Inhibition of Cytokine-Induced Microvascular Arrest of Tumor Cells by Recombinant Endostatin Prevents Experimental Hepatic Melanoma Metastasis

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ABSTRACT

We investigated effects of endostatin (ES) in the prometastatic microenvironment of inflammation occurring during the microvascular phase of cancer cell infiltration in the liver. We used a model of intrasplenic injection of B16 melanoma (B16M) cells leading to hepatic metastasis through vascular cell adhesion molecule-1 (VCAM-1)-mediated capillary arrest of cancer cells via interleukin-18 (IL-18)-dependent mechanism. We show that administration of 50 mg/kg recombinant human (rh) ES 30 min before B16M, plus repetition of same dose for 3 additional days decreased metastasis number by 60%. A single dose of rhES before B16M injection reduced hepatic microvascular retention of luciferase-transfected B16M by 40% and inhibited hepatic production of tumor necrosis factor α (TNF-α) and IL-18 and VCAM-1 expression by hepatic sinusoidal endothelium (HSE). Consistent with these data, rhES inhibited VCAM-1-dependent B16M cell adhesion to primary cultured HSE receiving B16M conditioned medium, and it abolished the HSE cell production of TNF-α and IL-18 induced by tumor-derived vascular endothelial cell growth factor (VEGF). rhES abrogated recombinant murine VEGF-induced tyrosine phosphorylation of KDR/flk-1 receptor in HSE cells, preventing the proinflammatory action of tumor-derived VEGF on HSE. rhES also abolished hepatic production of TNF-α, microvascular retention of luciferase-transfected B16M, and adhesion of B16M cells to isolated HSE cells, all of them induced in mice given 5 µg/kg recombinant murine VEGF for 18 h. This capillary inflammation-deactivating capability constitutes a nonantiangiogenic antitumoral action of endostatin that decreases cancer cell arrest within liver microvasculature and prevents metastases promoted by proinflammatory cytokines induced by VEGF.

INTRODUCTION

The capillary arrest of circulating cancer cells at target organs (1) constitutes an early stage of the metastasis process that precedes proliferation of cancer cells along a subsequent angiogenesis-dependent vascular stage (2). Proinflammatory cytokine-induced cell adhesion molecules play a role in this microvascular phase of metastasis (3), and specific blockade of such molecules causes metastasis inhibition (4, 5). This implies the critical role of microvascular arrest of cancer cell in the initiation of metastasizes and suggests that metastasis prevention is feasible if one targets the microvascular stage of cancer cell dissemination. Little information exists on endogenous molecules that are able to down-regulate the early stages of tumor dissemination leading to arrest and metastasis. Endostatin (ES), a potent endogenous regulator of vascular quiescence (6), increases in the blood of cancer patients at high risk of progression (7, 8) and has also been reported to increase upon onset of experimental hepatic colonization of cancer cells (9). Despite many studies on antitumoral effects of exogenous ES (6), specific action of ES at early preangiogenic stages of organ colonization by cancer cells has not been reported.

The aim of this work was to investigate effects of ES on the prometastatic microenvironment caused by inflammation during the microvascular phase of cancer cell infiltration at target organs (3, 10). We used a well-established hepatic metastasis model where the majority of intrasplenically injected B16 melanoma (B16M) cells generate metastases through a vascular cell adhesion molecule-1 (VCAM-1)-mediated capillary arrest (11), which is up-regulated by a tumor-induced cytokine cascade leading to hepatic sinusoidal cell production of interleukin (IL)-18 (12). In this study, a potent inhibitory activity of recombinant human (rh)ES on the early microvascular stage of the hepatic colonization of B16M cells is shown. This effect depends on the ability of ES to abrogate interleukin (IL)-18-dependent endothelial cell activation induced by tumor-derived vascular endothelial growth factor (VEGF; Refs. 13, 14). We report for the first time that early administration of rhES before full blown angiogenesis and inflammation can decrease microvascular arrest of circulating cancer cells preventing metastasis development.

MATERIALS AND METHODS

Culture of B16M Cells. B16M cells (B16F10 subline) were cultured in endothxin-free DMEM supplemented with 10% FCS and penicillin-streptomycin (Sigma Chemicals Co., St. Louis, MO). Cultures were maintained and passaged as described previously (5). B16M-conditioned medium (B16M-CM) was prepared as follows: 5 × 10⁶ cells were plated in a 25-cm² T-flask and cultured in the above conditions for 12 h. B16M cell supernatants were collected, 25% fresh serum-free medium supplemented, and 0.22-µm filtered, and endotoxin tested by the Limulus lysate assay.

Hepatic Metastasis Model. Syngeneic C57BL/6j mice (male, 6–8 weeks old) were obtained from IFFA Credo (L’Arbresle, France). Animal housing, their care, and experimental conditions were conducted in conformity with institutional guidelines that are in compliance with the relevant national and international laws and policies. Hepatic metastases were produced by the intrasplenic injection into anesthetized mice (Nembutal, 50 mg/kg i.p.) of 3 × 10⁵ viable B16M cells suspended in 0.1 ml of HBSS (5). Mice were killed by cervical dislocation on the twelfth day after the injection of cancer cells. Liver tissue was processed for histology. Fifteen 4-µm thick tissue sections of formaldehyde-fixed liver (five groups, separated 500 µm) were stained with H&E. An integrated image analysis system (Olympus Microimage 4.0 capture kit) connected to an Olympus BX51TF microscope was used to quantify the number, average diameter, and position coordinates of metastases. Percentage of liver volume occupied by metastases and metastasis density (foci number/100 mm³) were also determined (5). Each experiment was carried out three times.

rhES Treatment Schedule. rhES was kindly supplied by EntreMed, Inc. (Rockville, MD). It was produced in yeast with complete biochemical characterization described previously (15). Control recombinant protein was expressed in the same yeast system, purified by similar processes, was of similar molecular size to rhES and has been described previously (15). Mice were...
given injections of either vehicle [100 μl of citrate-phosphate buffer: 66 mM sodium phosphate, 17 mM citric acid, 59 mM NaCl (pH 6.2)] or rhES (50 mg/kg) s.c. at different time periods relevant to the intrasplenic injection of B16M cells.

**Luciferase Assay.** B16M cells were stably transfected by lipofection as previously described (16), using plasmid pRc/cytomegalovirus-luciferase, a construct containing the *Photinus pyralis* luciferase gene coding sequence under transcriptional control of the cytomegalovirus promoter and the neomycin-resistance gene encoding resistance to the G418 antibiotic (Life Technologies, Inc., Gaithersburg, Maryland). A total of 300,000 viable luciferase-transfected B16M cells was intraspinically injected into C57BL/6J mice (*n* = 30). Some of the mice (*n* = 15) received 50 mg/kg rhES 30 min before luciferase-transfected B16M cells. All mice were killed by cervical dislocation 18 h later, and livers were processed to measure luciferase activity by chemiluminescence using the standard luciferase assay kit (Promega Co., Madison, WI), as described previously (16). Production of light was measured using a luminometer designed to read individual sample tubes (Bio-Rad, LKB Wallace; Turku, Finland) after the addition of 100 μl of luciferase assay reagent to 20 μl of each liver homogenate. Light detector measurements were expressed in relative light units, which were proportional to photon numbers. Linearity and sensitivity of light detection in liver homogenates and influence of hepatic microenvironment on luciferase activity of luciferase-transfected B16M cells were also evaluated as described previously (16).

**Isolation of Hepatic Sinusoidal Cells and Enriched Primary Culture of Endothelial Cells.** Syngeneic C57BL/6J mice (male, 6–8 weeks old) were used as above. HSE cells were separated from these mice, identified, and cultured as described previously (17). Briefly, hepatic tissue digestion was carried out by sequential perfusion of Pronase and collagenase plus Pronase solutions. The liver was then minced and stirred in another solution containing Pronase, collagenase, and DNsase. Sinusoidal cells were separated in a 17.5% (w/v) metrizamide gradient and incubated in glutaraldehyde-treated human albumin-coated dishes for 30 min as a selective adherence step for Kupffer cells. Nonadherent sinusoidal cells were replated on type I collagen-coated 24-well plates, at 1 × 106 cells/ml/well, and 2 h later were rewarshed. HSE cell purity of resulting sinusoidal cells was around 95%, as checked by previously used identification parameters (17): positive endocytosis (acetylated low-density lipoprotein; ovalbumin); negative phagocytosis (1–2 μm latex particles and CD45 antigen; positive lectin binding site expression (wheat germ and *N*annexin); and late-stage CD31 antigen. Immunoreactive bands were visualized using a chemiluminescent substrate. Concentrations were measured in serum from hepatic blood and in supernatants of liver homogenates. Light detector measurements were expressed in relative light units, which were proportional to photon numbers. Linearity and sensitivity of light detection in liver homogenates and influence of hepatic microenvironment on luciferase activity of luciferase-transfected B16M cells were also evaluated as described previously (16).

**Western Blot Analysis of VCAM-1.** Freshly isolated HSE cells were washed with PBS and disrupted with radiomimunoprecipitation assay buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 2 mM EDTA, 10 mM NaF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonfluor fluoride]. Then, 40 μg of protein from cell lysates were separated by 12% SDS-PAGE under reducing conditions and blotted to a hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom). Blots were blocked using a 5% solution of nonfat dry milk in PBS and incubated with rat anti-VCAM-1 monoclonal antibody and with anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 with PBS-5% milk during 2 h. Then, blots were washed three times (PBS/0.1% TWEEN), and the secondary antibody, peroxidase-conjugated goat antirabbit IgG (Jackson ImmunoResearch, Baltimore, PA) diluted 1:5000 with PBS-5% milk, was added for another 2 h. Finally, after washing the blots, VCAM-1- and β-tubulin proteins were visualized using chemiluminescence (West LumiGlo System, DuPont) on X-ray film. Blots were post hoc analyzed using Adobe Photoshop scanning and densitometrically analyzed using the NIH image analysis program for Macintosh.

**B16M Cell Adhesion Assay to Primary Cultured HSE.** B16M cells were labeled with 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein-acetoxy-methylester solution (Molecular Probes, Eugene, OR) and added (2 × 104 cells/well) to 24-well plate primary culture of HSE cells, and 8 min later, wells were washed three times with fresh medium. The number of adhering cells was determined using a quantitative method based on a previously described fluorescence measurement system (5). In some experiments, HSE cells were treated with B16M-CM, preincubated or not with 1 μg/ml antimurine VEGF monoclonal antibody (R&D Systems, Minneapolis, MN) at 37°C for 30 min. In other experiments, HSE cells were incubated with 10 μg/ml rhES for 30 min and then, B16M-CM or 10 ng/ml recombinant murine VEGF165 or 1 ng/ml recombinant murine IL-18 (R&D Systems, Minneapolis, MN) were added for 12 h. In addition, some wells received 10 μg/ml antimouse IL-18 antibody (Peprotech Ltd., London, United Kingdom) 30 min before VEGF treatment. Finally, 10 μg/ml anti-VCAM-1 antibody (Serotec Ltd., Oxford, United Kingdom) were added to some HSE cells 30 min before B16M cell addition. Anti-VCAM IgG was added at a similar concentration and time to check the specificity of the anti-VCAM-1 antibody.

**Measurement of Tumor Necrosis Factor α (TNF-α) and IL-18 Concentration in Hepatic Blood and HSE Supernatants.** TNF-α and IL-18 concentrations were measured in serum from hepatic blood and in supernatants from primary cultured HSE cells using an ELISA kit based on specific murine TNF-α and IL-18 monoclonal antibodies, as suggested by the manufacturer (R&D Systems, Minneapolis, MN).

**Immunoprecipitation and Western Blotting of KDR/FK-1.** Freshly isolated HSE cells were incubated with 10 μg/ml rhES for 30 min, and then, cells were stimulated by the addition of 10 ng/ml recombinant murine VEGF165 for 20 min. Untreated HSE cells received basal medium. After stimulation, cells were lysed in 1 ml of lysis buffer [50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 15 mM magnesium chloride, 0.5% NP40, 10 mM sodium fluoride, 1 mM Na3VO4, 10% glycerol, 1% Triton X-100, and protease inhibitor mixture tablets as suggested by the manufacturer (Roche Diagnostics, Mannheim, Germany)]. Lysates were clarified by centrifugation at 15,000 × g for 10 min, and the resulting supernatants were immunoprecipitated with 1 μg/ml anti-KDR/FK-1 polyclonal antibody (Santa Cruz Biotechnology) at 4°C for 2 h, followed by the addition of protein G-Sepharose (Amersham Pharmacia, Uppsala, Sweden) beads at 4°C for 2 h. Then, cell immunoprecipitates were washed three times with lysis buffer, solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, run in SDS-PAGE gel, and transferred to a nitrocellulose membrane (Amersham Life Science, Little Chalfont). The blocked membranes were then incubated with rabbit anti-phosphotyrosine monoclonal antibody (Upstate, Lake Placid, NY) or mouse anti-KDR/FK-1 polyclonal antibody (Santa Cruz Biotechnology). Then, blots were washed three times (PBS/0.1% Tween) and incubated with the secondary antibodies, horseradish peroxidase-conjugated goat antirabbit, or antiimmunoglobulin, respectively. Immunoreactive bands were visualized using a chemiluminescent substrate.

**Statistical Analyses.** Data were expressed as means ± SD. Statistical analysis was performed by SPPS statistical software for Microsoft Windows, release 6.0 (Professional Statistic, Chicago, IL). Homogeneity of the variance was tested using the Levene test. If the variances were homogeneous, data were analyzed by using one-way ANOVA test with Bonferroni’s correction for multiple comparisons when more than two groups were analyzed. For data sets with nonhomogeneous variances, ANOVA test with Tamhane’s post hoc analysis was applied. Individual comparisons were made with Student’s two-tailed, unpaired test (program Statview 512; Abacus Concepts, Inc., for Macintosh). The criterion for significance was *P* < 0.05 for all comparisons.

**RESULTS**

**Reduced Microvascular Arrest of Circulating Melanoma Cells Accounts for Early Antimetastatic Effects of ES in the Liver.** A s.c. administration of 50 mg/kg rhES 30 min before intrasplenic injection of B16M cells plus repetition of same dose for 3 additional days notably decreased metastasis occurrence and burden in liver (Fig. 1A). Morphometrical sorting of metastatic foci by number and size to determine metastasis density (number of foci/100 mm2) and volume (percent organ occupancy) revealed that rhES significantly (*P < 0.01*) decreased metastasis numbers by 60% and the liver volume occupied by metastases by 80%, as compared with controls (Fig. 1B). Because microvascular arrest plays a critical role in the metastatic implantation of circulating cancer cells (1), the possible effect of rhES on this mechanism was next evaluated in the liver microvasculature from
mice given 50 mg/kg rhES 30 min before luciferase-transfected B16M cell injection. Consistent with metastasis data, the intrahepatic retention of luciferase-transfected B16M cells also significantly (*P < 0.01) decreased by 40% on the 18th h after injection, as compared with mice given vehicle alone (Fig. 1C).

ES Inhibits Hepatic Sinusoidal Cell Expression of VCAM-1 and Production of TNF-α and IL-18 Induced by Melanoma-Derived VEGF. VCAM-1 expression that mediates cytokine-induced B16M cell adhesion to HSE cells (11) increased by 40% in HSE cells that had been isolated from B16M cell-injected mice, as compared with HSE cells from vehicle-injected mice (Fig. 1D). This was completely abrogated in mice given 50 mg/kg rhES 30 min before B16M cells, indicating that a mechanism blocked by rhES accounted for the proadhesive endothelial cell response to B16M cell infiltration.

Previously, we have reported that TNF-α-dependent IL-1β up-regulates VCAM-1 expression via IL-18-dependent mechanism in HSE cells activated by the supernatant of cultured B16M cells (12), and that IL-18 augmentation in hepatic blood during hepatic colonization of B16M cells accounts for increased VCAM-1 steady-state mRNA and protein synthesis in HSE cells isolated from B16M cell-injected mice under similar conditions as in Fig. 1D (18). On this basis, the effect of rhES treatment on hepatic production of TNF-α and IL-18 was next analyzed in vivo and in vitro. As shown in Fig. 2, A and B, production of hepatic TNF-α and IL-18 induced by infiltrating tumor cells was completely abrogated in mice given 50 mg/kg rhES 30 min before B16M cell injection, although baseline production of these cytokines was not affected. Therefore, exogenous ES prevented production of major proinflammatory cytokines from cancer cell-infiltrated livers in vivo.

We also found that TNF-α and IL-18 increased by 6- and 3-fold, respectively, in the supernatant of primary cultured HSE cells given the CM from B16M cells (B16M-CM; Fig. 2, C and D). Again, rhES (10 μg/ml) completely abrogated cytokine production from B16M-CM-treated HSE cells without affecting basal secretion in untreated cells. This confirms that a mechanism blocked by rhES accounted for TNF-α and IL-18 production in tumor-activated HSE cells in vitro. In this regard, B16M-CM was devoid of detectable levels of IL-18, TNF-α, and other proinflammatory cytokines. However, similar to other melanoma cell lines (14), VEGF can be detectable at an appreciable concentration (70 ± 5 pg/l × 10^6 cells) in the supernatant of basal condition-cultured B16M cells. Interestingly, complete neutralization of tumor-derived VEGF by preincubation of B16M-CM with
1 μg/ml antimume VEGF antibody for 30 min before addition to HSE suppressed both TNF-α and IL-18 production from B16M-CM-treated HSE cells, indicating that release of these cytokines from tumor-activated HSE was tumor VEGF dependent. More importantly, the treatment of HSE cells with 10 ng/ml recombinant murine (rm)VEGF also resulted in 8- and 3-fold increase (P < 0.01) of TNF-α and IL-18 concentration, respectively, in the supernatant. However, addition of rhES before rmVEGF prevented these effects (Fig. 2, C and D).

ES Inhibits IL-18-Induced VCAM-1 through KDR/Flk-1 Tyrosine Phosphorylation Blockade in Hepatic Sinusoidal Endothelium Activated by Recombinant and Tumor-Derived VEGF

We had previously reported that B16M-CM contains an IL-18-inducing activity, which increases VCAM-1 expression from HSE cells and enhances their adherence to B16M cells in vitro (11, 12). Herein, primary cultured HSE cells given 10 μg/ml rhES 30 min before B16M-CM incubation for 12 h completely abrogated its proadhesive activity (Fig. 3A). Enhanced B16M cell adhesion to B16M-CM-treated HSE cells was also completely abrogated when B16M-CM was preincubated with 1 μg/ml antimume VEGF antibody for 30 min before be added to HSE cells. These data suggest that VEGF is involved in the reported IL-18-inducing activity of B16M cells and that rhES is blocking the proadhesive effect of tumor-derived VEGF on HSE. As shown in Fig. 3B, rhES abolished the increased adhesion of B16M cells to rmVEGF-treated HSE cells. As expected, the increased adhesion was also abrogated by 10 μg/ml antimume IL-18 antibody. In addition, IL-18-dependent B16M cell adhesion to rm-
VEGF-treated HSE was VCAM-1-mediated, as demonstrated in HSE cells given antimurine VCAM-1 antibody before rmVEGF (Fig. 3B). Moreover, exogenously added rmIL-18 (1 ng/ml), but not rmVEGF (10 ng/ml), reversed the inhibitory effect of rhES on B16M-CM-treated HSE cells (Fig. 3C). Conversely, rhES was not able to decrease B16M cell adhesion to HSE cells given 1 ng/ml rmIL-18 for 6 h. Therefore, the proadhesive effect of B16M-CM on HSE cells was produced through VEGF-induced IL-18, and rhES inhibited this mechanism through the selective blockade of VEGF-induced IL-18 but without interfering with the subsequent proadhesive action of paracrine IL-18 (Fig. 3C). Interestingly, rmVEGF induced tyrosine phosphorylation of KDR/Flk-1 receptor expressed on primary cultured HSE cells. However, this phosphorylation was prevented in HSE cells receiving rhES 30 min before rmVEGF, indicating that rhES blocks VEGF-mediated signaling via direct or indirect interaction with its KDR/Flk-1 receptor in tumor-activated HSE cells (Fig. 3D).

ES Inhibits VEGF-Induced Hepatic Production of TNF-α, Microvascular Retention of B16M Cells, and ex Vivo Adhesion of B16M Cells to HSE. The capillary inflammation-deactivating capability of ES was additionally confirmed in vivo (Fig. 4). Mice received 50 mg/kg rhES 30 min before being treated with 5 μg/kg i.p. rmVEGF for 18 h. Similar to B16M cell-injected mice (Fig. 2A), hepatic production of TNF-α significantly (P < 0.01) increased in response to rmVEGF treatment compared with vehicle administration, and rhES injection before rmVEGF completely abrogated its TNF-α-stimulating effect in vivo (Fig. 4A). rhES also neutralized enhanced microvascular retention of luciferase-transfected B16M cells occurring in rmVEGF-treated mice (Fig. 4B). Moreover, ex vivo adhesion of B16M cells to primary cultured HSE cells isolated from VEGF-treated mice also significantly (P < 0.01) increased by a rHES-inhibitable mechanism (Fig. 4C). This confirms that VEGF-induced hepatic microvascular retention of B16M cells was due to its proadhesive effects on HSE cells, as above reported in vitro (Fig. 3).

DISCUSSION

ES is a fragment of collagen XVIII that inhibits angiogenesis and tumor growth (6, 15). The present study was undertaken to analyze the effect of ES during the microvascular phase of cancer cell arrest in a target organ occurring in the process of metastasis before angiogenesis activation. We demonstrate that ES also significantly decreases development of metastatic lesions at this early nonangiogenic phase. The action of early ES treatment involves a decrease of both proinflammatory cytokine production and VCAM-1 expression by tumor-infiltrated livers that correlate with decreased cancer cell retention within liver microvasculature. Consistent with in vivo data, ES abolished TNF-α and IL-18 production from primary cultured HSE given CM from B16M cells and prevented proadhesive response of tumor-activated HSE. This antimetastatic effect of ES resulted from its specific inhibitory action on TNF-α and IL-18 production by HSE in response to tumor-derived VEGF. It appears that ES prevents VEGF-induced tyrosine phosphorylation of KDR/Flk-1 receptor expressed on primary cultured HSE cells, blocking VEGF-mediated signaling. This capillary inflammation-deactivating capability of ES was additionally confirmed in a model of hepatic melanoma metastasis induced by mouse pretreatment with recombinant VEGF before cancer cell injection. Thus, this study shows for the first time that rhES’s tumor inhibitory capabilities include mechanisms other than those directly affecting angiogenic endothelial cells per se such as apoptosis, migration, and proliferation. The ability of rhES to abrogate proinflammatory cytokine production from endothelial cells activated by melanoma-derived VEGF may have major implications in deactivating the prometastatic microenvironment of tumor-induced inflammation. In addition, this may have a broader significance if we consider that as the expression of VEGF increases, the probability of metastasis also increases in most of malignant tumors (19).
Despite its reported antitumoral (20) and antiangiogenic (21) activities, we found that IL-18 plays a major prometastatic role in this murine melanoma model (18). Production of IL-18 by tumor-activated HSE cells leads to up-regulation of VCAM-1 expression via H2O2-dependent mechanism, which promotes B16M cell adhesion to HSE cells and accounts for oxidative stress-dependent hepatic B16M metastases (11). IL-18 also up-regulates the expression of Fas ligand, which jeopardizes antitumoral effects of lymphocytes and promotes the immune escape of B16M cells (22). Moreover, IL-18 increases proliferation rate of both human and murine melanoma cells (18) and increases endothelial cell migration (23). Our current data suggest that these IL-18-dependent mechanisms are inhibited in rhES-treated mice, resulting in early regression of preangiogenic micrometastases. Similar colonization events were developed under VEGF/IL-18 effects by other murine tumors, including C26 and 51b colon adenocarcinoma and PAM-LY2 squamous carcinoma, and were also inhibited by rhES (unpublished observations). However, rhES did not completely inhibit metastatic activities of B16M and other studied tumors. This may be because of the phenotypic heterogeneity of these tumors with respect to metastatic behavior (24). In fact, IL-1-dependent and independent melanoma metastases are formed in the liver and other target organs (25). Moreover, an incomplete hepatic B16M metastasis eradication was also obtained in IL-18 binding protein-treated mice (18) and in IL-1β-converting enzyme-KO mice lacking mature IL-1β and IL-18 (12). However, the fact that rhES decreased metastasis volume by 80% and metastasis number by 60% while it only decreased microvascular arrest of metastatic cells by 40% suggests that ES’s effects block the hepatic colonization mechanism used by cancer cell variants possessing the highest metastatic potential.

We do not know how rhES prevents cytokine production from tumor-activated HSE cells. ES binds to several cell surface molecules, including heparan sulfate proteoglycans, glypicans, and integrins. Recent studies have pointed to integrins αvβ3 and αvβ5 (26, 27) as functional receptors mediating ES action on endothelial cells. Freshly isolated HSE cells basally express αvβ5 mRNA (as shown by reverse transcription-PCR, unpublished observations). This integrin may be blocked by rhES, which would prevent intracellular signaling of KDR/Fk1-receptor bound to its ligand, as reported previously (28). Another unexplored possibility comes from our recent observation that antinmouse LFA-1 antibodies inhibit HSE cell attachment to an immobilized rhES substrate. Interestingly, IL-18 production from either rmVEGF- or B16M-CM-treated HSE cells is soluble ICAM-1-dependent, and antinmouse LFA-1 antibody given to HSE cells before either B16M-CM or rmVEGF also completely abrogates IL-18 production (unpublished observations). Thus, the possibility that rhES interferes with the proinflammatory HSE activation by VEGF through a soluble ICAM-1-mediated mechanism should be investigated.

These rhES’s inhibitory effects on hepatic endothelial cell response to VEGF may have clinical implication for melanoma metastasis chemoprevention in the liver and other organs developing inflammation-dependent metastases (25). Not surprisingly, increased VEGF expression may aid in predicting patients at risk for metastasis from colon cancer (29, 30). Moreover, development of pulmonary metastases was also strongly inhibited in DC-Fk1-immunized mice challenged with B16M (31). According to present results, this may be because of the VEGF’s capability to induce adhesion molecule expression on endothelial cells via cytokine-dependent mechanism (13). However, this mechanism of early dissemination was inhibited by rhES in the current model, suggesting that administration of ES to patients bearing VEGF-overexpressing tumors at high risk of progression may help in preventing metastasis. Other candidate patients may be those surgically treated from primary tumor and those with no lymph node involvement but having detectable levels of circulating cancer cells.

This article suggests a new direction for better understanding of the biology of this endogenous product of collagen XVIII cleavage and provides impetus to address other methods to enhance tumor inhibition and regression in general. The fact that rhES potently affects the powerful arm of the inflammatory response induced by VEGF implies that ES’s clinical efficacy extends beyond angiostatic properties. Future studies should address antiadhesive and antiproteolytic effects of endostatin in the microenvironment of invasive tumors.

REFERENCES


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