DDR2 inhibition reduces migration and invasion of murine metastatic melanoma cells by suppressing MMP2/9 expression through ERK/NF-κB pathway

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Abstract

Metastatic melanoma is one of the most deadly and evasive cancers. Collagen I in the extracellular matrix promotes the migration and invasion of tumor cells through the production of matrix metalloproteinase (MMP) 2 and 9. Discoidin domain receptor (DDR) 2 is a collagen receptor that is implicated in several cancer types including breast and prostate cancers. However, the role of DDR2 in the migration and invasion of murine melanoma cells is less studied. In the present study, we investigated the effects and underlying mechanisms of DDR2 in migration and invasion of B16BL6 melanoma cells in response to collagen I. Results demonstrated that DDR2 is expressed and is phosphorylated by collagen I in the cells. Upon down-regulation of DDR2 using small-interfering RNA (siRNA) approach, both of the cell migratory and invasive phenotypes were significantly attenuated when compared with the control cells. This effect was mediated via suppression of MMP2/9 upon DDR2 inhibition. Furthermore, inhibition of DDR2 by specific siRNA markedly reduced the activation of extracellular regulated kinase (ERK) 1 and 2 and nuclear factor of kappa B (NF-κB) in the cells when compared with the control cells. Overall, these data demonstrated that DDR2 siRNA-mediated suppression of ERK1/2 and NF-κB could down-regulate the expressions of MMP2/9 in response to collagen I to reduce the migratory and invasive phenotypes of the cells.

Key words: B16BL6, collagen I, discoidin domain receptor 2, migration, invasion, ERK, NF-κB

Introduction

Metastatic melanoma (MM) is one of the most deadly and evasive cancers. It contributes to ~4% of skin cancers and is responsible for 75% of skin cancer-related deaths [1]. Lack of understanding of the molecular pathways that regulate the MM has prevented the development of efficient treatment strategy; as a result, MM patients have poor survival rate [1,2]. Therefore, elucidation of the molecular targets underlying the metastasis and invasiveness of melanoma cells may aid in improving treatment methodology for patients with MM.

Cancer cell metastasis involves several events, including enhanced proliferation of cells, tissue remodeling, and evasion [3]. During migration and invasion of the cells, there is increased production of matrix metalloproteinases (MMPs) such as MMP2/9 [4,5] which degrade extracellular matrix components such as collagen I to enhance migration and invasion [6]. In addition, collagen I fibers are known to act as highways for promotion of tumor migration and metastasis.

Discoidin domain receptor (DDR) 2 is a receptor tyrosine kinase that is activated by collagen I [7]. DDR2 is implicated in various...
Role of DDR2 in melanoma metastasis and invasion

Materials and Methods

Cell culture and transfection

B16BL6 cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin/streptomycin. Depletion of target protein in transfectants was analyzed by western blot analysis after 24 h.

Cell migration assay

After 24 h of transfection, cells were harvested and cultured in 48-well plates. After 16 h, a linear wound was gently created in the ≥90% confluent monolayer cells using a sterile yellow pipette tip, followed by washing with the complete medium DMEM, 10% FBS, and 1% P/S to remove the cellular debris in order to yield an acellular line per well. To initiate migration, cells transfected with or without DDR2 siRNA were treated with or without collagen I (20 µg/ml) in the medium. Cells were then allowed to migrate during 16 h incubation. At 0 and 16 h, cells were photographed with an Olympus IX71 microscope (Olympus Optical Co., Ltd, Tokyo, Japan) and distance between the wounded regions was measured using ImageJ software.

Cell invasion assay

Invasion assay was performed with 6-well transwell plates (Becton Dickinson, Bedford, USA) according to the manufacturer’s instructions. Cells were cultured in medium with or without collagen I (20 µg/ml) and then seeded onto inner well, and cells that invaded through the transwell were stained with 0.1% crystal violet (HARLECO DIVISION, Philadelphia, USA) and photographed as in migration assay using the Olympus IX71 microscope and were enumerated as an average in at least three fields.

Reverse transcription and real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. The total RNA (2 µg) was used for cDNA synthesis using Super Script™ III kit (Invitrogen). Then, mRNA expression was quantitatively determined by ABI Real-Time Polymerase Chain Reaction (PCR) system (Applied Biosystem, Inc., Forster City, USA) using SYBR Green PCR Master Mix (Life Technologies, Carlsbad, USA). GAPDH was the invariant control. Reverse transcription-PCR (RT-PCR) amplification was performed as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s. Final PCR products were separated on a 1.2% agarose gel and photographed. The primer sequences used for reverse transcription and real-time PCR are shown in Table 1.

Western blot analysis

Cells were lysed in ice-cold RIPA buffer for 40 min and centrifuged (12,000 g) for 20 min at 4°C. Protein concentration was measured using a bicinchoninic acid method. Totally, 30 µg of lysate was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, New Jersey, USA). Then, blocking was performed with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and the membranes were probed with primary antibodies as indicated at 4°C overnight, washed with TBST for four times, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min. After being washed with TBST for three times, proteins were visualized using an enhanced chemiluminescence detection kit (Millipore, Billerica, USA). Protein assay kit (RIPA buffer), rabbit and mouse secondary antibodies, and antibodies against DDR2 (sc-7555), ERK (sc-292838), phosphorilated ERK (sc-16982), NF-xB p65 (sc-8008), poly ADP ribose polymerase-1 (PARP-1) (sc-8007), and β-actin (sc-47778) antibodies were obtained from Santa Cruz Biotechnology. Antibody against phopho-tyrosine (#9411) was obtained from Cell Signaling (Beverly, USA).

Electrophoretic mobility shift assay

Eletrophoretic mobility shift assay (EMSA) was performed as previously described [11]. In brief, nuclear extract was prepared and incubated for 20 min at room temperature with 20 µg of bovine serum albumin, 2 µg of poly (dI-dC) (Pharmacia, Uppsala, Sweden), 2 µl of buffer C (20 mM Hepes/KOH, 20% glycerol, 100 mM KCl, and 0.5 mM PMSF, pH 7.9), 4 µl of buffer F (20% Ficoll-400, 100 mM Hepes/KOH, 300 mM KCl, 10 mM DTT, and 0.5 mM PMSF, pH 7.9), and 20,000 cpm of 32P-labeled probe encoding the consensus sequence for NF-xB-binding sites (5’-CAGAGGGACTTTCCGA GAG-3’) in a final volume of 20 µl. DNA–protein complexes were resolved in a native 4% polyacrylamide gel at 180 V for 4 h, dried, and exposed to an X-ray film.

Statistical analysis

All values were expressed as the mean ± SEM. Statistical significance was determined using the Student’s t-test. A P-value of <0.05 was considered statistically significant.
Results

DDR2 is expressed and is phosphorylated in response to collagen I in B16BL6 murine melanoma cells

To investigate whether DDR2 plays a role in melanoma metastasis and invasion, we examined the gene and protein expression levels of DDR2 in response to collagen I in B16BL6 cells. As shown in Fig. 1A–D, DDR2 protein and mRNA expressions were observed in the B16BL6 cells and collagen I significantly enhanced the expression of DDR2 protein (Fig. 1A,C) and mRNA (Fig. 1B,D) when compared with control. Also, collagen I phosphorylated DDR2 in a dose-dependent manner (Fig. 1A,C). Collectively, these data demonstrated that DDR2 is expressed by B16BL6 cells and the expression is regulated by collagen I, and DDR2 can also be phosphorylated upon collagen I treatment in a dose-dependent manner.

Inhibition of DDR2 reduces the migration of B16BL6 melanoma cells

To define the role of DDR2 in melanoma metastasis, we examined the metastatic properties of the cells transfected with DDR2-specific or control siRNA in the presence or absence of collagen I for indicated time points. Migration activities of cells treated with DDR2-specific or control siRNA were examined in the presence or absence of collagen I by wound-healing assay. Our data (Fig. 2A,B) showed that there was statistically non-significant difference in migration of the cells when compared with collagen I treated and non-treated cells. As the objective of our experiment was to evaluate the role of DDR2 in migration of cells, and the same concentration (20 µg/ml) of collagen I was sufficient to induce maximum activation of DDR2 (Fig. 1A,B), we performed migration assay using DDR2 inhibited or control cells in the presence or absence of collagen I and results showed that DDR2 suppression could attenuate migration of the cells treated with or without collagen I (Fig. 2A,B). Western blot analysis results showed that DDR2 protein expression was down-regulated by DDR2-specific siRNA, and there was ∼50% inhibition in DDR2 expression upon DDR2-specific siRNA transfection when compared with control siRNA transfected cells (Fig. 2C,D).

Down-regulation of DDR2 suppresses invasiveness of B16BL6 cells

As we observed from migration assay that there was marked suppression of migration of cells that were DDR2-inhibited in the presence of collagen I, we assessed the effect of DDR2 inhibition in the presence of collagen I on invasion phenotype of the cells. For this, the cells were transfected with DDR2-specific or control siRNA and then treated with or without collagen I in transwell plates of 8 µm size pores. It was found that DDR2 inhibition could substantially suppress cell invasion activity of the melanoma cells (Fig. 3A,B).

Table 1. Primer sequences for RT-PCR and qPCR

<table>
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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Method</th>
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<tbody>
<tr>
<td>DDR2</td>
<td>5'-CAAGGACCCAAACCATCCATCC-3'</td>
<td>5'-GATGCATCATCCTGGCTCC-3'</td>
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<tr>
<td>GAPDH</td>
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<td>5'-GTTCGACAGTCAGCCGCATC-3'</td>
<td>RT-PCR</td>
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<td>5'-CAGGGATGGAAGGATCTGCTCGTATGTA-3'</td>
<td>5'-AAXCTTGATAGGCTGGATGGG-3'</td>
<td>q-PCR</td>
</tr>
<tr>
<td>MMP9</td>
<td>5'-ACTCTCTTGGACAGTCTCGTATGTA-3'</td>
<td>5'-AGCCGAGGAGGAGGAGGAGG-3'</td>
<td>q-PCR</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GTTCGACAGTCAGCCGCATC-3'</td>
<td>5'-GGAATTTGCATGGTGGA-3'</td>
<td>q-PCR</td>
</tr>
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Figure 1. Expression of DDR2 and its phosphorylation in the murine melanoma cells

(A) Western blot analysis of DDR2 and its phosphorylated (p-DDR2) form in B16BL6 cells treated with indicated doses of collagen I (Coll) for 1 h. (B) RT-PCR to detect the DDR2 mRNA expression in the cells treated with or without 20 µg/ml Coll for 4 h was performed. (C) Graph shown indicates the ratio of p-DDR2/DDR2 as estimated using GelQuant.NET (version 1.8.2) software. (D) Graph represents the relative level DDR2 versus GAPDH of RT-PCR experiments as estimated using the same software as above. The results are expressed as the mean ± SEM (n = 3). *P < 0.05.
We investigated the mechanisms of migration and invasion phenotype of B16BL6 cells by determining the involvement of MMP2/9 expression upon DDR2-specific or control siRNA treatment in the presence of collagen I. Reductions in the expressions of both MMP2 and MMP9 were observed in DDR2 siRNA transfected cells when compared with control siRNA transfected cells or non-treated cells in the presence of collagen I (Fig. 4A,B).}

**Figure 2.** Inhibition of DDR2 in murine melanoma cells reduces their migratory phenotype. (A) Cells were transfected with DDR2-specific or control (Ctrl) siRNA at indicated doses for 24 h. Then, the cells were cultured in 48-well plates for 16 h. After this, linear wound was created using pipette tip and wells were washed twice, followed by treatment with or without 20 µg/ml collagen I (ColI). At 0 and 16 h, cells were photographed using Olympus IX71 microscope at x10 objective. (B) Graph represents the distances of the wounded regions as measured at 0 and 16 h using I’MEASURE software. (C) Western blot analysis of DDR2 protein expression after 24 h transfection of Ctrl or DDR2-specific siRNA in the cells. (D) Graph indicates the relative level of DDR2 versus actin as estimated using the same software in Fig. 1. The results are expressed as the mean ± SEM (n = 3). *P < 0.05.

**Figure 3.** Down-regulation of DDR2 inhibits invasive potential of murine melanoma cells. (A) After 24 h transfection with control (siCtrl) or DDR2-specific siRNA (siDDR2) at mentioned doses, cells were seeded onto inner wells in a transwell plates (8 µm pore size) with or without 20 µg/ml collagen I (ColI) for 12 h. Cells that invaded into the bottom side of the filter were stained with crystal violet. (B) Graph represents the number of cells per field in representative areas under a light microscope. The results are expressed as the mean ± SEM (n = 3). *P < 0.05.

**DDR2 modulates MMP2/9 expression in B16BL6 cells**

We investigated the mechanisms of migration and invasion phenotype of B16BL6 cells by determining the involvement of MMP2/9 expression upon DDR2-specific or control siRNA treatment in the presence of collagen I. Reductions in the expressions of both MMP2 and MMP9 were observed in DDR2 siRNA transfected cells when compared with control siRNA transfected cells or non-treated cells in the presence of collagen I (Fig. 4A,B).

**DDR2 regulates activation of ERK1/2 and NF-κB pathways**

Numerous studies have shown that MMP2/9 expression is regulated by MAPKs and NF-κB signaling pathways. In particular, previous study showed that DDR2 regulates ERK signaling in breast cancer metastasis [12,13]. In order to investigate the possible role of DDR2 in regulation of ERK and NF-κB signaling in B16BL6 cells, we evaluated the relative protein levels of phosphorylated and total ERK, and p65.
subunit of NF-κB in nucleus and cytoplasm. During western blot analysis, PARP and actin were used as a loading control for nuclear and cytoplasmic extracts, respectively. We also analyzed the NF-κB DNA-binding activity manifesting in the nuclear extracts of either DDR2 siRNA or control siRNA transfected cells in the presence of collagen I. We applied tumor necrosis factor alpha (TNF-α) as a positive control in these experiments.

Results demonstrated that DDR2 inhibition with siRNA substantially decreased the phosphorylation of ERK and activation of NF-κB when compared with control cells (Fig. 5A–C). Furthermore, nuclear extracts from the DDR2 siRNA-treated cells exhibited significant reduction in NF-κB-binding activity, as determined by the EMSA using labeled oligonucleotides containing a consensus NF-κB site (Fig. 5D). The nuclear extracts from control siRNA transfected cells
and TNF-α-treated cells showed enhanced NF-κB binding activities when compared with the DDR2 siRNA transfected cells, thereby suggesting that DDR2 may modulate the NF-κB activation in B16BL6 cells (Fig. 5D). Overall, these data indicate that DDR2 siRNA-mediated inhibition of ERK and NF-κB activation could regulate MMP2/9 expression in B16BL6 cells to modulate migration and invasive phenotype of the cells.

Discussion

MM is a highly aggressive cancer that confers resistance to current therapeutic approaches. About 20% of melanoma patients develop metastasis at the time of diagnosis. As there is increasing evidence of melanoma patients, novel molecular approaches for the treatment of MM are crucial to improve patient’s health [14]. It was found that the B16BL6 cells expressed DDR2. This prompted us to investigate the role of DDR2 in migration and invasion of B16BL6 cells. DDR2 has been extensively studied in immune cell activation [15,16]. However, the role of DDR2 in various types of cancers needs to be elucidated.

In the present study, we observed that MM cells express DDR2 which is phosphorylated upon exposure to collagen I. Therefore, gene silencing approach was used to understand the role of DDR2 in murine MM cells in vitro. Two phenotypes (migration and invasion) were tested to study the effects of DDR2 on murine MM cells. It was found that under DDR2 silencing condition, the rates of migration and invasion of the cells were significantly inhibited when compared with the control cells. Similar to our results, recent reports have indicated that DDR2 is crucial for the migration and invasion of breast and prostate cancer cells [17,18]. In addition, DDR2 silencing reduced experimental liver metastasis of human A375 cells [2]. Although it has been reported that collagen I enhances migration of cancer cells [6], our data showed no significant difference in migration of cells treated with or without collagen I. This effect could be due to the higher confluency of the highly aggressive cancer cells [1] in wound-healing assay that might have underestimated the direct effect of collagen I concentration used in cellular migration assay.

Moreover, DDR2 phosphorylation was detected in injury tissues, accompanying increased collagen deposition and resulting in the production of MMPs, which mediates collagen degradation during pathophysiological events, such as skeletal formation, cellular migration, inflammation, wound healing, arthritis, and cancer [9]. MMP2/9 exhibit a unique ability to cleave the basement membranes such as collagen I [19]. Previous reports have indicated that MMP2/9 are crucial for the migration and invasion of melanoma, and osteosarcoma cells [18,19]. In line with these studies, inhibition of DDR2 was found to significantly reduce MMP2/9 expression in the MM cells in response to collagen I. These data implicated that reduced expression of MMP2/9 due to down-regulation of DDR2 could reduce tumor cell migration and invasion.

Previous study showed that ERK1/2 are important for the pancreatic cancer cell invasion and cellular scattering in response to collagen I [20]. Furthermore, DDR2 has been implicated in signaling through ERK in regulation of breast cancer metastasis [13]. Therefore, we focused on role of ERK1/2 upon DDR2 inhibition in collagen I exposed MM cells. Similar to an earlier report [13], DDR2 inhibition was found to significantly reduce the activation of ERK1/2 upon collagen I treatment in the MM cells. In addition, ERK1/2 and NF-κB activation have been implicated in enhancing MMP2/9 production [21,22]. In case of dendritic cells, DDR2 inhibition has been implicated in the attenuation of NF-κB activation, suggesting that DDR2 could interact with NF-κB to signal various cellular responses [16]. Our data demonstrated that inhibition of DDR2 markedly reduced the activation of NF-κB in the MM cells. These data implicated that DDR2-mediated suppression of ERK1/2 and NF-κB could suppress MMP2/9 expression of the MM cells in response to collagen I to attenuate their migratory and invasive phenotypes (Fig. 6).

Taken together, this study provides evidence for DDR2 expression and its function in murine MM cells. Our data demonstrated that DDR2 regulates MMP2/9 expression in MM cells via ERK1/2 and NF-κB signaling pathways to suppress migration and invasion of the cells in response to collagen. Further in vivo studies are essential to confirm if DDR2 could act as a therapeutic target in MM patients.

Funding

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References

7. Ruiz PA, Jarai G. Discoid domain receptors regulate the migration of primary human lung fibroblasts through collagen matrices. Fibrogenesis Tissue Repair 2012, 5: 3.