Research Paper

Inhibition of Basigin Expression in Glioblastoma Cell Line via Antisense RNA Reduces Tumor Cell Invasion and Angiogenesis

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INTRODUCTION

Gliomas are the most common of brain neoplasms and exhibit a high degree of diffuse local invasion of normal nervous tissue. Tumor cells often infiltrate beyond any obviously defined tumor margin and contribute to the high incidence of recurrence. In the invasive and metastatic process of malignant tumor, molecules existing in extracellular matrix (ECM) and receptors or ligands existing on the surfaces of tumor cells play critical roles. CD147, also named extracellular matrix metalloproteinase inducer (EMMPRIN), is originated from human lung cancer cell line LX-1. EMMPRIN expressed by cultured tumor cells stimulates fibroblasts to produce very high levels of collagenase activity, which likely facilitates tumor metastasis. Previous studies demonstrated that EMMPRIN concentrated on the surfaces of most tumor cells, promoted invasion of tumor cells by stimulating stromal cells to produce elevated levels of several matrix metalloproteinase (MMPs) which play very important roles in several aspects of tumor progression, including growth, invasion, metastasis, and angiogenesis. Moreover, supporting its key role in the processes of tumorigenesis and metastasis, EMMPRIN was reported as one of the most constantly upregulated mRNA in metastatic cells. Our interest in gliomas let us to consider the role of CD147 in glioma invasion, metastasis, and angiogenesis. Here, we constructed a vector of antisense RNA of CD147 and investigated its inhibitory effects on invasion and angiogenesis of glioblastoma cells in vitro.

MATERIALS AND METHODS

Cell culture. The human glioblastoma cell line U251 was maintained in DMEM medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 atmosphere.

Construction and transfection of antisense vector. Full-length cDNA fragment coding for basigin/CD147 was cloned in the antisense orientation into the XbaI and XhoI site of the eukaryotic expression vector PCI-neo. The antisense vector of basigin, named as PCI-147.

Empty vector PCI-neo and antisense vector PCI-147 were transfected into human glioblastoma cell line U251 via cation liposome Lipofectamine2000 (Gibco) according to the manufacturer’s description and the two kinds of transfected cells were named as U251 neo and U251/147 respectively. Stable antisense transfectants were generated and characterized.

Western blot. U251, U251/neo and U251/147 were cultured for 72 h respectively. Then cells were harvested, washed twice with cold PBS and lysed in buffer (150mM NaCl, 50mM Tris-HCL, 2 mM EDTA, 1% NP-40, and pH7.4), containing protease inhibitors. Equal amount of protein (30 μg/lane) from lysate were subjected to SDS-PAGE under reducing conditions on 10% acrylamide...
gels. After SDS-PAGE, proteins were transferred to an ImmobilonTM-P Transfer Membrane (Millipore Corporation, USA). Then, to block non-specific binding, the membrane was incubated in PBS with 0.1% Tween-20 (T-PBS) containing 5% non-fat skim milk for 1 h. Subsequently, the membrane was incubated with antibody against CD147 overnight at 4˚C, washed in T-PBS and incubated with peroxidase-conjugated anti-mouse antibody (Sigma) for 2h. Protein on the membrane was visualized using enhanced chemiluminescence (ECL plus western blotting detective system, Amersham, UK). For loading control, the membrane was probed with monoclonal antibodies for β-actin as standard protocols.

Wound-induced migration assay. 3 x 10^5 cells suspended in DMEM were plated onto 12-well culture plates and incubated for 24 h. Then a single wound was created in the center of the cell monolayer. Debris was removed with two washings with DMEM and the medium was replaced. After 12h, the distance that the advancing cells had moved into the wound area was measured, after which the cells were fixed and stained with HE, then photographed using an inverted photomicroscope (Olympus).

Gelatin zymography. Gelatin zymography was assayed as described elsewhere. Briefly, three experimental groups of cells were U251, U251/neo and U251/147, which were cultured respectively. The supernatants were precipitated with 800 g/L saturated (NH4)2SO4. Precipitations were dissolved in 10 mmol/L Tris-HCl, pH 7.5 and dialyzed. Dialed samples were determined with SDS-PAGE that was modified in four points. Gelatin (Sigma) was added into the separating gel with 1.0 g/L, concentration of the stacking gel was 5%, samples were not boiled and sample buffers did not contain DTT. After electrophoresis, the gel was washed with 0.1 mol/L NaCl and incubated for 24 hours at 37˚C. Finally, the gel was dyed and decolorized.

Boyden chamber invasion assay. Invasion in vitro was measured in Boyden chamber assay (Millipore). Matrigel (main component was type IV collagen, purchased from Cell-biology Department of Pecking University) was added onto the inner surface of Boyden chambers to form the reconstituted basement membrane. Three groups of cell suspensions (U251, U251/neo and U251/147, 200 µL, 3 x 10^5 cells per mL) were added on the reconstituted basement membrane respectively. Then the chambers were put in the 24-well plates and cultured overnight. Cells infiltrated through the reconstituted basement membrane and appeared on the outer surfaces of the membrane were stained with HE. The numbers of the cells were counted under high-power microscope.

ELISA analysis of VEGF release. 2 x 10^5 cells were cultured in 6-well culture plates until 90% confluence. Then the spent medium was removed, dishes were washed three times and incubated with serum-free medium for 24 h. The conditioned medium was collected and VEGF concentration was determined using an ELISA kit (R and D, USA).

**RESULTS**

Effect of antisense vector on CD147 levels in total cell extracts. Basigin/CD147 has been shown to play significant roles in ECM degradation. Transfection of U251 cells with the antisense vector strongly inhibited the expression of CD147 as compared to U251, U251/neo (Fig. 1).

Downregulation of CD147 and inhibition of cell migration. Since CD147 has implicated in the invasiveness of tumor cells, we evaluated the migration of parental and stable transfectants. In scrape-wound-migration assay, the mobility of stable antisense transfectants was decreased as compared to parental- and empty vector-transfected cells (Fig. 2).

Downregulation of CD147 and inhibition of cell invasion and pro-angiogenic molecules. The secretions of MMP-9 and MMP-2 in the antisense transfectants were inhibited compared to parental- and empty vector-transfected cells in Gelatin zymography assay (Fig. 3). And the stable transfectants infiltrated through the reconstituted basement membrane were less than those of U251/neo and intact U251 (Fig. 4). Furthermore, down-regulation of CD147 by antisense transfection in U251 cells resulted in a
reduction of VEGF secreted into media by antisense tumor cells compared to parental- and empty vector-transfected cells, as determined by quantitative VEGF ELISA (Fig. 5).

DISCUSSION

ECM degradation is critical to malignant progression of tumor cells. Matrix metalloproteinases (MMPs) play an important part in tumor progression and tumor cell survival, with a positive correlation between MMP expression and the invasive and metastatic potential of malignant tumors, including colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian, and gastric carcinomas. CD147 is a heavily glycosylated transmembrane glycoprotein containing two immunoglobulin superfamily domains, which induces MMPs production in the adjacent stromal cells. Some results implied that a CD147 counter-receptor might existed on the fb cell surface, but such a counter-receptor has not been identified. Antisense RNA is a convenient approach for the selective down-regulation of protein expression. The principle of antisense technology is the sequence-specific binding of an antisense oligonucleotide to target mRNA, resulting in the prevention of gene translation. The specificity of hybridisation makes antisense treatment to be an attractive strategy selectively modulating the expression of genes involved in the pathogenesis of diseases. Since 1998, the first antisense drug (fomiviren) had been approved by the US Food and Drugs Administration (FDA) for the treatment of cytomegalovirus-induced retinitis in patients with AIDS, several antisense oligonucleotides have been under clinical trials, including oligonucleotides targeting the mRNA of Bcl-2, protein-kinase-C alpha, RAF kinase, H-ras, C-myb, DNA methyltransferase and RI-α regulatory subunit of protein kinase A (PKA). Antisense oligonucleotides are well tolerated and might have therapeutic activities.

Cell migration is a highly regulated process, which is critical for physiologic and pathologic tissue remodelind. The results here demonstrate that suppression of CD147 expression in human glioblastoma cells by CD147 transfection reduces cell invasion,
migration and angiogenic potential. The antisense vector transfection-mediated downregulation of CD147 was associated with inhibition of MMPs activity and reduced VEGF protein in culture supernatants. Gelatin zymography demonstrated that secretions of MMP-2 and MMP-9 of U251/147 were lower than those of U251.neo and U251. Boyden chamber invasion assay indicated that invasion of U251/147 cells was inhibited significantly. All of the results have shown that antisense RNA targeting CD147 mRNA interfered with translation and expression of CD147, weakened the productions of MMPs, and inhibited the invasion of glioblastoma cells through reconstructed basement membrane in vitro.

Angiogenesis plays a critical part in tumor development and progression, as it is the process by which a tumor increases its supporting vascular supply. The development of new blood vessels in tumors depends on the production of angiogenic factors released from the tumor cells and/or stromal cells. VEGF and MMP have been shown to be involved in angiogenesis in variety of experimental models. The progressive growth of glioblastomas is angiogenesis dependent. Glioblastoma cells secrete a variety of proangiogenic molecules including VEGF and type IV collagens. Expression of VEGF by glioblastoma cells has been shown to directly correlate with malignant potential and administration of anti-VEGF antibodies to mice injected with human glioblastoma cancer cells has been shown to inhibit neovascularization within the tumors. Therefore, it is not surprising that we also found a reduced VEGF protein in many areas of tumor progression including invasion, migration and angiogenesis. Intervention in CD147 function by agents, such as antisense RNA, may have potential therapeutic value in the prevention of glioma invasion and metastasis.

References