Recombinant fibromodulin and decorin effects on NF-κB and TGFβ1 in the 4T1 breast cancer cell line

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Abstract. Constitutive activation of nuclear factor-κB (NF-κB) stimulates cell proliferation and metastasis, and inhibits apoptosis in breast cancer. Transforming growth factor-β (TGF-β) signaling pathway is deregulated in breast cancer progression and metastasis. The aim of the present study was to investigate the inhibitory effects of the two small leucine rich proteoglycans fibromodulin (Fmod) and decorin (Dcn), overexpressed using adenovirus gene transfer, on NF-κB-activity and TGF-β1-expression in the highly metastatic 4T1 breast cancer cell line. The results demonstrate that Fmod and Dcn overexpression is associated with NF-κB and TGF-β1 downregulation, and that Fmod promotes this effect more effectively compared with Dcn.

Introduction

Breast cancer is the second most prevalent cause of cancer-associated mortality in females; metastasis in breast cancer is the primary cause of mortality and is a crucial factor in treatment (1-3). Constitutive activation of nuclear factor-κB (NF-κB), a family of transcription factors (4), stimulates proliferation and metastasis, and inhibits apoptosis in breast cancer (5). These proteins form homo- or heterodimers and have similar structural characteristics, including the Rel homology domain, which is necessary for dimerization, binding to cognate DNA elements and nuclear localization signals (4,6). In non-stimulated cells, NF-κB complexes in an inactive form interact with a monomer of an inhibitory protein called inhibitor of NF-κB (IκB) (6). NF-κB activity stimulating signals cause dissociation of IκB, allowing NF-κB dimers to locate to the nucleus and alter gene expression (6). Additionally, NF-κB signaling is essential for epithelial-mesenchymal transition (EMT), and the therapeutic inhibition of NF-κB may be an effective strategy to control tumor invasion and metastasis (7).

Transforming growth factor β (TGF-β) is a pleiotropic cytokine that is found in three isoforms (TGF-β1, TGF-β2 and TGF-β3), which are structurally and functionally associated (8). TGF-β isoforms are secreted as biologically latent precursor molecules and are stimulated by proteolytic cleavage interactions with integrins or by pH alterations in the local microenvironment; intracellular TGF-β signaling is complex and is activated by numerous signaling pathways (9). TGF-β1 is involved in the occurrence and development of breast cancer (10) and the TGF-β signaling pathway is deregulated in breast cancer progression and metastasis (11,12).

In invasive breast cancer, certain alterations have been observed in the stromal structure, including a reduction in the expression of two small leucine rich proteoglycans, fibromodulin (Fmod) and decorin (Dcn), and the acquisition of TGF-β antagonist activity *in vitro* and *in vivo* has been identified (13-16). Dcn is a dermatansulfate proteoglycan that reduces the growth of tumors, including gliomas, breast, lung, colon and squamous cell carcinoma, and has an anti-angiogenic role through the binding TGF-β, epidermal growth factor receptor (EGFR) and inducing the expression of p21 (13,17-21). Fmod, a keratan sulfate proteoglycan, functions as a modulator of TGF-β activity in scarless wound healing and is involved in collagen assembly in skin development (22,23). In addition, Fmod exerts a potent TGF-β-antagonist activity, compared with Dcn, in the inhibition of neointimal hyperplasia in saphenous vein graft (15). Fmod is also implicated in the inhibitory effect of NF-κB signaling thorough the suppression of IκBα protein in 3T3-L1 fibroblasts (24).

In the current study, the inhibitory effects of Fmod and Dcn overexpression on NF-κB and TGF-β1 were investigated using adenovirus-mediated gene transfer in the 4T1 breast cancer cell line.

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Materials and methods

Recombinant adenovirus construct. The recombinant adenovirus (Rad) Fmod and Dcn expression cassettes were constructed by Dr Paul Kingston (Gene Therapy Unit, University of Manchester, Manchester, UK), and contain the major immediate-early murine cytomegalovirus enhancer/promoter, Woodchuck hepatitis virus regulatory element and a fragment of the rabbit smooth muscle myosin heavy chain promoter, which produces increased transgene expression compared with other expression vectors. Rad vectors are E1/E3-deleted first-generation adenoviruses that have a recombinant transgene and promoter inserted instead of possessing an E1 region (13). The efficiency of these vectors was confirmed in a previous study (13).

Cell culture. The highly metastatic 4T1 breast cancer cell line was obtained from Pasture Institute of Iran (Tehran, Iran) and cultured in RPMI-1,640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5% CO_2 at 37˚C. Cells were not allowed to be >80% confluent, and $4x10^5$ cells were treated with Rad-Fmod, Rad-Dcn or Rad-LacZ at a multiplicity of infection (MOI) of 1,000, which was considered an appropriate MOI for this adenovirus (25). Cells were incubated for 4 h at 37˚C and medium was regularly replaced with fresh RPMI-1,640 (Sigma-Aldrich; Merck KGaA). After 72 h, cells were used for further analysis. Uninfected cells cultured in the same conditions were used as a negative control.

Reverse transcription-polymerase chain reaction (RT-PCR). The 4T1 cell line total RNA was extracted using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was reverse-transcribed with a OneStep RT-PCR kit (Qiagen GmbH, Germany) at 50˚C for 30 min with initial PCR activation at 95˚C for 15 min. cDNA was amplified by 35 cycles, each consisting of three steps: Denaturation at 94˚C for 45 sec, annealing at 63˚C for 45 sec, extension at 72˚C for 1 min and final extension at 72˚C for 10 min. Specific primers (Metabion GmbH, Steinkirchen, Germany) for Dcn (forward primer, 5'-CCCAGAAGTTCC TGATGAC-3'; reverse primer, 5'-CAGAGCGCACGTAGA CAC-3'), Fmod (forward primer, 5'-TGAAGGCAGCACCTG ACCGC-3'; reverse primer, 5'-ACGCCTTGGCTTCTCCTG CC-3') and β-actin as a control (forward primer, 5'-ATATCG CTGCGCTGGTCGTC-3'; reverse primer, 5'-AGGATGGCG TGAGGGAGAGC-3') were used in this experiment. PCR products were separated on 1% agarose gel with 0.5 μg/ml ethidium bromide for Dcn and 2% agarose gel for Fmod.

RT-quantitative PCR (RT-qPCR). RT-qPCR was used for the detection of Fmod and Dcn inhibitory effects on TGF-β1 expression in the 4T1 breast cancer cell line. Total RNA was extracted using an RNeasy Mini kit (Qiagen GmbH), then cDNA was amplified with the QuantiTec Reverse transcription kit (Qiagen GmbH) and TGF-β1 mRNA expression was quantified using a QuantiFast SYBR-Green Master PCR kit (Qiagen GmbH) in triplicate on an Applied Biosystems 7300 using the 'standard curve method' (26). Standard curves for TGF-β1 and GAPDH were generated via five serial dilutions with cDNA. Cq values from each gene were measured from the curve and were quantified relative to GAPDH as the control housekeeping gene (27). All experiments were performed in three independent experiments with 60˚C as the annealing temperature. The amplification process included 95˚C for 5 min, followed by 35 cycles at 95˚C for 10 sec and 60˚C for 30 sec. The primers were as follows: Mouse TGF-β1 forward, 5'-GGTAACCGGCTGCTGACC-3; mouse TGF-β1 reverse, 5'-GCCCTGTATTCCGTCTCCTTG-3'; mouse GAPDH forward, 5'-GGCCTTCCGTGTTCCTAC-3'; mouse GAPDH reverse, 5'-TGTCATCATACTTGGCAGGTT-3'.

Nuclear extract and NF-κB transbinding assay. A total of $1x10^6$ cells/ml 4T1 cells were collected and nuclear extracts were isolated using the Nuclear Extract kit from Active Motif GmbH (Regensburg, Germany) according to the manufacturer's protocol. Nuclear extracts were stored at -80˚C until they were used, and their concentration was measured using a Bradford assay. NF-κB DNA binding activity was determined using an Trans-AM P65-NF-κB ELISA-based kit (cat number 40096; Active Motif GmbH), which is a sensitive assay that measures the quantity of activated NF-κB in the nuclear extracts from the 4T1 breast cancer cell line, prior to and following Fmod and Dcn expression. In total, 10 μg nuclear extract was added to a biotinylated oligonucleotide containing the NF-κB consensus site attached to the streptavidin-coated 96-well plates. Plates were washed with wash buffer (cat number 40096; Active Motif GmbH) to remove all the unbound reagents; to visualize NF-κB DNA binding, an anti-p65 primary antibody (dilution, 1:2,000; cat number 40096; Active Motif GmbH) was added for 1 h at room temperature without agitation, followed by a goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (dilution, 1:5,000; cat number 40096; Active Motif GmbH) at room temperature without agitation. Subsequent to an incubation for 1 h at room temperature, 100 μl developing solution (cat number 40096; Active Motif GmbH) was added to all wells for 5 min at room temperature protected from direct light. The blue color development in the sample wells was monitored until it turned medium to dark blue. Subsequently, 100 μl stop solution (cat number 40096; Active Motif GmbH) was added and the blue color turned yellow. Finally, the absorbance value was ascertained using a spectrophotometer at a wavelength of 450 nm. For the p65 positive control: 2.5 μg of Jurkat nuclear extract was provided (1 μl of nuclear extract in 19 μl of complete lysis buffer per well according to the Active Motif kit protocol). For blank wells: 20 μl complete lysis buffer was added per well (according to the Active Motif kit protocol).

Statistical analysis. Statistical analysis was performed using one-way analysis of variance to compare replicates with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rad-Fmod and Rad-Dcn expression levels in the 4T1 breast cancer cell line as evaluated using RT-PCR. Expression of

replication-defective adenovirus containing bovine Fmod cDNA (Rad-Fmod) and human Dcn cDNA (Rad-Dcn) were identified in the 4T1 breast cancer cell line using RT-PCR. The 4T1 breast cancer cell line is highly metastatic and was established to be negative for Fmod and Dcn transcripts (28,29). Following 4T1 cell line adenoviral infection, mRNA was extracted and RT-PCR using specific PCR primers was performed. Fmod and Dcn expression signals were detected in Rad-Fmod and Rad-Dcn infected cell lines, but were not identified in Rad-LacZ-transfected cells (control; Fig. 1). Therefore, these results demonstrate the lack of Fmod and Dcn expression in the extracellular matrix in 4T1 highly metastatic breast cancer cells.

TGF-β1 is suppressed by Rad-fmod and Rad-dcn expression in the 4T1 breast cancer cell line. Increased expression levels of TGF-β1 have been associated with malignant conversion and progression in breast cancer (30). In the present study, the inhibitory effects of Rad-Fmod, Rad-Dcn and Rad-LacZ (control) on TGF-β1 expression were examined using RT-qPCR. The 4T1 cell line was infected with recombinant adenoviral vectors for 72 h at an MOI of 1,000. Notably, the overexpression of Rad-Fmod and Rad-Dcn demonstrated a significant reduction ($P<0.05$) of TGF-β1 expression compared with the non-transfected 4T1 cell line (Fig. 2). Fmod may be considered as a more effective inhibitor than Dcn compared with the non-transfected 4T1 cell line and Rad-LacZ.

Fmod suppresses NF-κB DNA binding. NF-κB has been established to have an important role in breast cancer progression, control of cell proliferation and oncogenesis (7). To study the effects of Fmod and Dcn on NF-κB activity, the 4T1 cell line was transfected with Rad-Fmod, Rad-Dcn and Rad-LacZ (control) for 72 h at an MOI of 1,000, nuclear protein extraction was performed and p65 DNA binding activity was measured using a NF-κB transbinding assay. Extracellular signals stimulate NF-κB via the phosphorylation and degradation of IκB, and subsequent NF-κB nuclear translocation promotes the expression of numerous target genes (31). Rad-Fmod and Rad-Dcn infected cells demonstrated a 31 and 27% reduction, respectively, in NF-κB DNA binding activity compared with Rad-LacZ infected cells. These results suggest that Fmod expression may have the ability to reduce NF-κB activity more effectively compared with Dcn expression ($P>0.05$) (Fig. 3).

Discussion

NF-κB is a transcription factor that regulates the transcription of numerous target genes involved in angiogenesis, invasion, migration and apoptosis (32,33). NF-κB is normally sequestered in the cytoplasm by the inhibitory molecules of the IκB family (IκBα, IκBβ and IκBε) (32). In response to certain stimulatory agents, including viral infection, inflammatory cytokines and bacterial lipopolysaccharide, IκBs are rapidly phosphorylated and degraded to promote the nuclear transfer of NF-κB and the activation of a number of genes (34,35). Activation of NF-κB has been demonstrated in a variety of inflammatory, autoimmune diseases and human disorders (24,36), and the failure of cancer treatment due to the activation of NF-κB and resistance to chemotherapeutic agents, has been demonstrated (37,38). Therefore, NF-κB may be a potential therapeutic target.

TGF-β has exhibited bifunctional activities through its role in the regulation of cell growth, differentiation and migration (39), and it been established as important for cancer progression and EMT (39). The functional polymorphic TGF-β genes that are expressed in humans include TGF-β1-3 and TGF-β receptor (TGF-βR) types I-III (39). A number of previous studies have demonstrated an association between allele variants of TGF-β1 and invasive breast cancer (30,39,40). TGF-β is deregulated during tumor progression and its enhancement has been identified in numerous tumor types, including glioblastomas, melanoma cells, colorectal and prostate carcinoma (41). The TGF-β ligand is released in a latent form in the extracellular matrix and when activated binds to the TGF-βR types I-III (39). The phosphorylation of TGF-β receptors initiates a cascade of Smad-dependent and -independent proteins locating to the nucleus for transcriptional regulation (42).

Extracellular matrix macromolecules are involved in cellular physiologic events and pathological processes (43). Among these, Fmod and Dcn, extracellular matrix proteoglycans, have been designated as potent antitumor molecules and the lack of these proteins is permissive for tumorigenesis (44).

In the current study, the lack of expression of Fmod and Dcn has been demonstrated in non-transfected and Rad-LacZ infected 4T1 cells, and the expression of Rad-Fmod and Rad-Dcn was examined using RT-PCR. The lack of expression of Fmod and Dcn in this highly metastatic cell line demonstrated an inhibitory role in acquisition of metastatic phenotypes and the increased expression of Rad-Fmod and Rad-Dcn in transfected cells. In addition, the effects of Fmod and Dcn on NF-κB and TGF-β1 expression were analyzed in the highly metastatic 4T1 breast cancer cell line. Fmod and Dcn may control TGF-β antagonist activity with differing affinities for the isoforms of TGF-β (15). Using RT-qPCR, the present study demonstrated that Fmod is a more potent inhibitor of TGF-β1 expression in 4T1 breast cancer cells compared with Dcn. Fmod contributes to a significant reduction in TGF-β1 expression levels compared with Rad-LacZ. Therefore, Fmod-deficiency in adult animals may lead to delayed wound healing and increased scar size, and Fmod overexpression may contribute to a decrease in TGF-β1 expression levels (45,46). Fmod was considered as a dominant inhibitor of TGF-β1 compared with Dcn in cultured human saphenous vein cells, and TGF-β1 was identified at a lower expression level in cells treated with Ad5-Fmod or Ad5-Dcn compared with those receiving Ad5-LacZ or vehicle only (15). In addition, a previous study evaluated the effects of recombinant adenoviral Dcn gene transfer in the rat CNS-1 glioma model (13). These results determined that ectopic Dcn expression has the potential to slow glioma development (13). Additionally, it was demonstrated that exogenous TGF-β ligands inhibit lung branch morphogenesis in mouse embryonic lungs in *ex vivo* culture, and treatment with a recombinant adenovirus containing Dcn cDNA abrogated this effect (21). This study indicated that Dcn is involved in suppressing TGF-β-mediated negative regulation and may be used as a potential therapeutic approach to optimize the levels of TGF-β signaling (21).

A potential explanation of why Fmod has improved inhibitory activity compared with Dcn is that Fmod may

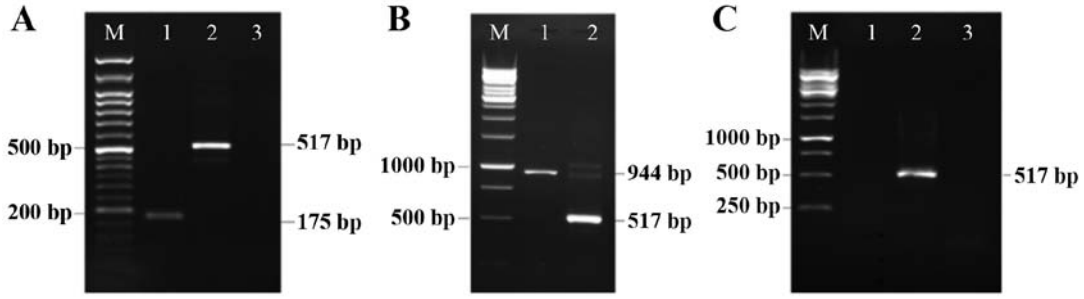

Figure 1. Detection of Rad-Fmod and Rad-Dcn expression in the 4T1 breast cancer cell line. (A) Rad-Fmod is expressed in the 4T1 breast cancer cell line (175 bp, lane 1). This image presents β-actin functioning as an internal control (517 bp, lane 2), the non-template control (lane 3) and the DNA marker (lane M). (B) This image presents Rad-Dcn gene expression (944 bp, lane 1), β-actin (517 bp, lane 2) and the DNA marker (lane M). (C) This image presents the 4T1 cell line infected with Rad-LacZ as a control, and was evaluated for fibromodulin (lane 1), β-actin (lane 2) and decorin (lane 3) expression. Rad-Fmod, recombinant adenovirus fibromodulin; Rad-Dcn, recombinant adenovirus decorin; Rad-LacZ, recombinant adenovirus LacZ.

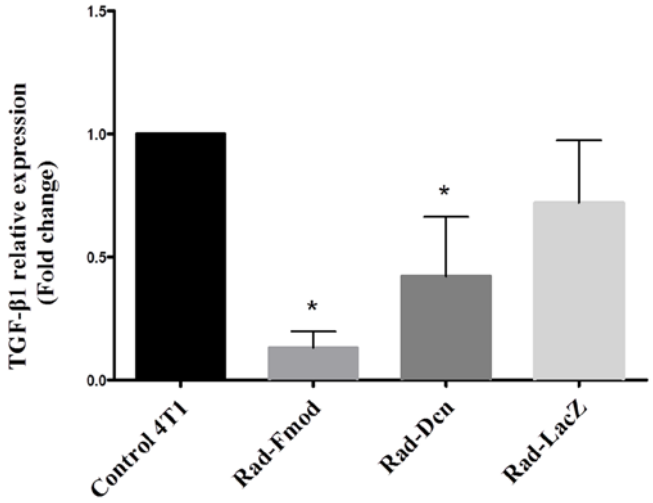

Figure 2. Assesment of inhibitory effects of Rad-Fmod and Rad-Dcn on TGF-β1 expression by RT-qPCR. The 4T1 breast cancer cell line was transfected with Rad-Fmod, Rad-Dcn and Rad-LacZ. Then TGF-β1 mRNA expression was quantified by RT-qPCR. These are the results of three independent experiments, each performed in duplicate (mean ± standard deviation). The asterisk indicates a significance difference was established between 4T1 control cells and Fmod and Dcn induced cells by one-way analysis of variance, respectively ($^{*}P<0.05$). Rad-Fmod, recombinant adenovirus fibromodulin; Rad-Dcn, recombinant adenovirus decorin; Rad-LacZ, recombinant adenovirus LacZ; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TGF-β1, transforming growth factor β1.

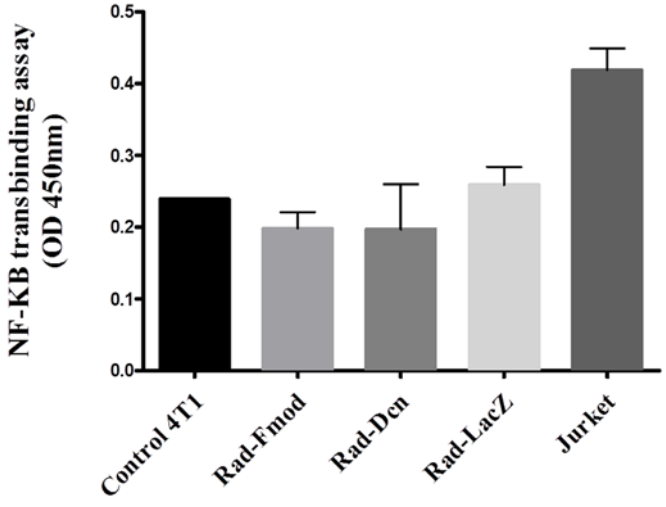

Figure 3. Characterization of NF-κB activity in the 4T1 breast cancer cell line prior to and following treatment. NF-κB DNA binding activity with the p65 subunit was evaluated in the 4T1 breast cancer cell line infected with Rad-Fmod, Rad-Dcn and Rad-LacZ. NF-κB DNA binding activity results in transfected cells were compared with the Rad-LacZ cell line, the non-transfected 4T1 cell line and Jurkat cell nuclear extract as a positive control. These are the results of four independent experiments (mean ± standard deviation). No significant differences were established between the 4T1 control cells and the Rad-LacZ cell line with Fmod and Dcn transfected cells. Rad-Fmod, recombinant adenovirus fibromodulin; Rad-Dcn, recombinant adenovirus decorin; Rad-LacZ, recombinant adenovirus LacZ; NF-κB, nuclear factor-κB.

have effective structural characteristics (47). Fmod and Dcn core proteins are composed of two disulfide-bonded domains flanking 10 leucine-rich repeats (LRR) (47). Dcn with a dermatansulfate chain in its amino terminal region may be secreted and has been identified to interact with EGFR through the activation of mitogen-activated protein kinase signaling pathway and this induces p21 cell cycle arrest (17). Additionally, Dcn interacts with collagen through the inner concave surface in the center of its arch-shaped structure with high affinity (48). In the case of Fmod, one to four keratan-sulfate chains may reside between the LRR domains and there are two collagen binding sites; therefore, LRRs 7 and 11 each may bind one collagen monomer (47). LRRs 11 on the C-terminus exhibited a higher affinity for collagen that may produce less spatial limitation for TGF-β1 binding in the center of the Fmod protein (49). Notably, five potential sites for sulfation of tyrosine residues, as a post-translational modification, are indicated in the N-terminus of bovine Fmod (50). Sulfation has been recognized to influence the half-life of Fmod and increase its stability (50).

The effect of Fmod and Dcn NF-κB activity was evaluated using a highly sensitive NF-κB transbinding assay. Despite the efficient expression of Fmod and Dcn proteins, there were no significant alterations in the levels of NF-κB in the nuclear extracts in comparison with the β-galactosidase transgene and the non-transfected 4T1 cell line. By contrast, in parental 3T3-L1 fibroblasts with high levels of NF-κB activity, Fmod inhibits NF-κB signaling by delaying the degradation of IκBα protein through the activation of c-Jun N-terminal kinase, the suppression of calpain and casein kinase 2 activity and the induction of fibroblast apoptosis (24).

In conclusion, the results of the present study indicate that Fmod binding to TGF-β1 is more effective compared with Dcn *in vitro*, but to further evaluate its effectiveness and therapeutic potential, this must be investigated *in vivo* and in combination with other antitumor agents.

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