Involvement of NF-κB-mediated expression of galectin-3-binding protein in TNF-α-induced breast cancer cell adhesion

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Abstract. Galectin-3-binding protein (G3BP) is highly expressed in various types of cancer and is thought to be involved in cancer malignancy; however, the role of G3BP in breast cancer cells is not fully understood. In this study, we investigated the role of NF-κB in the adhesion of breast cancer cells to a substrate by using (-)-DHMEQ, a specific inhibitor of NF-κB. (-)-DHMEQ inhibited both TNF-α-induced G3BP expression and cell adhesion in human breast cancer cell lines. We also found that knockdown of G3BP suppressed the adhesion, while its overexpression increased the adhesion. These data reveal that (-)-DHMEQ suppresses breast cancer cell adhesion by inhibiting NF-κB-regulated G3BP expression.

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among women (1). A woman born in the United States today has a 1 in 8 chance of having invasive breast cancer during her lifetime (2). Many factors are currently being studied to understand the causes of breast cancer. These include lifestyle, environmental, genetic, and biological factors (3). The most significant risk factors are gender (female) and age (growing older) (4). Despite earlier diagnosis and better management of locally confined breast cancer, 20-30% of patients will have recurrent disease in a distant organ (5). Successful treatment of advanced breast cancer with chemotherapy depends on finding an agent to which the tumor has not developed resistance and treating the patient with a sufficient dose of it to achieve a positive response (6).

NF-κB is a eukaryotic transcription factor that exists in virtually all cell types. It was first described in 1986 as a nuclear factor necessary for immunoglobulin κ light chain transcription in B cells (7). It exists in the cytoplasm of most cells in an inactive form bound to its inhibitor, IκB. When cells are treated with an inflammatory cytokine, such as TNF-α or IL-1β, NF-κB is released from IκB and is translocated to the nucleus where it upregulates the transcription of specific genes (8). NF-κB regulates many genes involved in the promotion of cancer, such as those orchestrating clonal expansion, growth, diversification, angiogenesis, adhesion, and extravasation. For example, NF-κB may regulate pro-inflammatory genes including TNF-α, IL-1β, iNOS, matrix metalloproteinase (MMP-9), urokinase-type plasminogen activator (uPA) and many other chemokines (9). Previously, we designed and synthesized a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin, abbreviated as DHMEQ (10). After the chiral separation of it, (-)-DHMEQ was shown to be 10 times more potent than (+)-DHMEQ (11). DHMEQ inhibits the translocation of NF-κB protein to the nucleus (12) and DNA binding activity of NF-κB by covalently binding to specific cysteine residues of the protein (13). DHMEQ suppresses the growth of prostate carcinoma, thyroid carcinoma, breast carcinoma, pancreatic carcinoma, multiple myeloma, and T-cell leukemia in nude or SCID mice without any toxicity (14).

Galectin-3-binding protein (G3BP) is a secretory glycoprotein, also designated as Mac-2BP or 90K, which was originally identified as a tumor-associated antigen in breast cancer cells (15). G3BP is present in the extracellular matrix of several tissues (16), tumor cell medium, milk, and serum; and it was shown to be a non-covalently linked oligomer with a molecular mass of >1000 kDa (17,18). Northern blotting and immunohistochemical analyses have revealed elevated expression of G3BP in pancreatic, breast and lung cancer (19-21). Elevated levels of G3BP are often observed in patients with different types of cancer and are associated with a poor survival and metastatic spread to the liver, as revealed by a study involving a series of 375 patients with operable breast cancer (22). Also, G3BP is expressed in about 30% of stage I non-small cell lung cancers; and its expression is associated with a significantly worse outcome (23). Therefore, G3BP is considered to be a tumor marker for these cancers; but its functions are not yet well understood. G3BP is extensively glycosylated and interacts with extracellular matrix components, such as fibronectin and β1-integrin, but not with collagen I (24). Accordingly, we

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hypothesized that G3BP mediates cell-fibronectin adhesion by interacting with β1-integrin and fibronectin.

In the present study, we assessed the involvement of NF-κB activation in the expression of G3BP and examined the function of G3BP in breast cancer cells.

Materials and methods

Materials. Recombinant human TNF-α was purchased from PeproTech (Rocky Hill, NJ). (-)-DHMEQ was synthesized in our laboratory as previously described (11).

Cell culture. Human breast carcinoma T47D and MCF-7 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 200 U/ml penicillin G, 200 mg/l kanamycin, 600 mg/l L-glutamine, and 2.25 g/l $NaHCO_3$ at 37˚C in a humidified incubator with 5% CO_2.

Electrophoretic mobility shift assay (EMSA). Cells were grown in 60-mm dishes and incubated with the desired chemicals. They were then harvested, washed with phosphate-buffered saline (PBS), suspended in 400 μl of buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF], and incubated on ice for 15 min. Nuclei were precipitated, resuspended in 40 μl of buffer C [50 mM HEPES (pH 7.8), containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% glycerol (v/v)], incubated on ice for 20 min, and centrifuged. The supernatant was used as the nuclear extract.

The binding reaction mixture included the nuclear extract (5 μg of protein), 2 μg poly(dI-dC), and 10000-cpm ^{32}P-labeled probe (oligonucleotide containing the NF-κB binding site) in a binding buffer (75 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 1.5% NP-40, 15 mM Tris-HCl; pH 7.0). DNA/protein complexes were separated from protein-unbound DNA on a 4% native polyacrylamide gel. The DNA probes used for NF-κB binding were purchased from Promega (Madison, WI). These oligonucleotides were labeled with [γ-^{32}P]-ATP (3000 Ci/mmol; GE Healthcare, Little Chalfont, UK) by use of T4 polynucleotide kinase (Takara, Otsu, Japan). Mouse monoclonal anti-p65 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (13).

Cell adhesion assay. Following, trypsin treatment and suspension in complete medium, T47D cells were resuspended in serum-free medium and seeded into the wells of a fibronectin-coated 24-well plate (BD Biosciences, Tokyo, Japan). After 1 h, the cells were gently washed twice with PBS followed by the addition of complete medium containing MTT to each well. After 2 h, the cells were dissolved in DMSO, and the amount of cell adhesion was measured in terms of the absorbance of the reduced MTT at 570 nm by using the iMark™ microplate reader (Bio-Rad Laboratories, Hercules, CA).

RNA isolation and semi-quantitative RT-PCR analysis. Total RNA was extracted from T47D or MCF-7 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was carried out at 37˚C for 120 min with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). The cDNA was used for PCR amplification with rTaq DNA polymerase (Takara). The number of PCR cycles for each product was determined after confirmation of the efficacy of amplification and after having defined the linear exponential portion of the amplification. The sequences of the primers used for semi-quantitative RT-PCR, the number of cycles, and the annealing temperatures were as follow: G3BP, 5'-ACCAATGAAACCAGGAGCAC-3' (forward) and 5'-GCATCCACACTCATGGTGAC-3' (reverse), 27 cycles, 59˚C; β1-integrin, 5'-GACCTGCCTTGGTGTCTGTGC-3' (forward) and 5'-AGCAACCACACCAGCTACAAT-3' (reverse), 25 cycles, 59˚C; and β-actin, 5'-TTCTACAATGAGCTGCGTGT-3' (forward) and 5'-GTCAGGTCCCGGCCAGCCAG-3' (reverse), 20 cycles, 55˚C.

Knockdown of G3BP by siRNA. The sequences of siRNAs against G3BP were the following: 5'-CAGACGUCACCGAUUUCGAdTdT-3' (G3BP#1) and 5'-CGCACCAUUGCCUACGAAAdTdT-3' (G3BP#2). The sequence of siRNA against luciferase, used as a negative control, was 5'-CGUACGCGGAAUACUUCGAdTdT-3'. Transfection of cells with siRNAs was performed by using X-tremeGENE transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

Establishment of G3BP-overexpressing stable cell lines. To obtain G3BP-overexpressing cell lines, we carried out gene transfection by using Lipofectamine LTX transfection reagent (Invitrogen). The human G3BP gene was cloned from the cDNA of T47D cells, and was inserted into the pCI-neo vector (Promega). Permanent cell lines stably expressing G3BP-MycHis$_6$ protein were established by transfecting T47D cells with pCI-neo-G3BP-MycHis$_6$ followed by G418 selection. The cells transfected with the pCI-neo vector were designated as T47D-Neo cells, and the clonal cells expressing high levels of G3BP-MycHis$_6$ protein were designated as T47D-G3BP cells.

Results

Inhibition of both TNF-α-induced cell adhesion and G3BP expression by (-)-DHMEQ. Exponentially growing T47D cells exhibited a low level of NF-κB activity; however, treatment with TNF-α enhanced the activity as assessed by the DNA-binding activity of nuclear NF-κB (Fig. 1A). (-)-DHMEQ inhibited the TNF-α-induced NF-κB activation in a dose-dependent manner (Fig. 1A). Concomitant with NF-κB activity, the number of T47D cells attached to the fibronectin-coated wells was increased by the treatment with TNF-α and this increase was inhibited by (-)-DHMEQ (Fig. 1B). Treatment with 10 ng/ml TNF-α induced G3BP mRNA expression (Fig. 1C), which was inhibited by (-)-DHMEQ (Fig. 1D). TNF-α treatment did not stimulate cell adhesion to type-I collagen as expected (data not shown), because G3BP is not a ligand of type-I collagen (24). Similar results were obtained when the MCF-7 human breast cancer cells were used (data not shown). Therefore, these results suggest that (-)-DHMEQ suppressed the TNF-α-induced adhesion of T47D cells to fibronectin by inhibiting NF-κB-regulated G3BP expression.

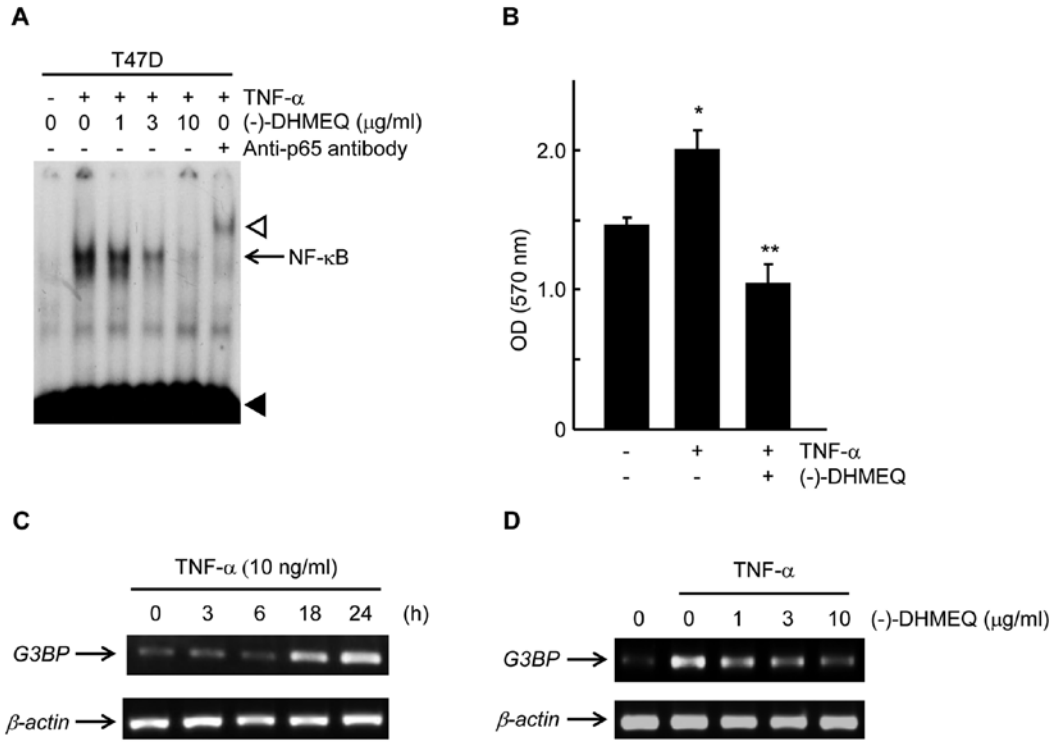

Figure 1. Inhibition of TNF-α-induced cell adhesion of and G3BP expression in T47D cells by (-)-DHMEQ. (A) Inhibition of TNF-α induced NF-κB activation by (-)-DHMEQ. T47D cells were pretreated with the indicated concentrations of (-)-DHMEQ for 1 h, and then stimulated with 10 ng/ml TNF-α for 1 h. Nuclear proteins were assayed by EMSA. Open and closed triangles are the super-shifted complex and free probe, respectively. (B) Inhibition of TNF-α induced cell adhesion by (-)-DHMEQ. T47D cells were pretreated with 10 μg/ml (-)-DHMEQ for 1 h, and subsequently stimulated with 5 ng/ml TNF-α. After 20 h of stimulation, the cells were examined for adhesion. The amount of cell adhesion was detected using the MTT assay. *,**P<0.05 (Student's t-test) for TNF-α-treated cells vs. the control cells and vs. the TNF-α/(-)-DHMEQ-treated cells, respectively. (C) Induction of G3BP expression by TNF-α. Total RNAs were extracted, and the expression of each gene was measured by semi-quantitative RT-PCR. (D) Inhibition of TNF-α-induced G3BP expression by (-)-DHMEQ. T47D cells were treated with various concentrations of (-)-DHMEQ for 1 h, and then stimulated with 10 ng/ml TNF-α for 24 h. Total RNAs were extracted, and the expression of each gene was measured by semi-quantitative RT-PCR.

G3BP regulates T47D cell adhesion to fibronectin induced by TNF-α. To determine the role of G3BP in breast cancer cell adhesion, we used 2 different siRNAs targeted against G3BP, i.e., siG3BP#1 and siG3BP#2, for avoiding the off-target effect. The efficacy of siG3BP#1 and siG3BP#2 was confirmed by performing semi-quantitative RT-PCR in the absence or presence of TNF-α (Fig. 2A). Moreover, treatment with siG3BP#1 or siG3BP#2 decreased the TNF-α-induced adhesion of T47D cells to fibronectin (Fig. 2B), thus indicating that the TNF-α-enhanced adhesion of T47D cells to fibronectin was mainly regulated by G3BP.

To confirm that inhibition of G3BP expression was responsible for (-)-DHMEQ-suppressed cell adhesion, we established G3BP-overexpressing T47D cells, designed as T47D-G3BP cells (Fig. 3A). Treatment with (-)-DHMEQ did not affect the expression of exogenous G3BP in T47D-G3BP cells, because this expression was regulated by the CMV promoter (Fig. 3B). Moreover, the expression of β1-integrin, which is a receptor of G3BP, was also not altered after (-)-DHMEQ treatment of T47D-G3BP and T47D-Neo cells (Fig. 3B).

Finally, we studied the effect of G3BP expression on cell adhesion to fibronectin. As shown in Fig. 3C, overexpression of G3BP increased the adhesion of T47D cells to fibronectin regardless of (-)-DHMEQ treatment. Therefore, the overexpression of G3BP overcame the (-)-DHMEQ-suppressed cell adhesion.

Discussion

The malignancy of breast cancer cells often depends on increased adhesion, migration, and invasion (25). NF-κB enhances the expression of many genes involved in cancer malignancy. For example, activation of NF-κB induces the expression of cell-adhesion molecules such as ICAM-1, ELAM-1, and VCAM-1 (26-28). In this study, we initially demonstrated that (-)-DHMEQ inhibited the activation of NF-κB in breast cancer T47D cells (Fig. 1A). (-)-DHMEQ also inhibited TNF-α-induced cell adhesion to fibronectin (Fig. 1B) but not to collagen I (data not shown). It has been reported that high expression of G3BP is associated with a poor survival of breast cancer patients (22). In agreement with these results, G3BP is known to bind to fibronectin, but not to collagen I (24). We then found that G3BP expression was regulated by NF-κB, since (-)-DHMEQ inhibited TNF-α-induced G3BP mRNA expression in breast cancer cells, as shown in Fig. 1C and D. After these observations, we studied the regulation of the G3BP gene transcription by performing a luciferase promoter assay. The human G3BP promoter region and first intron have 3 NF-κB consensus binding sites, i.e., from -1647 to -1637, -1679 to -1669, and from +1270 to +1279. Thus, we cloned these DNAs from T47D genomic DNA, and subcloned them into the pGL4.17 firefly luciferase reporter vector. After transfection, we examined

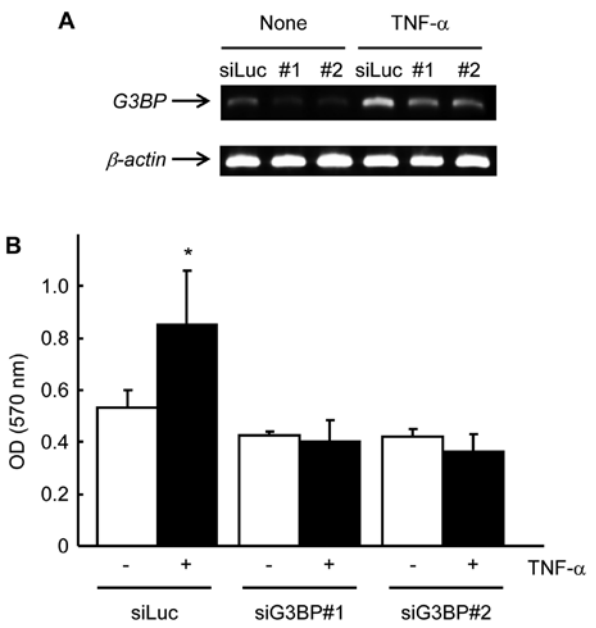

Figure 2. Involvement of G3BP expression in TNF-α-induced cell adhesion. (A) Knockdown of G3BP expression by G3BP-targeted siRNA. T47D cells were transfected with luciferase-siRNA (siLuc; used as control siRNA), G3BP-siRNA#1 (#1) or G3BP-siRNA#2 (#2) for 48 h, and thereafter stimulated or not with 5 ng/ml TNF-α. The expression levels of each gene were measured by semi-quantitative RT-PCR. (B) Suppression of cell-extracellular matrix adhesion of T47D cells by treatment with G3BP-siRNA. T47D cells were transfected with luciferase-siRNA (siLuc) or G3BP-siRNA#1 (siG3BP#1) or G3BP-siRNA#2 (siG3BP#2) for 48 h, and then stimulated with 5 ng/ml TNF-α for 20 h. The cells were thereafter seeded in serum-free medium into fibronectin-coated wells of a 24-well plate. Cell adhesion was assessed using the MTT assay. *P<0.05 (Student's t-test) between TNF-α-treated cells and control cells.

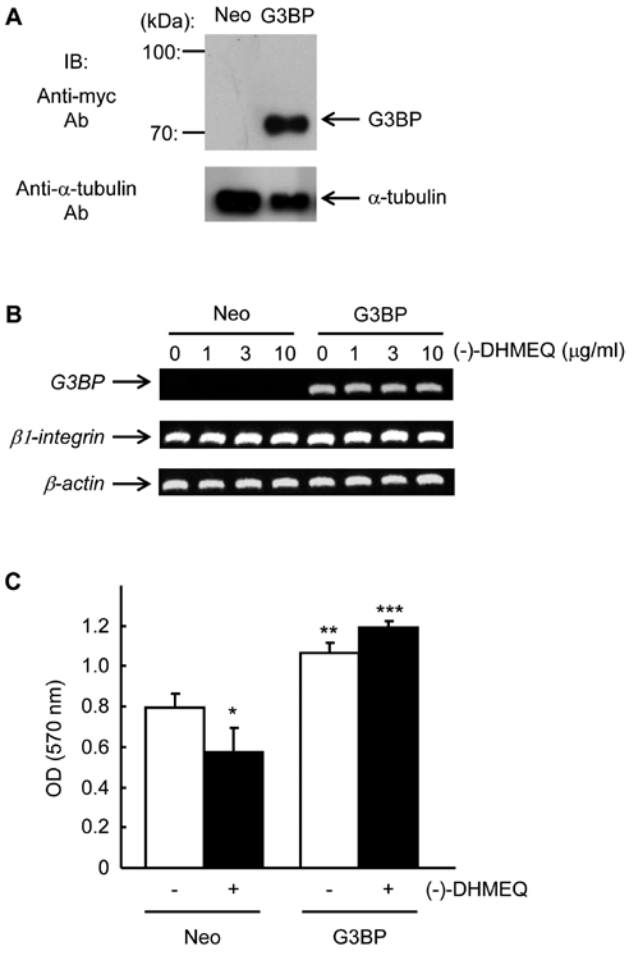

Figure 3. Circumvention of the (-)-DHMEQ-induced suppression of cell adhesion by overexpression of G3BP. (A) Establishment of T47D-Neo and T47D-G3BP cells. T47D cells were transfected with pCI-neo or pCI-G3BP-MycHis$_6$ vectors. After G418 selection, the cells were lysed, and aliquots of cell lysates were immunoblotted with the indicated antibodies. (B) Effect of (-)-DHMEQ on expression of G3BP and β1-integrin. T47D cells were treated with (-)-DHMEQ for 20 h. Total RNAs were then extracted, and the expression of each gene was measured by semi-quantitative RT-PCR. (C) Overexpression of G3BP overcomes (-)-DHMEQ-suppressed cell adhesion. T47D-Neo and T47D-G3BP cells were treated with 10 μg/ml (-)-DHMEQ for 20 h, and then the cells were seeded into fibronectin-coated wells of a 24-well plate. The amount of cell adhesion was assessed by the MTT assay. *,**,***P<0.05 statistically significant (Student's t-test) differences between (-)-DHMEQ-treated Neo cells and control Neo cells, G3BP-overexpressing cells and Neo cells, and (-)-DHMEQ-treated Neo cells and (-)-DHMEQ-treated G3BP-overexpressing cells, respectively.

whether treatment with TNF-α would activate G3BP expression and (-)-DHMEQ would inhibit it. Treatment with TNF-α enhanced the G3BP promoter activity; however, (-)-DHMEQ did not affect the TNF-α-activated G3BP promoter activity (data not shown). We thus speculate that inhibition of TNF-α-induced G3BP expression by (-)-DHMEQ is regulated by some other promoter region(s). Further studies are necessary to identify the responsible region(s).

The adhesive interactions between a cell and its surrounding extracellular matrix (ECM) regulate cell morphology, migratory properties, growth, and differentiation (29). Especially, cell-ECM adhesion has a role in metastasis to other organs. Fibronectin is a high-molecular-weight glycoprotein that plays major roles in cell adhesion, growth, migration and differentiation. The adhesion of lung carcinoma cells to fibronectin enhances their tumorigenicity and confers resistance to apoptosis-inducing chemotherapeutic agents (30). In this study, we found a new function of G3BP, i.e., its involvement in cell-fibronectin adhesion. As was shown in Figs. 2 and 3, G3BP knockdown decreased the adhesion of T47D cells to fibronectin; and overexpression of G3BP increased it. G3BP can bind to β1-integrin and fibronectin (24); and the expression of β1-integrin was detected in T47D, T47D-Neo, and T47D-G3BP cells but was not affected by the treatment with (-)-DHMEQ (Fig. 3B). Thus, these data suggest that the increase in T47D cell adhesion to fibronectin was due to the interaction of G3BP with β1-integrin and fibronectin.

In conclusion, our results show that NF-κB regulated the expression of G3BP and that G3BP increased the adhesion of T47D cells to fibronectin. These results reveal a mechanism for the NF-κB-dependent cell adhesion and the feasibility of G3BP as a novel molecular target of breast cancer therapy.

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