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ORIGINAL ARTICLE

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Histone deacetylase inhibitors such as sodium butyrate and trichostatin A inhibit vascular endothelial growth factor (VEGF) secretion from human glioblastoma cells

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Abstract We investigated the effects of histone deacetylase (HDAC) inhibitors such as sodium butyrate (SB) and trichostatin A (TSA) on the expression of vascular endothelial growth factor (VEGF) by human glioblastoma T98G, U251MG, and U87MG cells. The glioblastoma cells secreted three VEGF isoforms, VEGF (189), (165), and (121), although the expression levels of VEGF differed between the cell types. Treatment with either 5mM SB or 100ng/ml TSA reduced VEGF secretion in conditioned media and reduced VEGF mRNA expression. We also studied the expression of VEGF-B, -C, and -D mRNA in human glioblastoma cells and their modulation by HDAC inhibitors. The PCR products of VEGF-B (357bp), VEGF-C (501bp), and VEGF-D (484bp) were amplified in all glioblastoma cells examined. Treatment with SB reduced the expression of VEGF-D mRNA in U251MG cells and the expression of VEGF-B mRNA in U87MG cells. TSA treatment reduced the expression of VEGF-D in U251MG cells. These results suggest that HDAC inhibitors reduce VEGF secretion and modulate the expression of the other VEGF family members, and therefore may inhibit angiogenesis in glioblastoma tissues.

Key words Histone deacetylase inhibitors · Sodium butyrate · Trichostatin A · Glioma · VEGF

Introduction

Vascular endothelial growth factor (VEGF) has been shown to be a significant mediator of angiogenesis in a variety of normal and pathological processes, including tumor development.[1] Numerous proteins whose primary structure is closely related to that of VEGF have been reported that may also play roles in vascular biology, including placenta growth factor (PIGF),[2] VEGF-B,[3] VEGF-C,[4] and VEGF-D.[5] We previously showed that sodium butyrate (SB) treatment inhibited the G1-S transition that was associated with increased expression of p21 (WAF-1), and reduced pRb phosphorylation.[6] In addition, we reported that either SB or trichostatin A (TSA) induced apoptosis through an increase in Bad protein in human glioma cells in vitro.[7] Recently we transplanted human glioblastoma cells into the subcutaneous tissues of hereditary athymic mice, and the growing tumors were treated by intra-tumoral injection of SB or TSA. Apoptotic cells detected by TUNEL assay were increased by histone deacetylase (HDAC) inhibitors, and increased expression of p21 and Bad proteins was observed (data not shown). The effects of SB or TSA may result from their function as HDAC inhibitors. Core histones, which package DNA into the nucleosomes, can be acetylated on the ε-amino groups of specific lysine residues of the N-terminal tail. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase and deacetylase, respectively. We investigated whether HDAC inhibitors such as SB or TSA reduced VEGF secretion in conditioned media (CM) of glioblastoma cells. We investigated which HDAC inhibitors reduced VEGF secretion and also modulated the expression of the other VEGF family members. If these effects are confirmed, HDAC inhibitors may provide promising therapeutic approaches toward angiogenesis of human glioblastomas.

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Materials and methods

Reagents

SB was purchased from Wako (Osaka, Japan), dissolved in distilled water, and stored at −80°C as a stock solution until

use. TSA was obtained from Sigma-Aldrich Japan (Tokyo, Japan), dissolved in distilled water, and stored at −80°C as stock solution until use.

Cell culture and conditioned media

Human glioblastoma cells (T98G, U251MG, and U87MG) were obtained from the American Type Culture Collection (ATCC) and maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. Secretion of VEGF proteins into CM was analyzed by immunoblot. To generate CM, the human glioblastoma cells were grown to 80% confluence. The medium was changed to MEM containing 0.5% bovine serum albumin (Bio Whitaker, Walkersville, MD, USA) with either 5mM SB or 100ng/ml TSA for 16 or 30h. The medium was centrifuged at 2000rpm for 2min. The supernatant was concentrated with Ultrafree-4 Centrifugal Filter Unit, according to the manufacturer's protocol (Millipore, Bedford, MA, USA). The samples were added by 2× sample buffer (100mM Tris-HCl, pH 6.8/20% glycerol/1mM EDTA, and 10% 2-mercaptoethanol) and were heated at 95°C for 5min. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

Immunoblot analysis

Equal amounts of proteins (10μg per lane) were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the PVDF membranes. The membranes were incubated for 1h with 5% fat-free milk solution in phosphate-buffered saline (PBS) to quench the nonspecific bindings and were reacted for 1h at room temperature with the specific antibody against VEGF (1:2000, A-20, rabbit polyclonal, Santa Cruz, CA, USA). The membranes were washed three times with 50mM Tris HCl, 150mM NaCl (pH 7.6), and were reacted for 1h at room temperature with the horseradish peroxidase (HRP)-conjugated secondary antibody. The HRP reaction was performed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK), and was visualized with a Lumino image analyzer LAS-1000 (Fuji Film, Tokyo, Japan).

RNA isolation and RT-PCR

Total RNA was isolated from the cells by using the RNeasy Mini kit (Qiagen, Hilden, Germany). The first strand cDNA from the total RNA (2μg) was synthesized with SuperScript II RT (Invitrogen, Carlsbad, CA, USA) in a 20-μl reverse transcription step using Oligo (dT) 12–18 primers. The specific primers for VEGF, VEGF-B, VEGF-C, and VEGF-D were as follows: VEGF (F): 5′-ACCATGAACT TTCTGCTGTCT-3′(14–34), VEGF(R): 5′-TCACCGCC TCGGCTTGTCACA-3′(572–592), VEGF-B(F): 5′-AGC CAGTGTGAATGCAGA-3′(301–318), VEGF-B(R): 5′-ATAGCCTCTGAGGCAAGT-3′(640–657), VEGF-C(F): 5′-AGGCCACGGCTTATGCAA-3′(511–528), VEGF-C(R): 5′-TAGA CATGCATCGGCAGGAA-3′(992–1011), VEGF-D(F): 5′-CATCTCAGTCCACAT TGG-3′(604–621), VEGF-D(R): 5′-GGCAAGCACTTACAA CCT-3′(1070–1087). Thirty-five cycles of the polymerase chain reaction (PCR) at an annealing temperature of 62°C were carried out in a volume of 50μl containing 2μl cDNA as template. The standard PCR-mix contained 0.2mM deoxynucleotide triphosphates, 1.5mM $MgCl_2$, 1μM of each primer, 5% dimethylsulfoxide, 1× PCR buffer, and 1.75 units of Taq polymerase (Roche, Basel, Switzerland). The PCR products were run with 4% NuSieve 3:1 agarose (Takara, Osaka, Japan) containing 0.02% ethidium bromide and photographed with a Polaroid camera (Polaroid, Cambridge, MA, USA).

Results

VEGF expression in human glioblastoma T98G, U251MG, and U87MG cells

The VEGF proteins secreted into CM were analyzed by immunoblot. As shown in Fig. 1, all glioblastoma cells secreted three VEGF isoforms, VEGF (189), (165), and (121), although the expression levels of VEGF differed between the cell types. Treatment with either 5mM SB or 100ng/ml TSA reduced VEGF secretion in CM (Fig. 1). RT-PCR analysis using VEGF-specific primers described in Materials and methods showed three PCR products (567, 495, and 363bp) (Fig. 2). Among the three PCR products, the band of 495bp that was derived from VEGF (165) mRNA was weaker than those of 567 and 363bp. The protein levels of VEGF secreted in CM were VEGF (189) > VEGF (165) > VEGF (121), as shown in Fig. 1. The discrepancy between the results of VEGF protein and mRNA expression may be due to differences in post-translational processing of the three VEGF isoforms. Treatment with either 5mM SB or 100ng/ml TSA reduced VEGF mRNA expression, as shown in Fig. 2. Taken together, the present results clearly show reduction of the secreted VEGF and mRNA with treatment by HDAC inhibitors, such as SB and TSA.

Expression of VEGF-B, -C, and -D mRNA in human glioblastoma T98G, U251MG, and U87MG cells and their modulation by HDAC inhibitors

To further analyze the expression and modulation of the VEGF family members with HDAC inhibitors such as SB and TSA, RT-PCR using specific primers of VEGF-B, VEGF-C, and VEGF-D was performed as described in "Materials and methods." As shown in Fig. 3, the PCR products of VEGF-B (357bp), VEGF-C (501bp), and VEGF-D (484bp) were amplified in all glioblastoma cells examined. Treatment with SB reduced the expression of VEGF-D mRNA in U251MG cells and the expression of

VEGF-B mRNA in U87MG cells. TSA treatment reduced the expression of VEGF-D in U251MG cells, but not in T98G or U87MG cells (Fig. 3). The results suggest that the effects of HDAC inhibitors on the expression of VEGF family members differed between the cell types.

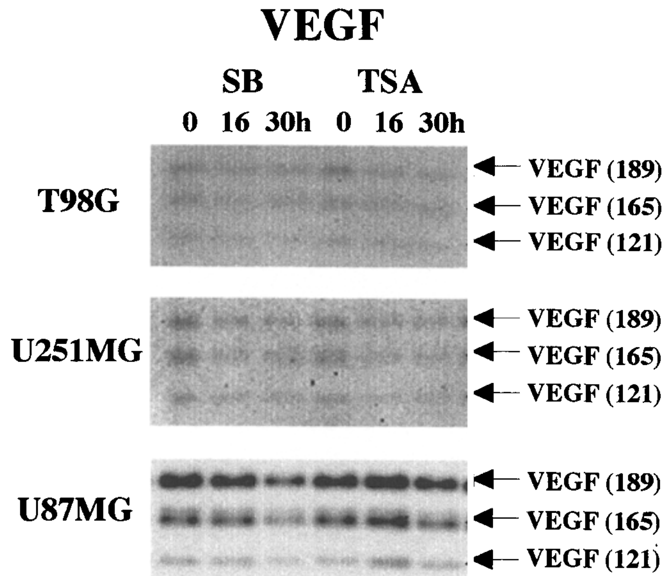

Fig. 1. Vascular endothelial growth factor (VEGF) expression in human glioblastoma T98G, U251MG, and U87MG cells. Notice the decrease in VEGF expression with histone deacetylase (HDAC) inhibitors, such as sodium butyrate (SB) or trichostatin A (TSA). The secretion of VEGF proteins into conditioned media (CM) was analyzed by immunoblot, as described in Materials and methods. This figure shows that all glioblastoma cells secreted three VEGF isoforms – VEGF (189), (165), and (121) – although the expression levels of VEGF differed between the cell types. Treatment with HDAC inhibitors such as SB or TSA reduced the secreted VEGF in CM

Discussion

It has been reported that VEGF and the closely related VEGF family members, including placenta growth factor (PlGF),[2] VEGF-B,[3] VEGF-C,[4] and VEGF-D,[5] may play roles in angiogenesis during a variety of normal and pathological processes, including tumor development. A previous report[8] showed that inhibition of VEGF expression by introducing the anti-sense VEGF construct into glioblastoma cells reduced tumorigenicity and angiogenicity in immunodeficient animals. These investigators suggest that VEGF may play a critical role in glioblastoma angiogenesis.

VEGF occurs in at least five different isoforms resulting from alternative splicing of the gene.[9] Little is known about the functional differences between these isoforms. Cheng et al.[10] reported that intracerebral tumor-associated hemorrhage was caused by overexpression of the VEGF isoform-121 and VEGF-165, but not VEGF-185. VEGF-B, which is approximately 43% identical in its amino acid sequence to VEGF, is mitogenic for endothelial cells.[3] VEGF-C was isolated as a ligand for the tyrosine kinase VEGFR-3 (Flt-4), and it may regulate angiogenesis of the lymphatic vasculature.[4] VEGF-D is closely related to VEGF-C in its primary structure.[5] A recent study[11] suggests that VEGF-C and VEGF-D stimulate lymphangiogenesis, and their receptor, VEGFR-3, has been linked to human hereditary lymphedema. It has been reported that VEGF-C expression is closely related to invasion phenotype, and that it affects the patient's survival in cervical carcinoma.[12] Skobe et al.[13] reported that induction of tumor lymphangiogenesis by VEGF-C might promote breast cancer metastasis. In this communication, we showed that HDAC inhibitors such as SB or TSA inhibited secretion of VEGF in the CM of glioblastoma cells. In addition, HDAC inhibitors reduced the expression of VEGF-D mRNA in U251MG cells and the expression of VEGF-B mRNA in U87MG cells. The

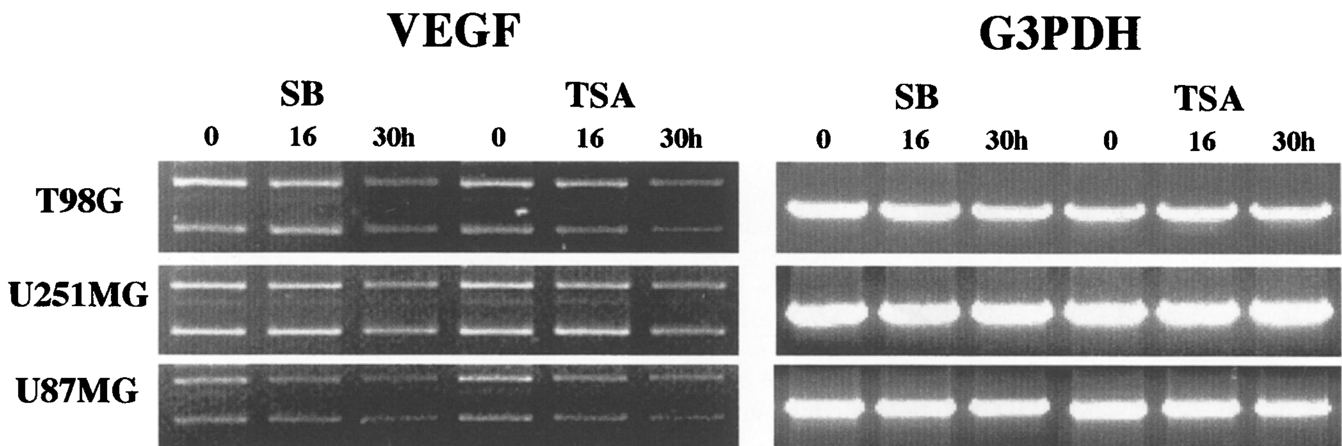

Fig. 2. RT-PCR analysis for VEGF mRNA in human glioblastoma T98G, U251MG, and U87MG cells. Notice the decreased expression of VEGF-mRNA with HDAC inhibitors. VEGF-specific primers amplified three PCR products (567, 495, and 363 bp) in all glioblastoma cells examined. Among the three PCR products, the 495-bp band that was derived from VEGF (165) mRNA was weaker than the 567-bp and 363-bp bands. This figure shows decreased expression of VEGF mRNA with treatment by HDAC inhibitors. The expression of G3PDH mRNA was not changed by treatment with HDAC inhibitors

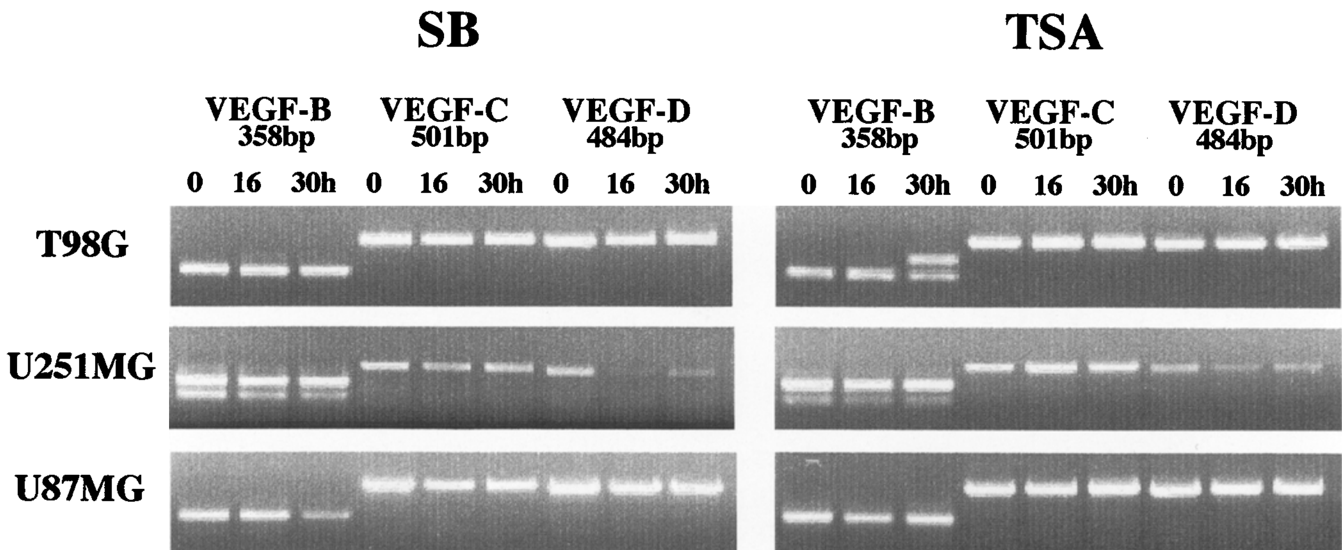

Fig. 3. Expression of VEGF-B, -C, and -D mRNA in human glioblastoma cells and their modulation by SB or TSA. The PCR products of VEGF-B (357 bp), VEGF-C (501 bp), and VEGF-D (484 bp) were amplified in all glioblastoma cells. SB reduced the expression of VEGF-D mRNA in U251MG cells and the expression of VEGF-B mRNA in U87MG cells. TSA reduced the expression of VEGF-D in U251MG cells, but not in T98G or U87MG cells. The expression of G3PDH mRNA was not changed by treatment with HDAC inhibitors (data not shown)

results suggest that HDAC inhibitors may also suppress angiogenesis of glioblastoma tissues. Kim et al.[14] reported that HDAC induces angiogenesis and that TSA specifically inhibits hypoxia-induced angiogenesis in the Lewis lung carcinoma model. Previously, we showed that HDAC inhibitors arrested the cell cycle in the G1 phase and induced apoptosis through an increase in Bad protein in human glioma cells. Since HDAC may play critical roles in regulation of the cell cycle, apoptosis, and angiogenesis, as mentioned, molecular target therapy using HDAC inhibitors is promising approach for therapy of intractable glioma.

Conclusion

We investigated the effects of HDAC inhibitors such as SB or TSA on VEGF expression by human glioblastoma T98G, U251MG, and U87MG cells. Treatment with SB or TSA reduced VEGF secretion in the CM and the expression of VEGF mRNA. Furthermore, treatment with SB reduced the expression of VEGF-D mRNA in U251MG cells and the expression of VEGF-B mRNA in U87MG cells. TSA treatment reduced the expression of VEGF-D in U251MG cells. These results suggest that HDAC inhibitors reduce VEGF secretion and modulate the expression of the other VEGF family members and, therefore, may inhibit angiogenesis in glioblastoma tissues.

References

1. Potorak Z, Cohen T, Sivan R, et al (1997) VEGF 145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem 272:7151–7158
2. Maglione D, Guerriero V, Viglietto G, et al (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci USA 88:9267–9271
3. Olofsson B, Pajusola K, Kaipainen A, et al (1996) Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 93:2576–2581
4. Kaipainen A, Korhonen J, Mustonen T, et al (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci USA 92: 3566–3570
5. Achen MG, Jeltsch M, Kukk E, et al (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinase VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 95:548–553
6. Ito N, Sawa H, Nagane M, et al (2001) Inhibitory effects of sodium butyrate on proliferation and invasiveness of human glioma cells. Neurosurgery 49:430–437
7. Sawa H, Murakami H, Ohshima Y, et al (2001) Histone deacetylase inhibitors such as sodium butyrate and trichostatin A induce apoptosis through an increase of the bcl-2-related protein Bad. Brain Tumor Pathol 18:109–114
8. Cheng SY, Huang HJ, Nagane M, et al (1996) Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc Natl Acad Sci USA 93:8502–8507
9. Yu JL, Rak JW, Klement G, et al (2002) Vascular endothelial growth factor isoform expression as a determinant of blood vessel patterning in human melanoma xenografts. Cancer Res 62:1838–1846
10. Cheng SY, Nagane M, Huang HS, et al (1997) Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF 121 and VEGF 165 but not VEGF 189. Proc Natl Acad Sci USA 94:12081–12087

11. Malinen T, Jussila L, Veikkola T, et al (2001) Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med 7:199–205
12. Ueda M, Terai Y, Yamashita Y, et al (2002) Correlation between vascular endothelial growth factor-C expression and invasion phenotype in cervical carcinomas. Int J Cancer 98:335–343
13. Skobe M, Hawighorst T, Jackson DG, et al (2001) Induction of tumor lymphangiogenesis by VEGF-C promotes breast carcinoma metastasis. Nat Med 7:192–198
14. Kim MS, Kwon HJ, Lee YM, et al (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 7:437–443