Epidermal Growth Factor (EGF)-enhanced Vascular Cell Adhesion Molecule-1 (VCAM-1) Expression Promotes Macrophage and Glioblastoma Cell Interaction and Tumor Cell Invasion*

Received for publication, July 2, 2013, and in revised form, September 13, 2013. Published, JBC Papers in Press, September 17, 2013, DOI 10.1074/jbc.M113.499020

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Background: The mechanism underlying EGF receptor (EGFR) activation-promoted glioblastoma (GBM) cell invasion remains elusive.

Results: EGFR activation resulted in PKCε- and NF-κB-dependent VCAM-1 up-regulation, which subsequently promoted the interaction between macrophages and GBM cells, as well as GBM cell invasion.

Conclusion: EGF-induced VCAM-1 up-regulation plays an instrumental role in EGFR activation-promoted macrophage-tumor cell interaction and tumor cell invasion.

Significance: VCAM-1 expression is a potential target for GBM therapy.

Activated EGF receptor (EGFR) signaling plays an instrumental role in glioblastoma (GBM) progression. However, how EGFR activation regulates the tumor microenvironment to promote GBM cell invasion remains to be clarified. Here, we demonstrate that the levels of EGFR activation in tumor cells correlated with the levels of macrophage infiltration in human GBM specimens. This was supported by our observation that EGFR activation enhanced the interaction between macrophages and GBM cells. In addition, EGF treatment induced up-regulation of vascular cell adhesion molecule-1 (VCAM-1) expression in a PKCε- and NF-κB-dependent manner. Depletion of VCAM-1 interrupted the binding of macrophages to GBM cells and inhibited EGF-induced and macrophage-promoted GBM cell invasion. These results demonstrate an instrumental role for EGF-induced up-regulation of VCAM-1 expression in EGFR activation-promoted macrophage-tumor cell interaction and tumor cell invasion and indicate that VCAM-1 is a potential molecular target for improving cancer therapy.

Glioblastoma (GBM)2 is the most aggressive brain tumor, and efficient therapies for it are lacking. Median survival durations in patients with GBM range from 12 to 15 months, and the majority of patients die within 2 years after diagnosis (1). The genetic alteration most often associated with GBM is overexpression or mutation of the EGF receptor (EGFR) gene, which plays a significant role in GBM progression and is correlated with the degree of malignancy of GBM (2–4). Aberrant EGF signaling promotes GBM cell growth and invasion via incompletely understood mechanisms (5, 6).

Macrophages are important inflammatory cells in human immune system (7). Macrophages have been implicated in the contribution of chronic inflammation to malignancy (8). The majority of malignant tumors recruit macrophages. These cells are part of the host’s immune system and are thought to defend against the tumor cells. However, clinical evidence has revealed a strong correlation between a high density of tumor-associated macrophages (TAMs) and poor prognosis for human cancers (9, 10). TAMs create an inflammatory microenvironment to promote tumor initiation and modulate angiogenesis and cell migration (10, 11). At metastatic sites, TAMs promote the extravasation, seeding, and persistent growth of tumor cells (12, 13).

Vascular cell adhesion molecule-1 (VCAM-1) is an inducible cell-cell adhesion protein that belongs to the immunoglobulin supergene family (14). It is a key receptor for the leukocyte-associated integrin α4β1 (VLA4), which is a crucial mediator of monocyte adhesion. By interacting with α4β1, VCAM-1 mediates the movement of leukocytes from blood to tissue (15, 16).

* This work was supported, in whole or in part, by National Institutes of Health Grants R01CA109035 and R01CA169603 (to Z. L.) from NCI. This work was also supported by MD Anderson Cancer Center Support Grant CA016672, Cancer Prevention and Research Institute of Texas Research Grants RP110252 and RP130389 (to Z. L.), American Cancer Society Research Scholar Grant RSG-09-277-01-CSM (to Z. L.), James S. McDonnell Foundation 21st Century Science Initiative in Brain Cancer Research Award 220020318 (to Z. L.), and Sister Institution Network Fund grants from the MD Anderson Cancer Center (to Z. L.) and the Cancer Institute and Hospital of Chinese Academy of Medical Sciences (to J. H.).

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VCAM-1 is generally expressed in activated endothelial cells in inflamed tissue and mediates endothelium-leukocyte interactions, leading to leukocyte diapedesis to the site of inflammation (17). Abnormal expression of VCAM-1 has been recently reported in various cancer cell types, including glioma cells (18–20). In addition, high levels of VCAM-1 expression correlate with high rates of leukocytic infiltration in tumors. VCAM-1 expressed on the surface of tumor cells tethers macrophages that express α4 integrins to tumor cells so that a favorable microenvironment for tumor angiogenesis, invasion, and metastasis is created (21, 22). However, whether growth factors regulate macrophage interaction with tumor cells via VCAM-1, thereby promoting tumor progression, is unknown.

In this study, we show that EGFR activation in tumor cells was correlated with macrophage infiltration in human GBM specimens. In addition, EGFR activation up-regulated VCAM-1 expression in a PKCε- and NF-κB-dependent manner. Up-regulated VCAM-1 expression enhanced the interaction between macrophages and GBM cells and EGF-induced and macrophage-mediated tumor cell invasion.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Conditions—The human GBM cell lines U251, A172, and D54 were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (HyClone, Logan, UT). THP-1 monocytes (American Type Culture Collection) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (HyClone, Logan, UT).

Materials—Rabbit anti-phospho-EGFR Tyr-1172 polyclonal antibody (1:100 dilution) was obtained from Signalway Biotechnology (Pearland, TX) and Signalway Antibody (College Park, MD). Mouse anti-human CD68 monoclonal antibody (M0876) was obtained from DakoCytomation (Glostrup, Denmark). Rabbit anti-VCAM-1 monoclonal antibody (3540-1) was purchased from Epitomics (Burlingame, CA). Anti-tubulin monoclonal antibody was from Sigma. Rabbit polyclonal antibodies for PKCε (sc-214) and IκBα (sc-371) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The DAB Plus substrate staining system (TA-060-HDX) was obtained from Fisher. The EGFR inhibitor AG1478 (658552) was obtained from Calbiochem. Matrigel (354234) was from BD Biosciences. Hoechst 33342 and 2′,7′-bis(2-carboxyethyl)-5′(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM; B1150) were purchased from Molecular Probes (Eugene, OR).

Immunohistochemistry—Immunohistochemical (IHC) analysis was performed as described previously (23). Briefly, 5-μm thick GBM tumor specimens were fixed in formalin and embedded in paraffin, and sections of them were placed on slides. The tissue sections on slides were melted for 1 h at 60 °C, deparaffinized in xylene and ethanol, and rehydrated in PBS. Afterward, antigen retrieval was performed by placing the slides in 10 mM citrate buffer (pH 6.0) and heating them at 95 °C for 30 min, followed by cooling for 30 min and washing with PBS. Endogenous peroxidase activity in the sections was blocked in 3% hydrogen peroxide in PBS and 0.05% Tween 20 for 30 min. Sections were then washed with PBS and blocked for 30 min in serum diluted to 10% in PBS, followed by incubation with the following primary antibodies: rabbit anti-phospho-EGFR Tyr-1172 polyclonal antibody (1:100 dilution) for active EGFR detection, mouse anti-CD68 monoclonal antibody (1:50 dilution) for macrophage cell staining, and rabbit anti-VCAM-1 polyclonal antibody (1:50 dilution) for VCAM-1 detection. The antibodies were diluted in PBS and 10% serum and applied to the tissue sections in a humid chamber overnight at 4 °C. The slides were rinsed and incubated for 2 h at room temperature with peroxidase-conjugated secondary antibodies and then rinsed with PBS and stained using the DAB Plus substrate staining system according to the manufacturer’s instructions. All tissue sections were counterstained with hematoxylin and mounted.

The tissue sections were scored quantitatively according to the percentage of positive cells and staining intensity. Proportion scores of 0 were assigned to a tumor section if 0% of the tumor cells were stained, 1 if >0% but <2% of the cells were stained, 2 if 2–10% were stained, 3 if 11–30% were stained, 4 if 31–70% were stained, and 5 if 71–100% were stained. The staining intensity was rated on a scale of 0–3: 0, negative; 1, weak; 2, moderate; and 3, strong. Next, the proportion and intensity scores were combined to calculate a total score (range of 0–8) as described previously (23). Use of the human GBM specimens and database was approved by The University of Texas MD Anderson Cancer Center Institutional Review Board.

Cell Binding Assay—A cell binding assay was performed as described previously (24). Briefly, THP-1 cells were labeled for 1 h at 37 °C with 0.1 μg/ml BCECF/AM (green) and washed twice with growth medium. GBM cells were treated with 100 ng/ml EGF for the indicated periods of time. The medium was removed from the wells, and BCECF/AM-labeled THP-1 cells (2.5 × 105 cells/ml) were added to a monolayer of GBM cells. After incubation for 1 h at 37 °C, the wells were gently washed twice with warm medium to remove non-adherent cells. The nuclei of both cell types were stained with Hoechst 33342 (blue). The cells were then photographed under a fluorescence microscope. The GBM cells and labeled THP-1 cells were quantified by flow cytometry. The ratio of adherent THP-1 cells to GBM cells was calculated as the ratio of BCECF/AM-positive cells to all cells minus BCECF/AM-positive cells.

Immunoblot Analysis—Proteins were extracted from cultured cells using modified lysis buffer and then immunoblotted with the corresponding antibodies as described previously (25).

DNA Constructs—pGIPZ-p65/RelA shRNA, pGIPZ-PKCe shRNA, and pGIPZ-VCAM-1 shRNA were generated using oligonucleotides GAGCATCATGAAGAAGTGCC, CAACATT-CGGAAGCCTTGTCC, and GCGGAGATATGAATGTTG-AAT, respectively.

Transfection—Cells were plated at a density of 4 × 105 cells/60-mm-diameter dish 18 h prior to transfection. Transfection was performed using HyFect reagent (Denville Scientific Inc., Metuchen, NJ) according to the manufacturer’s instructions. Stable cell lines were selected by treatment with puromycin (5 μg/ml) for 10–14 days at 37 °C. After the treatment, antibiotic-resistant colonies of the cells were picked, pooled, and expanded for further analysis under selective conditions.

In Vitro Invasion Assay—Invasion of cells through Matrigel-coated Transwell inserts was assessed (23, 25). Briefly, Tran-
swell inserts with 8-μm pores were coated with 100 μl of Matrigel in cold serum-free medium at a final concentration of 1 mg/ml. Cells were then trypsinized and resuspended in serum-free medium. A cell suspension (1 × 10⁶ cells in 100 μl of medium) was added to the Transwell inserts. After 24 h of incubation, cells that invaded the Matrigel and passed through the filters were stained with 0.1% crystal violet for at least 5 min. The non-invading cells were then wiped from the inside of the inserts with a cotton swab. The membrane was photographed using a digital camera mounted onto a microscope, dissolved in 4% deoxycholic acid, and read colorimetrically at 590 nm.

Statistical Analysis—The significance of correlations was determined using the Pearson product-moment correlation coefficient. The mean values obtained in the control and experimental groups were analyzed for significant differences. Pairwise comparisons were performed using two-tailed Student’s t test. p values <0.05 were considered significant.

RESULTS

EGFR Activation Is Correlated with Macrophage Infiltration in Human GBM Tumors—We performed IHC analysis of 40 human GBM specimens using antibodies against CD68, which is a membrane protein expressed specifically in TAMs (26), and phospho-EGFR Tyr-1172, the levels of which represented the levels of EGFR activation (Fig. 1A) (27). Staining of the specimens was scored according to both the percentage of stained cells and the intensity of staining. Statistical analysis revealed a strong positive correlation between levels of phospho-EGFR Tyr-1172 expression and macrophage staining (r = 0.59, p < 0.01) (Fig. 1B). These results suggest that EGFR activation results in macrophage infiltration into human GBM tumors.

EGF Stimulation Increases Macrophage Binding to GBM Cells—To further examine the relationship between EGFR activation in tumor cells and macrophage recruitment to tumors, we examined whether EGF induces binding of GBM cells to THP-1 immortalized monocytes, which were derived from an acute monocytic leukemia patient and have been used as models to mimic the function and regulation of monocytes and macrophages (28). We treated U251 cells with EGF for different periods and then incubated them with BCECF/AM-labeled THP-1 cells (green). We stained both U251 and THP-1 cells with Hoechst 33342 (blue). As shown in Fig. 2A, EGF treatment promoted the binding of THP-1 cells to U251 cells in a time-dependent manner. To determine whether this effect was EGFR activation-dependent, we treated U251 cells with AG1478, a specific EGFR inhibitor, and then stimulated them with EGF. Fig. 2B shows that AG1478 treatment largely blocked EGF-induced interaction between THP-1 and U251 cells. Similar results were also obtained using D54 (Fig. 2C) and A172 (Fig. 2D) human GBM cells. These results indicate that EGFR activation in GBM cells results in the binding of macrophages to the tumor cells.

EGF Activation Results in PKCe- and NF-κB-dependent VCAM-1 Expression—VCAM-1 is reported to mediate binding of TAMs to cancer cells (29). To determine the role of VCAM-1 in EGF-induced binding of macrophages to GBM cells, we examined the effect of EGFR activation on VCAM-1 expression in GBM cells. Immunoblot analysis demonstrated that treatment with EGF increased the VCAM-1 expression in U251 cells in a time-dependent manner and that pretreatment with AG1478 inhibited the up-regulation of VCAM-1 expression (Fig. 3A). Similar results were also obtained using D54 (Fig. 3B) and A172 (Fig. 3C) cells. In addition, IHC staining of 40 human GBM specimens demonstrated a positive correlation between the levels of phospho-EGFR Tyr-1172 and VCAM-1 expression (r = 0.75, p < 0.01) (Fig. 3, D and E). These results indicate that EGFR activation results in up-regulation of VCAM-1 expression in human GBM cells.

The VCAM-1 promoter contains binding sites for NF-κB transcription factors composed of p65/RelA and p50 proteins (30, 31), and NF-κB activation is required for VCAM-1 expression (22, 32). To determine whether NF-κB activation is required for EGFR activation-induced up-regulation of VCAM-1 expression, we treated U251 cells with a NF-κB inhibitor before stimulating them with EGF. As shown in Fig. 3F, treatment with the NF-κB inhibitor largely blocked EGF-induced up-regulation of VCAM-1 expression. In addition, depletion of RelA by expression of its shRNA in U251 cells abrogated EGF-enhanced VCAM-1 expression (Fig. 3G). These data indicate that NF-κB activation is essential for EGF-induced VCAM-1 overexpression in GBM cells.
We recently reported that EGFR activation induces PKCe monoubiquitylation (31). Monoubiquitylated PKCe recruits the cytosolic IkB kinase complex to the plasma membrane, where PKCe phosphorylates and activates IkB kinase β. IkB kinase β activation phosphorylates IkBa, leading to degradation of IkBa. Cytoplasmic RelA/p50 dimers are subsequently released from binding to IkBa and translocated to the nucleus, where they bind to NF-κB sites in the promoter region of PKM, leading to the activation of PKM2 transcription (31). To test whether PKCe is essential for EGF-induced VCAM-1 up-regulation via activation of NF-κB, we depleted PKCe by shRNA expression in U251 cells (Fig. 3A, left panels). As shown in Fig. 3A (right panels), depletion of PKCe blocked EGF-induced IkBa degradation for activation of NF-κB and NF-κB-enhanced VCAM-1 expression.

**VCAM-1 Mediates Binding of THP-1 Cells to GBM Cells**—To further determine whether VCAM-1 functions as a mediator of macrophage binding to GBM cells, we used shRNA interference to stably reduce the expression of VCAM-1 in U251 cells. As shown in Fig. 4A, VCAM-1 depletion significantly inhibited EGF-induced up-regulation of VCAM-1 expression in U251 cells. In addition, depletion of VCAM-1 expression significantly inhibited EGF-induced THP-1 cell binding to U251 cells (Fig. 4B). To support this finding, we quantified the macrophages and VCAM-1 expression in human GBM specimens. As shown in Fig. 4 (C and D), we observed a strong positive correlation ($r = 0.57, p < 0.01$) between the degree of macrophage infiltration and level of VCAM-1 expression. These results indi-
cate that EGFR activation induces VCAM-1 expression in GBM cells, which promotes macrophage infiltration of GBM tumors.

**VCAM-1 Expression Is Required for EGF-induced and THP-1 Cell-mediated GBM Cell Invasion**—Contact with macrophages enhances tumor cell extravasation (12). To determine whether direct cell-cell interactions between macrophages and GBM cells regulate GBM cell invasion, we performed a Matrigel invasion assay. We found that incubation of THP-1 cells with U251 cells promoted U251 cell invasion (Fig. 5A). EGF stimulation, which induced up-regulation of VCAM-1 expression and increased the interaction between THP-1 cells and U251 cells, further enhanced U251 cell invasion (Fig. 5B). In addition, depletion of VCAM-1 significantly inhibited EGF-induced and THP-1 cell-mediated U251 cell invasion (Fig. 5B). These results indicate that THP-1 cells promote GBM cell invasion in a VCAM-1 expression-dependent manner.

**DISCUSSION**

EGFR was found to be overexpressed or mutated in 61.8% of brain gliomas (33). EGFR activation increases brain tumor angiogenesis and promotes brain tumor cell migration and invasion (6, 34). In this study, we showed that macrophage infiltration correlated with EGFR activation in human GBM specimens. In addition, EGFR activation resulted in up-regulation of
VCAM-1 expression, which subsequently promoted the interaction between macrophages and GBM cells, as well as GBM cell invasion.

TAMs derived from circulating peripheral blood monocytes are recruited to the tumor vasculature, where they extravasate into the interstitium and differentiate (13, 35). Many factors produced in areas of hypoxia in tumors, such as monocyte chemoattractant protein-1, granulocyte/macrophage colony-stimulating factor (CSF), TGF-β1, and the monocyte chemoattractant protein family (e.g. CCL2, CCL7, and CCL8), are potent chemo-
EGF Enhances VCAM-1 Expression for GBM Cell Invasion

Kines that are chemotactic toward monocytes in nearby blood vessels (10, 35, 36). In addition, VCAM-1 expression is critical to the infiltration of monocytes into tumor sites (37). Upon binding to integrin α4β1 on monocytes (38), VCAM-1 can form a scaffold for monocyte migration from the blood into tumors.

Accumulating evidence suggests that a high level of TAM infiltration of tumors, which correlates with poor prognosis, is advantageous to the spread of certain cancers via enhancement of tumor angiogenesis and tumor cell migration and invasion (36). Genetic studies of mice demonstrated that the presence of macrophages is required for efficient metastatic seeding and growth and that decreased numbers of macrophages in the tumor bed are associated with large reductions in the rates of metastasis. In addition, ablation of the macrophage population inhibits established metastatic growth (12, 39). Multiphoton microscopic analyses detected interaction between macrophages and tumor cells in close proximity, which defined a microenvironment that is directly involved in the intravasation of cancer cells in mammary tumors (40). It was reported that macrophages in areas of hypoxia can alter the tumor microenvironment by releasing diverse growth factors (VEGF, basic FGF, EGF, and TGF-α), cytokines (IL-6, IL-8, and TNF-α), and tissue matrix-degrading enzymes (matrix metalloproteinase-2, -7, and -9), which in turn activate a broad array of genes to promote tumor cell invasion and tumor angiogenesis (10, 36). A paracrine interaction between tumor cells and macrophages involving EGF and the macrophage growth factor CSF-1 takes place in EGFR-mediated invasion of breast cancer cells. Specifically, EGF secreted by macrophages activates EGFR in cancer cells to secrete CSF-1. CSF-1 attracts macrophages and promotes EGF secretion by macrophages, thereby generating a positive feedback loop (41).

VCAM-1 was identified as an adhesion molecule whose expression is induced in endothelial cells by the inflammatory cytokine IL-1, TNF, LPS, and VEGF during inflammation (38). Our results demonstrate that treatment with EGF induced up-regulation of VCAM-1 expression in GBM cells in a NF-κB-dependent manner, which is in line with previous reports demonstrating that the promoter region of the VCAM-1 gene contains NF-κB-binding sites (30, 42) and that activation of NF-κB stimulates VCAM-1 expression (38, 43, 44). In addition, via direct interaction with tumor cells, macrophages significantly induce activation of STAT-3 (signal transducer and activator of transcription-3) in the tumor cells (45). Furthermore, VCAM-1-mediated macrophage-tumor cell interaction and clustering of VCAM-1 on the cell surface transmit survival signals in cancer cells via AKT activation and enhance establishment of lung and bone metastases of breast cancer cells (29).

In summary, we have provided evidence that EGFR activation induces up-regulation of VCAM-1 expression in human GBM cells and evidence of VCAM-1-dependent interactions between macrophages and GBM cells. These interactions enhance EGFR activation-induced tumor cell invasion. These results demonstrate an instrumental role for macrophage-GBM cell interaction in GBM progression and that VCAM-1 expression is a potential target for GBM therapy.

Acknowledgment—We thank Donald R. Norwood for critical reading of this manuscript.

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