Epigenetic Regulation of Tumor Endothelial Cell Anergy: Silencing of Intercellular Adhesion Molecule-1 by Histone Modifications

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Abstract

Tumors can escape from immunity by repressing leukocyte adhesion molecule expression on tumor endothelial cells and by rendering endothelial cells unresponsive to inflammatory activation. This endothelial cell anergy is induced by angiogenic growth factors and results in reduced leukocyte-vessel wall interactions, thereby attenuating infiltration of leukocytes into the tumor. This report describes a novel mechanism of endothelial cell anergy regulation. We recently reported that DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors have angiostatic activity. Here, we studied whether epigenetic mechanisms regulate this angiogenesis-mediated escape from immunity. We found that DNMT inhibitors 5-aza-2′-deoxycytidine and zebularine, as well as HDAC inhibitor trichostatin A, reexpressed intercellular adhesion molecule-1 (ICAM-1) on tumor-conditioned endothelial cells *in vitro*, resulting in restored leukocyte-endothelial cell adhesion. In addition, treatment with DNMT or HDAC inhibitors *in vivo* also restored ICAM-1 expression on tumor endothelial cells from two different mouse tumor models. Furthermore, leukocyte-vessel wall interactions in mouse tumors were increased by these compounds, as measured by intravital microscopy, resulting in enhanced leukocyte infiltration. We show that ICAM-1 down-regulation in tumor endothelial cells is associated with *ICAM-1* promoter histone H3 deacetylation and loss of histone H3 Lys^4 methylation but not with DNA hypermethylation. In conclusion, our data show that *ICAM-1* is epigenetically silenced in tumor endothelial cells by promoter histone modifications, which can be overcome by DNMT and HDAC inhibitors, suggesting a new molecular mechanism based on which novel therapeutic approaches for cancer can be pursued. (Cancer Res 2006; 66(22): 10770-7)

Introduction

Leukocyte rolling on, adhesion to, and diapedesis through the tumor vessel wall are processes of key importance to immune surveillance, as well as to immunotherapy, a well-established anticancer approach (1). Leukocyte-vessel wall interactions are mediated by endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and CD34 (2). Interference in the expression of endothelial cell adhesion molecules is one of the mechanisms tumors have developed to escape the immune response. We and others have shown previously that by producing angiogenic factors, such as vascular endothelial cell growth factors (VEGF) and basic fibroblast growth factors (FGF), tumors down-regulate vascular adhesion molecule expression (3–6). This angiogenesis-mediated endothelial cell anergy to inflammatory signals results in diminished leukocyte-vessel wall interactions and, therefore, decreased inflammatory infiltration (7, 8).

Epigenetic mechanisms play a crucial role in regulation of gene expression by affecting chromatin accessibility. Different epigenetic processes are interconnected in gene silencing (9). DNA methylation and histone modifications are two important epigenetic mediators of transcriptional repression (10, 11). Aberrant epigenetic regulation is a frequent event in cancer cells, where DNA hypermethylation and histone deacetylation within the promoters of tumor suppressor genes result in undesirable gene silencing (12–14). Due to the reversibility of epigenetic events, drugs that inhibit DNA methyltransferases (DNMT) or histone deacetylases (HDAC) can synergistically reactivate epigenetically silenced tumor suppressor genes, thereby suppressing tumor cells *in vitro* and *in vivo* (14, 15). Considerable promise lies in the further development of epigenetic therapies that already have shown antitumorigenic effects for several malignancies (16–18).

In contrast to the increasing knowledge on epigenetic aberrations in tumor cells, there is almost nothing known about the role of DNA methylation and histone modifications in regulation of gene expression in tumor endothelial cells. Recently, we and others have shown that DNMT and HDAC inhibitors are potent angiostatic agents that inhibit endothelial cell growth *in vitro* and *in vivo* (19, 20). Because regulation of adhesion molecule expression in tumor endothelial cells is pivotal to antitumor immunity, and because ICAM-1 is the key endothelial cell

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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adhesion molecule (21), we investigated whether epigenetic mechanisms are involved in the regulation of ICAM-1 expression in tumor endothelial cells. Here, it is reported for the first time that epigenetic events regulate adhesion molecule expression and leukocyte infiltration in tumors. We found that ICAM-1 expression in tumor endothelial cells and leukocyte-endothelial cells adhesion are restored by DNMT- and HDAC inhibitors, resulting in enhanced inflammatory infiltration. Our results show that *ICAM-1* is epigenetically silenced in angiogenically stimulated endothelial cells through promoter histone modifications.

Materials and Methods

Cells, cultures, and reagents. Human umbilical vein endothelial cells (HUVEC), mouse b.END5 brain endothelioma cells (European Collection of Animal Cell Cultures, Salisbury, United Kingdom), mouse B16F10 melanoma cells (kindly provided by Dr. J. Fidler, Houston, TX), and human LS174T colon tumor cells were cultured as previously described (7, 19). Quiescent endothelial cells were prepared by culturing for 3 days in the presence of 2% serum. Tumor conditions were mimicked by a 6-day exposure to 10 ng/mL basic FGF (bFGF; Peprotech, London, United Kingdom) and 10 ng/mL VEGF (Peprotech). During the last 3 days, tumor-conditioned endothelial cells were treated with the DNMT inhibitors 5-aza-2′-deoxycytidine (DAC; 200 nmol/L; ref. 14; Sigma, Zwijndrecht, the Netherlands) or zebularine (100 μmol/L; ref. 22; obtained from National Cancer Institute, Bethesda, MD), or with the HDAC inhibitor trichostatin A (TSA; 300 nmol/L; ref. 14; Wako, Neuss, Germany), replacing drugs and culture medium every 24 hours, as described previously (14, 19). Tumor-conditioned endothelial cells treated during the last 3 days with a combination of DAC and TSA were first treated with DAC (200 nmol/L) for 48 hours, with drug and medium replaced 24 hours after the beginning of the treatment, followed by medium replacement and addition of TSA (300 nmol/L) for a further 24 hours (14, 15). When applied, tumor necrosis factor-α (TNF-α; HUVEC, 4 ng/mL from Peprotech; b.END5, 40 ng/mL from Peprotech) was added 6 hours before harvesting (7).

Fluorescence-activated cell sorting analysis. The expression of ICAM-1 on HUVEC was determined by mouse anti-human ICAM-1 monoclonal antibody (MEM111, Monosan, Uden, the Netherlands), as described previously (8). ICAM-1 expression on b.END5 cells was determined using rat anti-mouse ICAM-1 monoclonal antibody (CD54; R&D Systems, Abingdon, United Kingdom), as described previously (7).

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from cultured cells or frozen tissue sections using the RNeasy RNA isolation kit (Qiagen, Hilden, Germany) according to the supplier's protocol. cDNA synthesis and quantitative real-time reverse transcription-PCR (RT-PCR) were done as described previously (23) using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Primer sequences are listed in Supplementary Table S1.

Adhesion assay. Human peripheral blood leukocytes were isolated by Ficoll density gradient centrifugation (Amersham, Uppsala, Sweden) and labeled with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands). Cells were washed twice and subsequently adhered for 1 hour at room temperature to confluent HUVEC cultures. Nonadhering cells were removed by washing with prewarmed culture medium. Cells were harvested and fixed in 1% paraformaldehyde (Merck, Amsterdam, The Netherlands) for 30 minutes at room temperature. Where indicated, confluent HUVEC cultures were pretreated with the F10.2 anti-ICAM-1 blocking monoclonal antibody (24) for 1 hour. Leukocyte-HUVEC adhesion was measured both by fluorescence activated cell sorting (FACS) analysis by detecting the number of CFSE-labeled leukocytes and by counting under an inverted microscope.

Mouse tumor models and intravital microscopy. All animal experiments were approved by the local ethical review committee. At day 0, 10^5 B16F10 cells or 10^6 LS174T cells were inoculated s.c. on the right flank of 6-week-old C57BL/6 and Swiss *nu/nu* mice (obtained from Charles River, Maastricht, The Netherlands), respectively. Between days 6 and 9 (B16F10) or between days 10 and 14 (LS174T), the tumors became visible, and treatments were initiated. Zebularine ($n = 5$) at doses of 1,000 mg/kg (25) and TSA ($n = 5$) at doses of 1 mg/kg (20) were given daily by i.p. injection in a solution of 0.9% saline for 7 days (B16F10) or 10 days (LS174T). Tumor volumes were measured as described previously (19). Intravital microscopic measurements of B16F10 flank tumors were done after 7 days of treatment. Mice were anesthetized by s.c. administration of a mixture of ketamine (0.1 mg/g body weight; Nimatek; Ad Usem Veterinarium, Cuijk, The Netherlands) and xylazine (0.02 mg/g body weight; Sedamun; Ad Usem Veterinarium). Intravital microscopy was done as described before (7). Body temperature was kept at 37°C by an IR heating lamp. To enable intravital microscopic observation of leukocytes, 10 to 20 μL of a Rhodamine 6G solution (Sigma Chemical Co., St. Louis, MO; 1 mg/mL) was injected into a tail vein. Images were recorded on DVD for offline analysis.

Vessel diameter, centerline blood flow velocity, reduced velocity, local blood flow, leukocyte rolling, and leukocyte adhesion were determined as before (7, 26) and are described in Supplementary Materials and Methods.

Immunohistochemistry. Frozen sections of tumor tissues were stained using rat anti-mouse CD45 (gift from Dr. A. Duijvesteijn, Maastricht, The Netherlands), which was detected by biotinylated donkey anti-rat Ig antibody (Jackson ImmunoResearch Laboratories, Inc., Suffolk, England) and avidin-biotin-horseradish peroxidase complex (DAKO, Heverlee, Belgium). The staining was visualized with 3,3′-diaminobenzidine, and the slides were counterstained with hematoxylin. $CD45^+$ cells were counted in three independent areas in each section (using a 0.25-mm^2 grid at a ×200 magnification) by two independent observers.

Bisulfite sequencing. Genomic DNA was isolated using the Puregene DNA Isolation kit (Gentra Systems, Biozym, Landgraaf, The Netherlands). Bisulfite modification of genomic DNA was carried out as described previously (19). Bisulfite-treated DNA samples were then purified with a Wizard Genomic DNA Purification kit (Promega, Leiden, The Netherlands) and desulfonated before ethanol precipitation. PCR products were cloned using the TA cloning kit (Invitrogen, Breda, The Netherlands), and single colonies were picked and sequenced. Primer sequences are listed in Supplementary Table S2.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays of an *ICAM-1* proximal promoter region (−230 to −56) were done essentially as described (27) using anti-acetyl-histone H3 (Lys^9 and Lys^{14}), or anti-dimethyl-histone H3 (Lys^4) antibody (both from Upstate Biotechnology, Lake Placid, NY). One primer set for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to amplify a 128-bp fragment of the genomic sequence to serve as an internal control (28). All PCR reactions were optimized with input DNA to ensure that PCR products were in the linear range of amplification. Primer sequences are listed in Supplementary Table S3. PCR products were size-separated by agarose gel electrophoresis, and bands were quantified using Molecular Analyst 2.1 software. Enrichment was calculated by taking the ratio between the net intensity of the ICAM-1 PCR product and the net intensity of the GAPDH PCR product for the bound sample and dividing this by the same ratio calculated for the input samples (28).

Statistical analysis. Data obtained from intravital microscopic experiments are presented as medians with interquartile ranges because of their nonsymmetrical distribution. Other data are presented as mean ± SE. Differences between two independent data groups were tested with the Mann-Whitney *U* test using SPSS 10.0.5 software. Correlation between variables was determined using Spearman's correlation test. Statistical analysis for the tumor volumes was done by means of the two-way ANOVA test.

Online supplementary material. Supplementary Tables S1, S2, and S3 show PCR primers used in this study. The Supplementary Materials and Methods describes determination of vessel diameter, centerline blood flow velocity, reduced velocity, local blood flow, leukocyte rolling, and leukocyte adhesion of the intravital microscopy experiments.

Results

DNMT and HDAC inhibitors restore ICAM-1 expression in tumor-conditioned endothelial cells. By releasing angiogenic factors, tumors suppress adhesion molecule expression on tumor endothelial cells, thereby reducing leukocyte-vessel wall interactions and inflammatory infiltration (3, 4, 7). To examine whether epigenetic mechanisms are involved in regulation of adhesion molecule expression on tumor endothelial cells, the effects of DNMT and HDAC inhibitors on endothelial cell adhesion molecule expression were studied *in vitro*. In tumor-conditioned HUVECs, ICAM-1 protein expression was down-regulated by 81% compared with that in quiescent HUVECs ($P < 0.01$; Fig. 1*A*). This is in agreement with previous results (3, 4). Treatment of tumor-conditioned HUVECs with the DNMT inhibitor DAC significantly restored ICAM-1 protein expression ($P < 0.01$). A similar effect was observed after treatment with zebularine, a recently discovered DNMT inhibitor that requires higher effective concentrations (22, 25), or with the HDAC inhibitor TSA ($P < 0.01$). Because DNMTs and HDACs cooperate in gene silencing (15), we further treated tumor-conditioned HUVEC with a combination of DAC and TSA (14). Combined treatment also induced ICAM-1 protein expression ($P < 0.01$), although no synergism was observed (Fig. 1*A*). Decreased protein expression of VCAM-1 and E-selectin in tumor-conditioned HUVEC was also restored by treatment with DNMT and/or HDAC inhibitors (data not shown). Further studies are focused on ICAM-1 because it has been shown that this is the most important endothelial cell adhesion molecule for leukocyte extravasation (21).

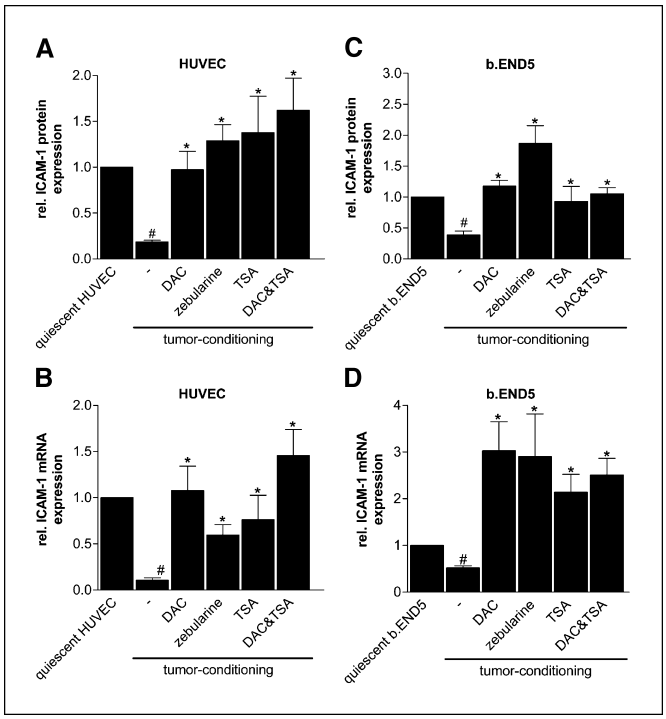

Figure 1. DNMT and HDAC inhibitors restore ICAM-1 expression in tumor-conditioned endothelial cells. *A,* ICAM-1 protein expression measured by FACS analysis in quiescent HUVECs, tumor-conditioned HUVECs, and tumor-conditioned HUVECs treated with DAC (200 nmol/L), zebularine (100 μmol/L), TSA (300 nmol/L), or a combination of DAC and TSA. *Columns,* mean of relative protein expression (quiescent HUVECs set to 1) of at least three independent experiments; *bars,* SE. #, $P < 0.01$ versus quiescent HUVEC; *, $P < 0.01$ versus tumor-conditioned HUVECs. *B,* ICAM-1 mRNA expression measured by quantitative real-time RT-PCR in HUVEC. *Columns,* mean of relative mRNA expression of six independent experiments; *bars,* SE. #, $P < 0.001$ versus quiescent HUVECs; *, $P < 0.05$ versus tumor-conditioned HUVECs. *C,* ICAM-1 protein expression in b.END5 mouse endothelial cells. #, $P < 0.05$ versus quiescent b.END5; *, $P < 0.05$ versus tumor-conditioned b.END5. *D,* ICAM-1 mRNA expression in b.END5 endothelial cells. #, $P < 0.001$ versus quiescent b.END5; *, $P < 0.05$ versus tumor-conditioned b.END5.

Quantitative real-time RT-PCR analysis of ICAM-1 showed similar results, indicating that ICAM-1 protein induction by DAC, zebularine, and TSA results from increased ICAM-1 mRNA levels (Fig. 1*B*). DAC, zebularine, and TSA also significantly restored ICAM-1 protein and mRNA expression in tumor-conditioned HUVECs treated with TNF-α (data not shown; i.e., normalizing the up-regulation to this inflammatory cytokine). We observed similar effects using b.END5 mouse endothelial cells (Fig. 1*C* and *D*). ICAM-1 up-regulation by DNMT and HDAC inhibitors was not observed in B16F10 mouse melanoma cells and normal cultured human fibroblasts (data not shown), indicating that it is not a general effect of these compounds.

DNMT and HDAC inhibitors restore leukocyte-endothelial cell adhesion *in vitro*. To show the functional effect of restored adhesion molecule expression on tumor-conditioned endothelial cells by using DNMT and HDAC inhibitors, we investigated the adhesion of fluorescein-labeled human peripheral blood leukocytes to endothelial cell monolayers. After adding these leukocytes to endothelial cell monolayers and removing nonadherent cells, the adherent leukocytes were counted both by using an inverted microscope (Fig. 2*A*) and by flow cytometry (Fig. 2*B*). In tumor-conditioned HUVEC, leukocyte adhesion was decreased by 75% compared with that using quiescent HUVEC ($P < 0.001$; Fig. 2*A* and *B*). Treatment of tumor-conditioned HUVEC with the DNMT inhibitor DAC or zebularine significantly restored leukocyte adhesion ($P < 0.01$). The same observation was made when endothelial cells were treated with TSA, or with a combination of DAC and TSA ($P < 0.01$). The restored leukocyte-endothelial cell adhesion by these compounds was mainly due to up-regulation of ICAM-1 because a blocking antibody significantly decreased the induction of adhesion by DNMT and HDAC inhibitors (Fig. 2*B*).

Zebularine and TSA induce leukocyte-vessel wall interactions in tumor vessels *in vivo*. To infiltrate a tumor, leukocytes must interact first with the tumor vessel wall. We recently showed that leukocyte-vessel wall interactions are reduced in tumors compared with those in healthy control vessels (7). Here, we examined whether restored leukocyte-endothelial cell adhesion by DNMT and HDAC inhibitors *in vitro* is also observed in tumor vessels *in vivo*. To quantify leukocyte-vessel wall interactions in tumor blood vessels, intravital microscopy was used on immunocompetent B16F10 melanoma-bearing C57BL/6 mice (Fig. 3*A-C*). In B16F10 flank tumors in mice treated with zebularine (the DNMT inhibitor of choice because of its lower toxicity profile and higher stability; ref. 25), both leukocyte adhesion (Fig. 3*A*) and leukocyte rolling (Fig. 3*B*) were significantly increased compared with those in untreated tumors ($P < 0.01$ and $P < 0.001$, respectively). The HDAC inhibitor TSA also significantly restored leukocyte adhesion and rolling in tumor vessels (Fig. 3*A* and *B*).

Vessel diameter and local blood flow did not differ between these groups (Table 1), indicating that observed effects from zebularine and TSA cannot be explained simply by changes in local fluid dynamic conditions. Centerline velocity and reduced velocity were significantly increased in TSA-treated mice compared with those from the control group ($P < 0.05$). However, no correlation between these variables and leukocyte adhesion or rolling could be found in

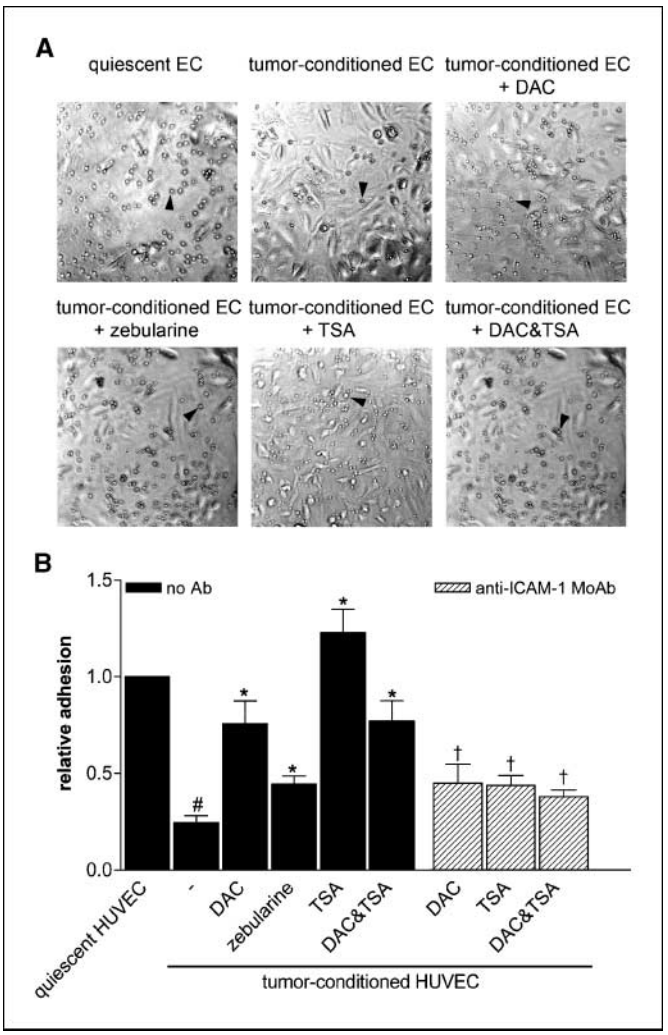

Figure 2. DNMT and HDAC inhibitors restore leukocyte-endothelial cell adhesion *in vitro*. *A,* adhesion of human peripheral blood mononuclear cells to monolayers of quiescent HUVECs, tumor-conditioned HUVECs, and tumor-conditioned HUVECs treated with DAC (200 nmol/L), zebularine (100 μmol/L), TSA (300 nmol/L), or a combination of DAC and TSA. *Arrowheads,* leukocytes. *B,* quantification of adhered CSFE-labeled leukocytes to endothelial cells by flow cytometry. *Columns,* mean of relative adhesion (quiescent HUVECs set to 1) of three independent experiments; *bars,* SE. #, $P < 0.001$ versus quiescent HUVECs; *, $P < 0.01$ versus tumor-conditioned HUVECs. Hatched columns represent relative adhesion after pretreatment of endothelial cells with an anti-ICAM-1 blocking monoclonal antibody. †, $P < 0.05$ versus without ICAM-1 antibody.

these mice. Therefore, observed differences in leukocyte-vessel wall interactions cannot be explained by differences in fluid dynamic variables.

To examine whether DNMT and HDAC inhibitor–induced increased leukocyte-vessel wall interactions *in vivo* were associated with enhanced expression of endothelial cell adhesion molecules, quantitative real-time RT-PCR was done on B16F10 tumor tissues. ICAM-1 expression was significantly induced in B16F10 tumors of mice treated with zebularine or TSA compared with that in untreated mice ($P < 0.001$; Fig. 3*D*). VCAM-1 was also up-regulated in both zebularine- and TSA-treated B16F10 tumors. For E-selectin, there was a significant induction upon zebularine treatment ($P < 0.001$) but not with TSA treatment, which only suggested a trend in the same direction (Fig. 3*D*). Because expression of VCAM-1 and E-selectin is restricted to the endothelial cells of the tumors, increased mRNA levels reflect effects of zebularine and TSA on the expression of these molecules on tumor endothelial cells. ICAM-1, however, is expressed by tumor endothelial cells, as well as by tumor and/or stromal cells. For this reason, enhancement of ICAM-1 expression observed in zebularine- and TSA-treated B16F10 tumor-bearing mice might have been due in part to expression in tumor cells. However, no effects of these compounds were observed on ICAM-1 expression in B16F10 cells *in vitro* (data not shown). We also investigated these effects in the human xenograft model of LS174T colon carcinoma in athymic mice. In this model, human tumors have recruited a vasculature of mouse origin. Using species-specific primers, we developed a technique to discriminate between human (tumor) and mouse mRNAs within the xenograft tumor (23). Using this technique, we found that with zebularine, expression of both ICAM-1 and VCAM-1 was significantly induced in the vasculature of LS174T tumors (Fig. 3*E*; $P < 0.001$). Treatment of LS174T tumor-bearing mice with TSA significantly increased expression of ICAM-1 ($P < 0.05$) but not VCAM-1. In this mouse model, E-selectin mRNA levels in endothelial cells were undetectable.

Leukocyte infiltration is enhanced by zebularine and TSA. Leukocyte-endothelial cell adhesion and leukocyte-vessel wall interactions preceed extravasation and infiltration into the tumor. To study the latter effect, we examined whether increased endothelial cell adhesion molecule expression and leukocyte-vessel wall interactions induced by using zebularine and TSA treatment contribute to an enhanced tumor leukocyte infiltration. Treatment of B16F10 or LS174T tumor-bearing mice with zebularine or TSA significantly decreased tumor growth (Fig. 4*A* and *B*) and microvessel density (data not shown), as we reported previously (19). The number of infiltrating leukocytes in both B16F10 and LS174T tumors was determined by staining for the pan-leukocyte marker CD45. In B16F10 tumors, both zebularine and TSA significantly enhanced the number of infiltrating leukocytes by ~2-fold (Fig. 4*C* and *D*; $P < 0.001$). Comparable results were observed in LS174T tumors ($P < 0.001$ for zebularine and $P < 0.01$ for TSA; data not shown).

ICAM-1 down-regulation in tumor-conditioned endothelial cells is associated with promoter histone H3 deacetylation and loss of H3 Lys^4 methylation. Because ICAM-1 is the primary endothelial cell adhesion molecule (21), regulation of its expression is pivotal to endothelial cell anergy. Reexpression of ICAM-1 by inhibitors of DNA methylation and histone deacetylation suggests that epigenetic mechanisms may be responsible for silencing of this gene in tumor endothelial cells. Therefore, epigenetic modifications in the *ICAM-1* promoter of quiescent and tumor-conditioned HUVEC were examined. Three 5′CpG islands (GC content >60%, ratio of CpG to GpC >0.6, and minimum length of 200 bp; ref. 29) were identified in the *ICAM-1* promoter region (Fig. 5*A*). DNA methylation of *ICAM-1* promoter CpG islands was evaluated by genomic bisulfite sequencing. Interestingly, only a few methylated CpG sites were present in the *ICAM-1* promoter of quiescent- and tumor-conditioned HUVEC (Fig. 5*A*). Furthermore, the *ICAM-1* promoter showed no major differences in methylation patterns between quiescent and activated endothelial cells in the region examined. We also examined DNA methylation of part of the *ICAM-1* promoter (−322 to −17) in tumor endothelial cells obtained from colorectal tumors by laser microdissection. Similar to tumor-conditioned endothelial cells, hardly any methylation was found in the region examined (Fig. 5*A*). These results show that

silencing of ICAM-1 in tumor endothelial cells occurs independently of direct dense promoter methylation.

To study whether ICAM-1 down-regulation in tumor-conditioned endothelial cells is associated with promoter histone deacetylation, we examined acetylation of histone H3 (Lys^9 and Lys^{14}) in the proximal *ICAM-1* promoter region (−230 to −56) by using chromatin immunoprecipitation. Interestingly, *ICAM-1* promoter histone acetylation was significantly decreased in activated HUVEC compared with quiescent HUVECs, correlating with the decreased gene expression (Fig. 5*B*; $P < 0.05$). Treatment of tumor-conditioned HUVEC with DAC, TSA, or a combination of both drugs greatly increased *ICAM-1* promoter histone acetylation, which is associated with gene reactivation induced by these compounds. We also examined another key gene activating histone modification (i.e., Lys^4 methylation of histone H3). This histone modification also was significantly decreased in tumor-conditioned endothelial cells and was increased by DAC and TSA (Fig. 5*B*; $P < 0.05$). This led us to conclude that ICAM-1 down-regulation in tumor-conditioned endothelial cells, and resulting endothelial cell anergy, is associated with loss of promoter histone H3 acetylation and of histone H3 Lys^4 methylation but not with DNA hypermethylation.

Discussion

Suppression of endothelial cell adhesion molecule expression that leads to reduced leukocyte-vessel wall interactions and leukocyte infiltration is one of the mechanisms tumors have developed to escape from immunity (5, 7, 8). This endothelial cell anergy is mediated by angiogenic factors like VEGF and bFGF (3, 4). The mechanism behind angiogenic factor–mediated silencing of tumor endothelial cell adhesion molecules was hitherto unknown. Here, we show that epigenetic mechanisms are involved in the regulation of endothelial cell anergy through repression of *ICAM-1* by promoter histone modifications.

In the present study, we found that DNMT and HDAC inhibitors reexpress ICAM-1 in tumor endothelial cells, both at protein and mRNA level, and restore leukocyte-endothelial cells adhesion *in vitro* and *in vivo*. Although tumor growth and angiogenesis were inhibited by zebularine and TSA, as we published recently (19), the amount of infiltrated leukocytes was enhanced markedly in both the syngeneic B16F10 mouse melanoma model and the human LS174T xenograft model. Overall, this provides functional meaning to the observed changes in leukocyte adhesion. This is consistent with what we observed on the molecular level; that is,

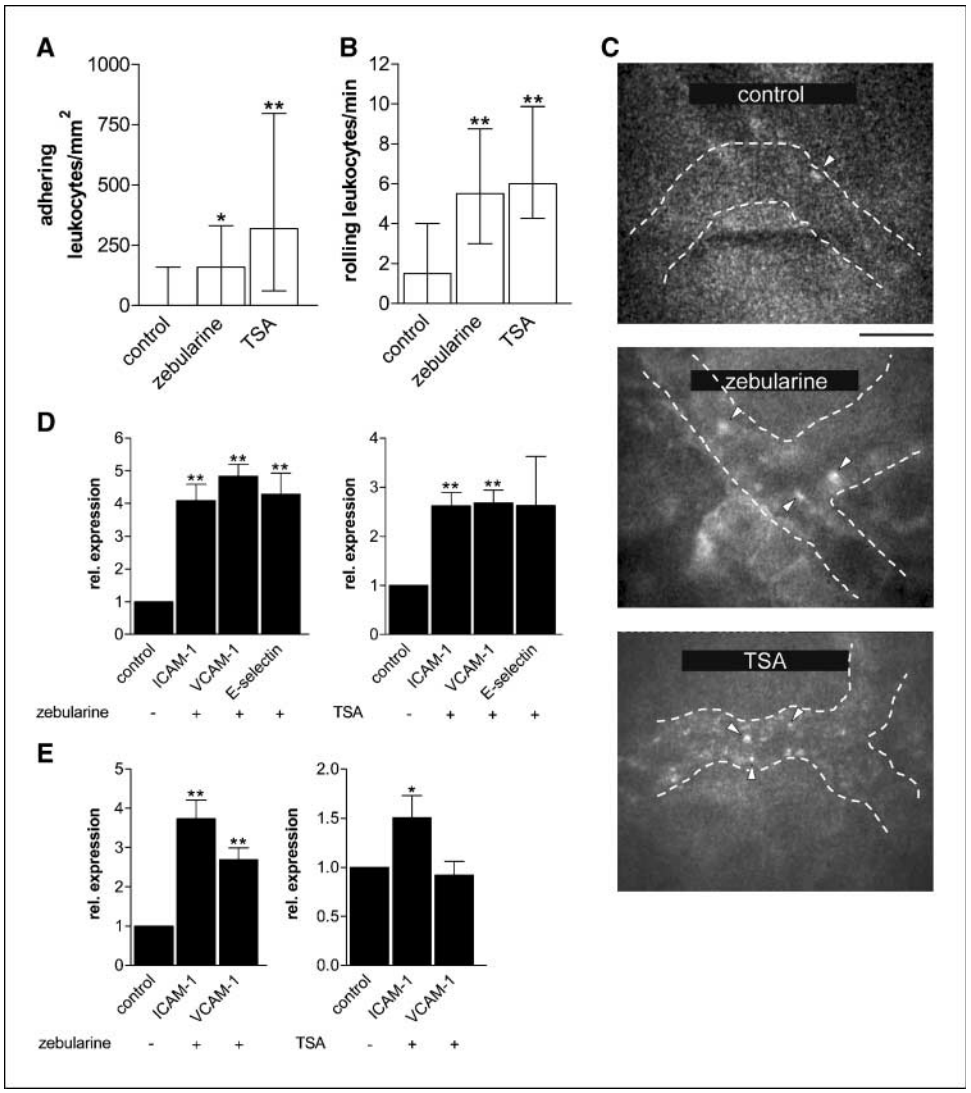

Figure 3. Increased leukocyte-vessel wall interactions and ICAM-1 expression in tumor vessels *in vivo* by zebularine and TSA. Adhering (*A*) and rolling (*B*) leukocytes in flank tumor vessels of C57Bl6 mice bearing B16F10 mouse melanoma tumors. Mice were treated with zebularine ($n = 5$) or TSA ($n = 5$). Medians with interquartile ranges. Statistical significance was assessed in comparison to values in tumor vessels of untreated mice ($n = 5$; control). *, $P < 0.01$; **, $P < 0.001$. *C,* typical intravital fluorescence microscopy images of an untreated, zebularine-treated, and TSA-treated tumor vessel. Leukocytes are fluorescently labeled with Rhodamine 6G. *Dashed lines,* vessels; *arrowheads,* examples of leukocytes. Bar, 25 μm. Because the stills have rather low resolution, the video recordings can be observed at http://www.fdg.unimaas.nl/AngiogenesisLab/mirrorsite/movies.htm. For numbers of mice and vessels, see Table 1. Expression levels of ICAM-1, VCAM-1, and E-selectin in B16F10 (*D*) and LS174T (*E*) tumor tissues of mice treated with zebularine or TSA measured by quantitative real-time RT-PCR. *Columns,* mean of relative mRNA expression compared with untreated control mice; *bars,* SE. *, $P < 0.05$; **, $P < 0.001$ versus control mice.

Table 1. Fluid dynamic variables in tumor vessels of treated and untreated mice

	Control	Zebularine	TSA
No. mice (n_m)	9	5	5
No. vessels (n_v)	30	29	22
Diameter (μm)	20 (20-30)	25 (20-35)	25 (19-25)
Centerline velocity (mm/s)	0.7 (0.5-0.9)	0.6 (0.4-0.8)	1.1* (0.9-1.5)
Reduced velocity U (s^{-1})	15.6 (11.7-35.2)	13 (9.4-25)	29* (23.8-42)
Flow Q (nL/s)	0.17 (0.08-0.30)	0.15 (0.07-0.34)	0.27 (0.17-0.44)

NOTE: Data are presented as median values and interquartile ranges. Statistical significance was assessed in comparison with values in untreated mice.
*$P < 0.05$.

ICAM-1 expression is significantly increased upon DNMT and HDAC inhibitor treatment of tumors in both these mouse models. Although up-regulation of other adhesion molecules, such as CD34, P-selectin, and CD44 (that are also involved in leukocyte-vessel wall interactions), can not be excluded, it is unlikely that enhanced leukocyte-vessel wall interactions result from increased expression of adhesion molecules on leukocytes (30) or changing fluid dynamic variables (Table 1). DNMT and HDAC inhibitors decrease tumor cell growth by reactivation of epigenetically silenced tumor suppressor genes (15). Therefore, increased leukocyte infiltration and leukocyte-vessel wall interactions by zebularine and TSA *in vivo* could result from their inhibitory effects on tumor cells (e.g., interfering in the production of angiogenic factors like bFGF and VEGF). The increased leukocyte-endothelial cell adhesion *in vitro*, however, where no other cells (tumor cells) are present, show that these compounds directly restore endothelial cell ICAM-1 expression and enhance leukocyte-endothelial cell adhesion.

Posttranslational modifications of histone NH_2-terminal tails are important epigenetic modifications, which together form the "histone code" (11, 31–33). This "histone code" is "read" by proteins that modulate chromatin structure, thereby regulating gene transcription (34). Hyperacetylation of histone H3 and H4 lysine residues is generally associated with active chromatin, whereas deacetylation has been correlated with inactive genes (35). Another histone modification that has been associated with transcriptionally active chromatin is methylation at Lys^4 of histone H3 (36, 37). In cancer cells, histone modifications work in concert with DNA methylation to silence tumor suppressor genes (13, 28, 38). In fact, DNA methylation seems to be dominant over histone deacetylation in maintaining transcriptional repression of tumor suppressor genes because these genes can be activated by DAC but not by TSA alone (15). Our data indicate that in tumor endothelial cells, histone modifications alone are responsible for ICAM-1 down-regulation. ICAM-1 can be reactivated by both DAC and TSA alone, through increasing *ICAM-1* promoter histone H3 acetylation and H3 Lys^4 methylation. Increased histone acetylation, H3 Lys^4 methylation, and/or gene expression by DNMT inhibitors independently of effects on DNA methylation have been described before (38–40). The potency of the DNMT inhibitors DAC and zebularine to reactivate ICAM-1 independently of promoter DNA methylation indicates that methylation-independent silencing activity of DNMTs might be essential for ICAM-1 down-regulation in tumor endothelial cells. Methylation-independent transcriptional repressor effects of DNMTs have been linked to the interaction of these enzymes with histone methyltransferases and HDACs (39, 41–43).

In tumor cells, induction of ICAM-1 by DNMT (44) and HDAC inhibitors (45), as well as *ICAM-1* promoter DNA hypermethylation (in a region within the area we examined by bisulfite sequencing; ref. 46), have been described. The suggested difference between tumor cells and tumor endothelial cells in the involvement of promoter DNA hypermethylation in ICAM-1 silencing is very interesting. Some studies have shown that DNA methylation, which is a more stable epigenetic modification compared with the more dynamic nature of histone modifications, serves to maintain

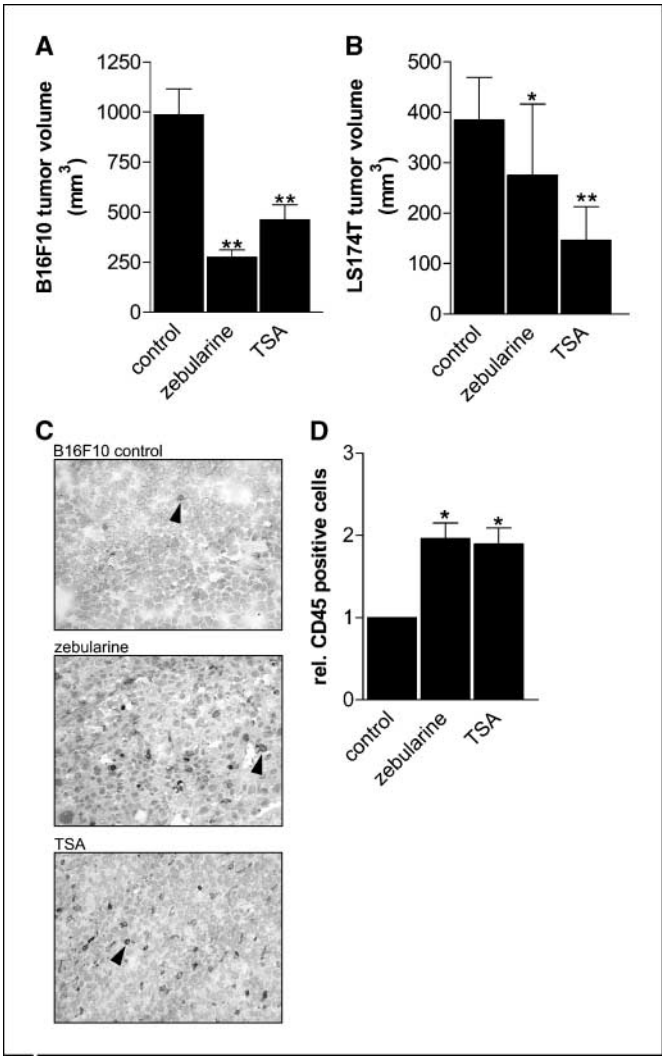

Figure 4. Leukocyte infiltration is enhanced by zebularine and TSA. Tumor size of B16F10 mouse melanoma tumors (*A*) and human LS174T colon carcinoma (*B*) after treatment with or without zebularine or TSA for 7 days (B16F10) or 10 days (LS174T). *Columns,* mean tumor volume; *bars,* SE. *, $P < 0.01$; **, $P < 0.001$. *C,* cryosections of B16F10 tumors from control mice and treated mice stained with CD45 antibody for leukocyte infiltration. *Arrowheads,* examples of leukocytes. *D,* quantification of CD45-positive leukocytes in B16F10 tumors treated with zebularine or TSA. *Columns,* relative mean values of CD45-positive leukocytes compared to control mice; *bars,* SE. *, $P < 0.001$.

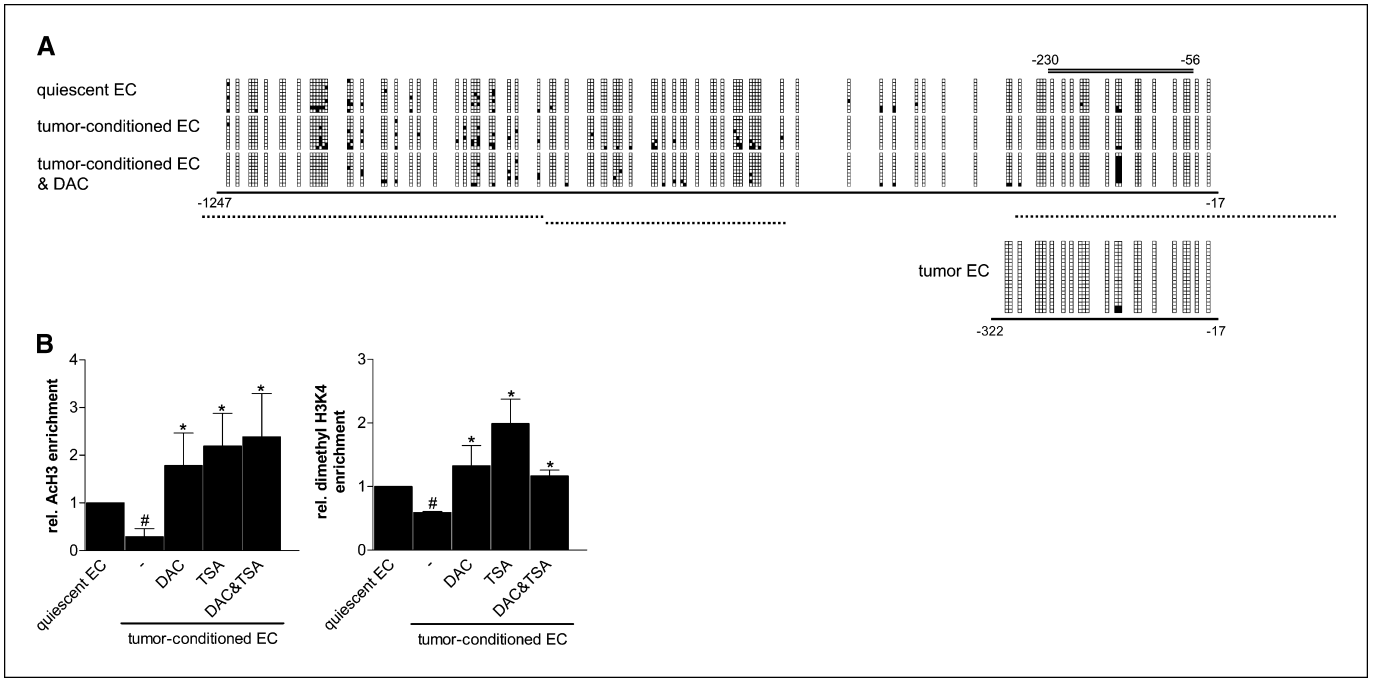

Figure 5. ICAM-1 down-regulation in tumor-conditioned endothelial cells is associated with promoter histone H3 deacetylation and loss of H3 Lys^4 methylation. *A,* genomic bisulfite sequencing of the ICAM-1 5′CpG island in quiescent HUVECs, tumor-conditioned HUVECs, and tumor-conditioned HUVECs treated with DAC (200 nmol/L). In each clone, the methylation status of each CpG dinucleotide is represented as a box. If a box is shaded, the position is methylated; if white, it is not. Numbers indicate the position relative to transcription start site. *Dotted lines,* locations of three promoter CpG islands (1283 to −867, −855 to −547, −268 to +207), according to the criteria of Gardiner-Garden and Frommer (29). *Doubled horizontal line,* region examined by chromatin immunoprecipitation. *Bottom right,* genomic bisulfite sequencing of part of the ICAM-1 promoter in microdissected tumor endothelial cells. *B,* chromatin immunoprecipitation assay of the ICAM-1 promoter using anti-acetyl-histone H3 (Lys^9 and Lys^{14}) and anti-dimethyl-histone H3 (Lys^4) antibody in quiescent HUVECs, tumor-conditioned HUVECs, and tumor-conditioned HUVECs treated with DAC (200 nmol/L), TSA (300 nmol/L), or a combination of DAC and TSA. The location of the PCR fragment done on DNA recovered from chromatin immunoprecipitation experiments is indicated by the doubled horizontal line in (*A*). PCR was done on nonimmunoprecipitated (input) DNA, immunoprecipitated DNA, and a no-antibody (*no Ab*) control DNA. Enrichment was calculated by taking the ratio between the net intensity of the ICAM-1 PCR product and the net intensity of the GAPDH PCR product for immunoprecipitated DNA and dividing this by the same ratio calculated for the input DNA. Relative acetylated H3 (AcH3) and methylated H3 Lys^4 (dimethyl H3K4) enrichment (quiescent HUVECs set to 1). *Columns,* mean from three independent chromatin immunoprecipitation experiments; *bars,* SE. #, $P < 0.05$ versus quiescent HUVECs; *, $P < 0.05$ versus tumor-conditioned HUVECs.

instead of initiate gene silencing (47, 48). It is attractive to speculate that transcription of ICAM-1 is irreversibly "locked" into a permanently silent state in tumor cells by promoter DNA hypermethylation, and that ICAM-1 down-regulation in tumor-conditioned endothelial cells is a more reversible phenomenon that only involves histone modifications. It could be argued that culturing HUVECs for 6 days with angiogenic growth factors is not sufficient to induce irreversible gene silencing by promoter DNA methylation. Therefore, we also examined *ICAM-1* promoter DNA methylation in tumor endothelial cells obtained from colorectal tumors by using laser capture microdissection. However, no meaningful promoter methylation was observed in these cells.

Several studies have reported on transcriptional regulators of basal- and cytokine-induced expression of ICAM-1 (49). Inflammatory cytokines, such as TNF-α, induce ICAM-1 transcription predominantly through activation of the transcription factor nuclear factor-κB (NF-κB; ref. 50).

However, preliminary results of electrophoretic mobility shift assay showed that NF-κB activity was not decreased in tumor-conditioned versus quiescent endothelial cells and was not increased by treatment with DAC and/or TSA (data not shown). These data suggest that regulation of ICAM-1 expression in tumor-conditioned endothelial cells by DNMT and HDAC inhibitors does not involve NF-κB activation. Nevertheless, involvement of other transcriptional mechanisms in regulating ICAM-1 expression during endothelial cell anergy, besides the epigenetic regulation of tumor endothelial cell ICAM-1 expression described in this study, cannot be ruled out. In addition, different (epi)genetic mechanisms can cooperate in ICAM-1 transcriptional activation and repression during tumor angiogenesis.

The potential therapeutic implications of this work are substantial. Until now, augmentation of antitumor immunity by DNMT and HDAC inhibitors was recognized but attributed mainly to induction of molecules on tumor cells, like cancer testis antigens, HLA class I antigens, and costimulatory/accessory molecules (51, 52). Here, we show a new mechanism by which DNMT and HDAC inhibitors might be used in anticancer therapy, for reversal of endothelial cell anergy. Together with our recent findings (19) and findings of others (20, 53), showing that these compounds are powerful inhibitors of endothelial cell growth and tumor angiogenesis *in vitro* and *in vivo*, our current data show that DNMT and HDAC inhibitors have direct effects on tumor endothelial cells. Therefore, the therapeutic targets of these compounds can be extended beyond merely tumor cells. Aside from the inhibitory effects of epigenetic therapy on tumor cell growth through reexpression of previously silenced tumor suppressor genes, the potential use of DNMT and HDAC inhibitors as angiostatic and immunotherapeutic agents makes them promising anticancer drugs.

In conclusion, we have shown a role of epigenetics in regulation of endothelial cell anergy. We show that *ICAM-1* is epigenetically repressed in tumor endothelial cells by promoter histone

modifications, and that DNMT and HDAC inhibitors reinduce expression of this gene by reversal of histone modifications in the *ICAM-1* promoter, thereby restoring leukocyte-vessel wall interactions and leukocyte infiltration. This work has laid the foundation for a novel anticancer approach, whereby DNMT and HDAC inhibitors may be used to modulate leukocyte infiltration into tumors.

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Epigenetic Regulation of Tumor Endothelial Cell Anergy: Silencing of Intercellular Adhesion Molecule-1 by Histone Modifications

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