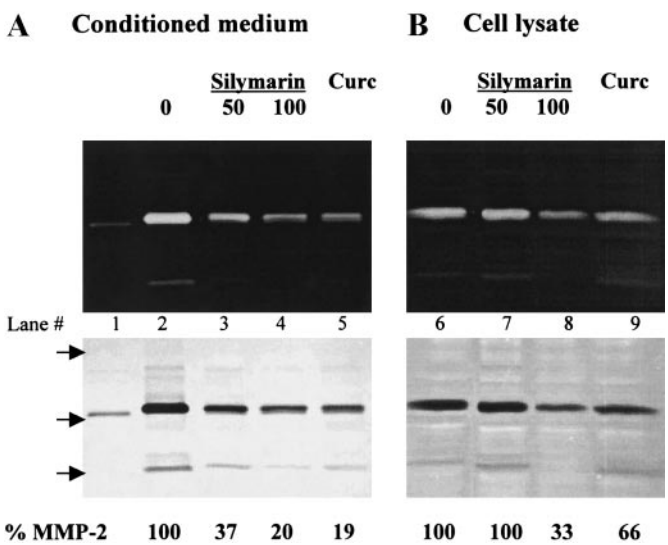

FIG. 2. Effect of silymarin or curcumin on secreted (A) and cell-associated (B) matrix metalloproteinase-2 detected by gelatin substrate gel zymography. HUVEC were treated in serum-free medium supplemented with 100 μg/ml of ECGS with silymarin or curcumin for 6 h. The conditioned media (A, lanes 2–5) and cell lysates (B) were analyzed on gelatin I impregnated substrate gels. Silymarin concentrations were 50 and 100 μg/ml. Curcumin treatment concentration was 25 μM. Inverted images of the zymograms (lower panels) were used for densitometric quantitation. The relative pixel density for the 72 kD gelatinase A/MMP-2 was shown below each lane. Arrowheads on the left mark position of molecular weight standards corresponding to (from top) 97, 66, and 47 kD. Lane 1 was serum-free medium as a blank control.

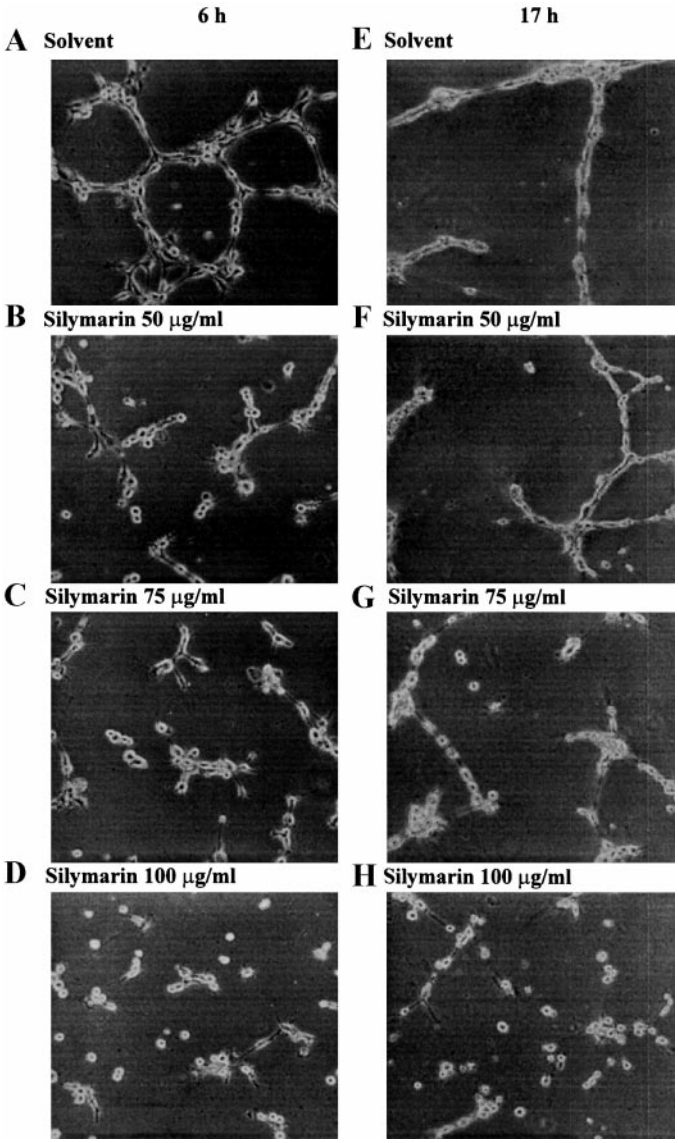

FIG. 3. Effect of silymarin on HUVEC capillary tube formation (*in vitro* differentiation) on Matrigel. HUVEC (20,000 cells/per well) in medium containing 10% serum was seeded into Matrigel pre-coated 24-well plate and treated with DMSO/ethanol solvent vehicle (A, E) or increasing concentrations of silymarin (B–D, F–H). Representative phase contrast photomicrographs (100× magnification) were taken at 6 h (A–D) and 17 h (E–H) after seeding. Each experiment condition was performed in duplicate wells and the experiments were repeated twice.

concentration-dependently inhibited tube formation at both 6 (Figs. 3A–D) and 17 h (Figs. 3E–H), achieving almost a complete block at the 100 μg/ml dose. Silymarin exposure of pre-formed tubes led to the retraction of cells and capillary disintegration (Fig. 4B versus 4A). The efficacy of silymarin at 100 μg/ml was comparable to that of 25 μM curcumin (Fig. 4C).

VEGF secretion by cancer epithelial cells. Silymarin treatment of DU145 human prostate carcinoma cells for 6 h decreased the secreted (in conditioned medium) VEGF content in a concentration dependent manner, resulting in a complete block by the 100 μg/ml dose (Table 1). Such inhibitory effect was observed in the absence of a reduction of the cell lysate VEGF content (Table 1). In human breast cancer cells, silymarin exposure reduced VEGF level in conditioned media in both MDA-MB 468 and MCF-7 cell lines (Table 1). The impact of silymarin on the cellular VEGF content was similar to that on DU145 cells, i.e., in MCF-7 cells as well as MDA-MB 468 cells at low to intermediate exposure levels, a reduction of secreted VEGF level was not associated with decreased cellular

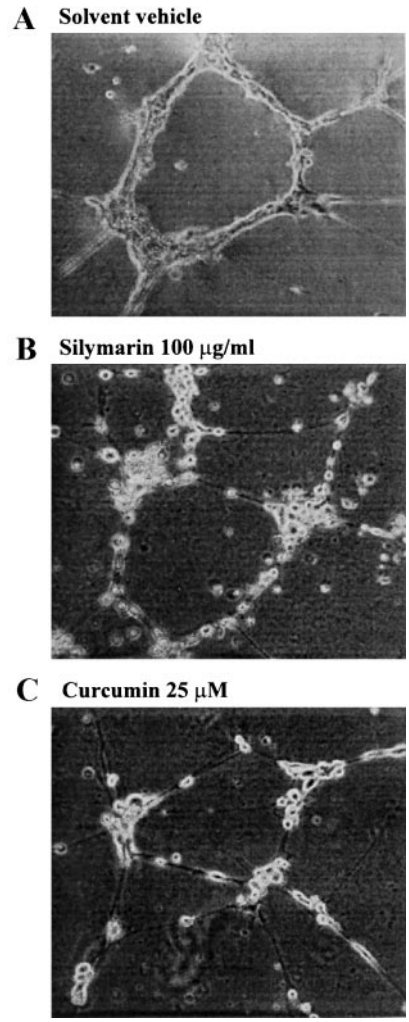

FIG. 4. Effect of silymarin or curcumin treatment on preformed HUVEC tubes. HUVEC (40,000 per well) were seeded into Matrigel pre-coated 24-well plate for 6 h for tube formation. The unattached cells and conditioned medium were removed and the tubes washed in fresh medium. The preformed tubes were treated with DMSO/ethanol vehicle (A), silymarin (B, 100 μg/ml) or curcumin (C, 25 μM). The cells were fixed in 1% glutaldehyde at 20 h after seeding and representative phase-contrast photomicrographs (100× magnification). Each experiment condition was performed in duplicate wells and the experiment was repeated twice.

TABLE 1

Effects of Silymarin Treatment on Vascular Endothelial Growth Factor (VEGF) Content in Conditioned Media (Secreted) and in Prostate and Breast Cancer Cell Lysates

Cell line	Silymarin μg/ml	Exposure time, h	VEGF in medium pg/flask	VEGF in lysate pg/flask
DU145	0	6	4272 ± 516[a1,2]	640 ± 54[c]
	25	6	4128 ± 480[a]	760 ± 16[b]
	50	6	2658 ± 264[b]	894 ± 10[a]
	100	6	72 ± 1[c]	758 ± 22[b]
MDA-MB-468	0	5	7815 ± 480[a]	1112 ± 24[a]
	25	5	6150 ± 165[b]	1222 ± 42[a]
	50	5	5300 ± 105[c]	1140 ± 26[a]
	100	5	2590 ± 65[d]	742 ± 14[b]
MCF-7	0	6	3420 ± 120[a]	455 ± 24
	50	6	3156 ± 246[a]	447 ± 9
	100	6	2178 ± 102[b]	501 ± 22

[1] Mean ± sd; $n = 3$ replicates.
[2] Data were analyzed by one-way ANOVA. Dissimilar superscripts indicate significant difference between means ($P < 0.05$).

VEGF content (Table 1). The exception was MDA-MB cells treated with 100 μg/ml silymarin where cellular VEGF content was decreased. In time course experiments, the secretion of VEGF was significantly decreased at 1 h of exposure to silymarin in both DU145 and MDA-MB 468 cells (Fig. 5). The inhibitory effects in all three cell lines were observed in the absence of morphological changes such as cell retraction, rounding, detachment or cytoplasmic vaccuolation.

DISCUSSION

A number of recent studies by Agarwal and associates (15–24) have shown that silymarin possesses significant chemopreventive and anti-cancer activity. Although cell culture studies have revealed many insights concerning the potential direct effects of silymarin exposure on cancer epithelial cells with respect to growth and survival signaling and cell cycle regulation, there has been no published work to address the potential impacts of silymarin on vascular endothelial cells and angiogenesis. The results of the present study support a potential anti-angiogenic activity of silymarin. Because tumor epithelial cells *in vivo* depend on angiogenesis to provide nutrients for their growth and survival, it is plausible that an anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity of silymarin.

In the present study, first, silymarin inhibited endothelial cell growth and survival through induction of apoptosis in a concentration dependent manner (Fig. 1). Because angiogenic factor-stimulated proliferation of endothelial cells is crucial for capillary sprouting, growth inhibition and apoptosis induction can be one mechanism for silymarin to inhibit angiogenic response. Second, silymarin inhibited endothelial MMP-2 secretion and expression (Fig. 2) and such an effect occurred rapidly prior to the onset of any morphological changes. Because matrilytic activity of angiogenically-stimulated endothelial cells via MMP-2 is another important requirement for capillary sprouting (28–31), the inhibition of MMP-2 secretion and expression by silymarin may provide an inhibitory mechanism on angiogenesis independent of and/or in addition to endothelial growth arrest and apoptosis. Furthermore, silymarin inhibited *in vitro* capillary formation on Matrigel, a process requiring cell-matrix interaction, inter-cellular communications as well as cell motility, to name a few. It was noteworthy that the inhibitory effect on tube formation manifested whether the treatment was initiated simultaneous with seeding cells on the Matrigel (Fig. 3) or when the tubes had preformed (Fig. 4). These results support an anti-

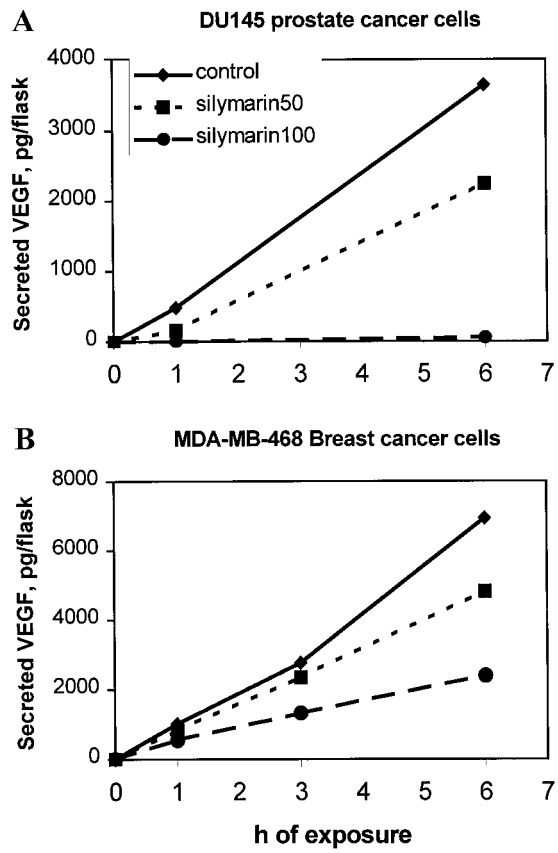

FIG. 5. Representative time course of silymarin effects on VEGF secretion by DU145 prostate cancer cells (A) and MDA-MB-468 breast cancer cells (B) in serum-free media. Confluent cells in T25 flasks were treated with solvent vehicle (DMSO/ethanol) or silymarin in 6 ml serum-free medium. At designated time points, 1-ml aliquots of conditioned media were taken for VEGF assay by ELISA. Each data point represents the mean of triplicate measurements. SD < 5% of respective means.

angiogenic potential for silymarin through multifaceted effects on endothelial proliferation and survival and matrix degradation activity and the capillary differentiation process. Work is in progress to further substantiate an anti-angiogenic effect of silymarin in other human endothelial cells in culture and in *in vivo* models.

In addition to these inhibitory effects on endothelial responses and *in vitro* angiogenesis, silymarin also exerted a rapid inhibitory action on the secretion of VEGF by cancer epithelial cells (Table 1 and Fig. 5). VEGF, also known as vascular permeability factor (VPF) (32, 33), plays several critical roles in vasculogenesis as well as angiogenesis. Its expression is so crucial that germ-line knockout of even one VEGF allele leads to embryonic lethality and homozygous knockout embryonic stem cells are incapable of forming tumors (34, 35). Whereas overexpression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior (36, 37), anti-angiogenic interventions based on VEGF antibodies or interference of signal transduction through its receptors (38–42) have been shown to result in the inhibition of tumor growth and induction of endothelial apoptosis. Transformed epithelial cells have been shown to be the major source of VEGF expression in many types of solid cancers (43–46), however, recent data suggest that stromal cells and even vascular endothelial cells may also express VEGF in the hypoxic angiogenic microenvironment of tumors (47). These findings are supported by the observations that certain oncogenic mutations constitutively upregulate VEGF expression (48–51), and that cancer epithelial hypoxia, as a result of dysregulated cellular proliferation (5), is a potent *in vivo* inducer of VEGF expression (52, 53). The inhibitory effect of silymarin on secretion of VEGF in cancer epithelial cells, therefore, may be an important mechanism to negatively regulate the angiogenic switch of avascular lesions, further contributing to the overall control of lesion growth and progression.

The manners by which epithelial VEGF and endothelial MMP-2 were inhibited by silymarin are noteworthy and suggestive of a commonality with regard to the mechanisms of action by silymarin on these secretory proteins. In DU145 and MCF-7 cancer cell lines, silymarin exposure decreased secreted VEGF in the conditioned media without a reduction of cellular VEGF protein level (Table 1). In the MDA-MB468 cell line, exposure at low to intermediate levels of silymarin (25 or 50 μg/ml) decreased secreted VEGF level without lowering the cellular VEGF content, and only at the higher exposure level (100 μg/ml) a reduction of cellular VEGF level was observed (Table 1). This pattern was similarly to that observed for HUVEC MMP-2 expression in that an intermediate level of silymarin exposure (50 μg/ml) significantly decreased secreted MMP-2 level without a change in cellular MMP-2 (Fig. 2). These results from both epithelial and endothelial cells suggest that a primary action of silymarin may involve preferential targeting of the secretion and/or export (exocytosis) of these proteins critical for angiogenic switch regulation. We are currently investigating such mechanisms.

When the results of the present study showing cell death effect of silymarin on HUVEC were compared to those published by us showing anti-proliferative, but not cytotoxic and apoptotic effects, in several different human carcinoma and normal epithelial cells (20–24), it is important to emphasize here that apoptotic effect of silymarin is possibly specific to vascular endothelial cells. Based on these results, there is a possibility that on one hand silymarin is an anti-proliferative and a differentiation-inducing agent for cancer epithelial cells and on the other hand is both an anti-proliferative and an apoptogenic agent for vascular endothelial cells that are involved in neo-vascularization. These dual effects of silymarin possibly make it a useful agent for the prevention and therapy of epithelial cancers in humans.

In summary, this study, for the first time, documents the inhibitory actions of silymarin on several angiogenic responses, including growth and survival, MMP-2 expression and *in vitro* angiogenesis, of vascular endothelial cells as well as an inhibitory effect on the secretion of a primary angiogenic cytokine VEGF by cancer epithelial cells. The anti-angiogenic activity reported in this paper combined with the previously published multi-faceted broad spectrum anti-cancer effects of silymarin support the merit of further investigations to assess and define its cancer chemopreventive and/or therapeutic potential for humans.

ACKNOWLEDGMENTS

This work was supported, in part, by U.S. Army Medical Research and Material Command Grants 99-1-9061 (to J.L.) and 98-1-8588 (to R.A.).

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