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Fusion Expression of DDR2 Extracellular Domain in Insect Cells and its Purification and Function Characterization

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Abstract Discoidin domain receptor 2 (DDR2) is a kind of protein tyrosine kinases associated with cell proliferation and tumor metastasis, and collagen, a ligand for DDR2, up-regulates matrix metalloproteinase 1 (MMP-1) and MMP-2 expression in extracellular matrix (ECM). To investigate the role of DDR2 in cartilage destruction in rheumatoid arthritis (RA), we expressed the extracellular domain (ECD) of DDR2 (without signal peptide and transmembrane domain, designated DR) in insect cells, purified and characterized DR, hoping to use it as a specific antagonist of DDR2. By using Bac-To-Bac Expression System with a His tag, we successfully obtained the recombinant bacularvirus containing DDR2 ECD, purified it and characterized its function. The soluble fraction of DR was about 12% of the total fused protein. After chromatographic purification, DR with 92% purity was obtained. Competitive inhibition assay demonstrated that DR blocked the binding between DDR2 and natural DDR2 receptors on NIH3T3 and synovial cells. Results of RT-PCR, Western blotting, and gelatinase zymography showed that DR was capable of inhibiting MMP-1 and MMP-2 secretion from NIH3T3 and RA synoviocytes stimulated by collagen II. For MMP-1, inhibition was displayed at the levels of mRNA and protein, whereas for MMP-2 it was at the level of protein. These findings suggested that the expressed DR inhibited the activity of natural DDR2 and relevant MMP-1 and MMP-2 expression in RA synoviocytes and NIH3T3 cells provoked by collagen II. J. Cell. Biochem. 102: 41–51, 2007.

Key words: discoidin domain receptor 2 (DDR2); extracellular domain (ECD); baculovirus; matrix metalloproteinase (MMP); antagonist

Protein tyrosine kinases (PTKs) constitute a large family of protein kinases, which play important roles in proliferation and differentiation of multicellular organisms. A major class of PTKs is receptors (receptor tyrosine kinases, RTKs) of some growth factors, such as epidermal growth factor receptor (EGFR), and insulin-like growth factor-1 receptor (IGF-1R). Discoidin domain receptor 2 (DDR2) is a kind of collagen II receptor. RTKs usually have an extracellular domain (ECD), ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular ligand-stimulated kinase domain that initiates signal transduction. All PTKs make themselves or other proteins to be phosphorylated, and such phosphorylation

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will act as a new signal and be transmitted to downstream pathways [Hunter, 1996].

DDRs are wide-distributed receptor tyrosine kinases possessing discoidin domains, and their ligands are collagens. Two members in this family exist in the mammals: DDR1 and DDR2 [Vogel, 1999]. They are similar on the protein structure and function but with different amino acid sequences. DDR1 usually expresses in tumor cells, while DDR2 expresses in normal tissue matrix including human articular synovial tissues [Wang et al., 2001]. Ligand of DDR2 is mainly fibrillic collagen (such as type I, II, or III), and their binding to DDR2 might up-regulate cell proliferation and MMP-1 and MMP-2 expression in normal cells [Vogel et al., 1997a; Enjoji et al., 2000]. Interestingly, DDR2-defected mice appear dwarfism [Labrador et al., 2001]. It was also found that DDR2 was over-expressed in some tumors, and supposed to be related to metastasis of tumor cells [Lai and Lemake, 1994].

In RA patients, over-proliferation of synovial fibroblasts (SF) are the main reason for cartilage destruction [Aupperle et al., 1998]. Matrix metalloproteinases (MMPs) produced by synovium are the main direct reason for destruction of collagen II in cartilage, among which MMP-1, MMP-2, and MMP-9 are more predominant in RA. Many cytokines and their signal pathways are involved in up-regulation of MMPs, for example, TNFα-NF-*k*B-AP-1 and TGFβ-NF-*k*B-AP-1 pathways in up-regulation of MMP-2 and MMP-9 [Cheon et al., 2002].

Our previous work indicated that there possibly existed a vicious cycle in the joints of RA patients [Wang et al., 2002]. Under self-immunoreactivity state in RA, DDR2 in SF and other cells were activated by native or fallen collagen II, and such DDR2 activation up-regulated MMP-1 expression to degrade collagen II in joint cartilage. Sustained stimulation of collagen II continuously made DDR2 activated, and made synoviocytes secrete MMP-1 continuously as well. Thus, large amount of MMP-1 contributed to serious cartilage destruction. The injured cartilage released more collagen, further inducing DDR2 activation and MMP-1 secretion again, thus so did the vicious cycle continue. For this reason, collagen II was also a promoter for irreversible joint destruction during late period of RA. If this vicious cycle existed, blocking the interaction of collagen II and DDR2 could potentially become a new strategy for treatment of RA patients.

However, there are no known specific DDR2 antagonists or any homologues so far. Thus, it is necessary for research purpose to find out such antagonists or agonists either natural or synthetic. So we tried to express DDR2 ECD in a baculovirus expression system to testify its blocking function with collagen II.

MATERIALS AND METHODS

Materials

RA synoviocytes and NIH3T3 cells, *Escherichia coli* DH10Bac strain, pFBHTa vector, recombinant plasmid pRsetA-DDR2-DR and sf9 insect cells were all preserved in our department. TC-100 powder was product from Invitrogen Corporation (Carlsbad). Restriction endocucleases, T_4 DNA ligase, Taq DNA polymerase, IPTG and X-gal were purchased from Promega Corporation (Madison). Protease K, low melting agarose for cell culture was purchased from Clontech Laboratories, Inc. (Mountain View); Plasmid Miniprep System and Lipofectamine^{TM}2000 Reagent were products from Gibco. Mouse anti-His monoclonal antibody (mAb) and NTA chromatographic column stuff were purchased from Qiagen (Valencia); FITC labeled goat anti-mouse secondary IgG and HRP labeled goat anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz). Western chemiluminescent substrates and fetal bovine serum (FBS) without mycoplasma were products from Pierce Biotechnology, Inc. (Rockford). Mouse anti-MMP1 monoclonal antibody was from Calbiochem (La Jolla).

Methods

Cell culture. RA synoviocytes and NIH3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS in a humidified 5% CO_2 atmosphere at 37°C.

Construction of recombinant pFBHTa-DR plasmid. pRsetA-DDR2-DR was digested by *Eco*RI and *Sal*I, electrophoresed in 1% agarose, and the 1.1 kb DNA segment (DDR2-DR) was recycled and purified. The DDR2-DR fragment was then linked to pFBHTa vector also digested by *Eco*RI and *Sal*I, electroporated to transform *E. coli* JM109. Single colony was randomly selected, cultured, and plasmid DNA was

extracted, and later conformed by agarose electrophoresis after *Eco*RI and *Sal*I digestion.

Recombinant plasmid transformation of *E. coli* DH10Bac. Recombinant vector pFBHTa-DR containing DR was transformed in *E. coli* DH10Bac by calcium chloride method, and cultured on 2 × YT medium. One hundred microliters cultures from different dilution were evenly streaked on 2 × YT plates containing 1.2% agar (containing 50 μg/ml Kan, 7 μg/ml Gen, 10 μg/ml Tet, 100 μg/ml X-gal, 40 μg/ml IPTG) and cultured at 37°C for 24 h. White colonies were the ones that needed.

Extraction and characterization of recombinant bacmid DNA. Single white colony selected above was inoculated onto 2 × YT medium (containing 50 μg/ml Kan, 7 μg/ml Gen, 10 μg/ml Tet), and shaken at 37°C overnight. Plasmid DNA of Bacmid was extracted. Templated by Bacmid, PCR identification was assayed to see if foreign genes were correctly inserted. The sequences of PCR primers are: upstream: 5′CGGAATTCAATCCAGCTATATGCCGCTATCCTCTG3′; downstream: 5′GGTCGACTTAAGTGTTGCTGTCATCAACTTTAAGCATTGG3′. Bacmid DNA segments from positive clones were further purified for transfection.

sf9 cell culture. sf9 cells were cultured at 27°C in TC-100 culture fluid containing 10% FBS and penicillin and streptomycin, and passaged when confluent monolayer cells formed.

sf9 cell transfection. Ten microliters Bacmid DNA segments and 50 μl TC-100 culture fluids without serum and antibiotics were evenly mixed. Five microliters Lipofectamine^{TM}2000 reagent and TC-100 culture fluid without serum and antibiotics were mixed. After 5 min incubation at room temperature (RT), these two liquid mixtures were mixed thoroughly, added with sf9 cells after placed 20 min at RT, and incubated at 27°C for 6 h with 900 μl TC-100 culture fluid without serum and antibiotics. Then culture fluid was replaced by 10% FBS TC-100 culture medium, incubated at 27°C overnight, refreshed culture medium the next day, and continued culture for 72–120 h. Supernatants (containing virus particles) were finally collected, centrifugated at 500*g* for 10 min, and stored at −70°C for future use.

Purification of recombinant baculovirus. Harvested recombinant baculovirus in supernatant was diluted in TC-100 medium, and infected sf9 cells in 6-well culture plates. After incubation at 27°C for 1 h, supernatants were removed, and added with 1 ml TC-100 culture medium containing 1% agarose. After solidification, 1 ml TC-100 culture fluid containing 10% FBS was added and incubated at 27°C for 4–5 days for plaque formation. Single plaque was dissolved in TC-100 culture fluid, and infected sf9 cells after placed at 4°C for 12 h. After incubation at 27°C for 3 days, the sf9 cells and supernatants were collected. Baculovirus in supernatants was thus purified.

PCR characterization of recombinant baculovirus. Recombinant baculovirus (MOI 1-5) was inoculated to confluent monolayer sf9 cells, and incubated at RT for 1 h virus adsorption. Then supernatant was discarded, refreshed with new culture fluid, and cultured at 27°C for 72 h. After two PBS washes, baculovirus-infected sf9 cells were suspended in 250 μl TE solution, and with addition of 250 μl lysis buffer, 12.5 μl 10 mg/ml protease K and 2 μl 10 mg/ml RNase at 37°C for 30 min. DNA segments was extracted by commensurate phenol: chloroform mixtures and ethanol precipitation. Recombinant baculovirus was then characterized by PCR.

Baculovirus expression product detected by IFA. Baculovirus-infected sf9 cells were resuspended at 96 h post-infection, dropped onto cover glasses pre-treated with poly-L-lysine at 27°C for 1 h, and then fixed by cold acetone for 20 min. 1:50 diluted mouse anti-His monoclonal antibody was added, incubated at 37°C for 40 min in a humidified box; then 1:100 diluted FITC labeled goat anti-mouse IgG containing 0.2 mg/ml Evans blue after PBS washes were covered and incubated at 37°C for 30 min in a humidified box. Fluorescent microscopy was finally applied.

Characterization of baculovirus expression product by Western blotting. Recombinant baculovirus infected sf9 cells (1.0×10^6) were resuspended in 50 μl PBS at 96 h post-infection after washes twice with 0.01 M PBS (pH 7.4). The pellet was resuspended in 50 μl PBS with addition of 12.5 μl 5× sample buffer, water-bathed at 95°C for 5 min, and centrifugated at 14,000*g* for 10 min. Fifteen microliters sample solution was subject to SDS–PAGE and Western blotting.

Chromatographic purification of DDR2-DR protein. Three bottles of baculovirus-infected sf9 cells (1.0×10^8) were blown off, washed by PBS, centrifugated at 500*g* for 5 min,

and the pellets were resuspended in 3.0 ml lysis buffer (50 mM NaH_2PO_4, 300 mM $NaCl_2$, 10 mM imidazole, 0.05% Tween-20, NaOH adjusted pH to 8.0). The solution was then subject to freeze-thaw three times, centrifugated, and the supernatant was taken for chromatographic purification. NTA-Ni^{2+} column was equilibrated by 10 ml lysis buffer, and loaded with the supernatant obtained as above. 5.0 ml lysis buffer was used to wash the column, then washed again by 15.0 ml washing buffer (50 mM NaH_2PO_4, 300 mM $NaCl_2$, 20 mM imidazole, 0.05% Tween-20, NaOH adjusted pH to 8.0), and DDR2-DR protein was diluted by 3.0 ml elution buffer (50 mM NaH_2PO_4, 300 mM $NaCl_2$, 250 mM imidazole, 0.05% Tween-20, NaOH adjusted pH to 8.0). Eluents were collected.

Competitive binding inhibition assay. RA cells were coated onto 96-well plates, fixed in 4% paraformaldehyde for 24 h, washed by PBS, and added double-diluted DR solution. Negative control group was set as purified His protein in 0.1 mg/L with equivalent collagen II (dispersed by ultrasonic) in PBS after incubation at 37°C for 1 h and three times of washing by PBS. Then added antibody against type II collagen for incubation at 37°C for 1 h, and later added HRP labeled goat anti-mouse secondary antibody for incubation at 37°C for 0.5 h. Finally, substrate ABTS was added, and absorbance on 410 nm was read on an ELISA reader.

Preparation of cell culture plates coated with collagen II. Collagen-coated dishes were prepared by incubating 20 μg/ml of collagen II solution in 0.1 mol/L of acetic acid at 37°C for 24 h and washing twice with DMEM before cell seeding.

Blocking assay of DR on collagen II stimulation. DR solution was diluted to 0.035 μg/ml in DMEM, added into 6-well culture plates pre-coated with collagen II, and incubated at 37°C for 1 h, while purified His protein was set as the negative control. After washing with DMEM, cultured synoviocytes and NIH3T3 cells at 4.0×10^5 were passaged. When confluent cells reached to 70-80%, DMEM with 10% FBS was replaced by pure DMEM, and the cells were collected for further experiments after 72 h incubation.

Cellular expression of MMP-1 detected by RT-PCR. Total cellular RNA was extracted by TRIzol. First-strand cDNA was synthesized with 1μg of total RNA and oligo-dT primer. PCR reactions were performed using specific human MMP-1 isoform primers as: upstream: 5′TTCTACCCGGAAGTTGAGCTCAATTTCATT3′, and downstream: 5′TTAGTAATGTTCAATTTTTCCTGCAGTTG3′.

MMP-1 expression in cell culture supernatants detected by Western blotting. Cell culture supernatants were collected by 1,000*g* centrifugation for 5 min, and expressed DR was condensed by ultra-centrifugation. Protein concentrations were determined by BCA protein assay. Ten micrograms condensed supernatant protein was used for Western blotting. The primary antibody was mouse monoclonal anti-MMP1, and β-actin was set as control. The scanned images were quantified by Kodak Digital Science 1D software.

MMP-2 and MMP-9 activity detected by gelatinase zymography. Culture samples were prepared in non-reducing loading buffer and separated on 10% SDS–PAGE gel containing 1 mg/ml of gelatin. After electrophoresis, gels were washed three times in washing solution (150 mmol/L NaCl and 2.5% Triton X-100) for 30 min, rinsed in water, and incubated for 12–16 h in collagenase buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 10 mmol/L $CaCl_2$, pH 7.5). Gels were subsequently fixed and stained in Coomassie Blue fixative solution (25% methanol, 7% acetic acid, 0.25% Coomassie Blue R250) for 2 h at RT and destained with washing solution (25% methanol and 7% acetic acid in water) for 4–5 h.

RESULTS

Construction and Characterization of Recombinant Transfer Plasmid and Recombinant Baculovirus

DR gene segment was obtained from recombinant plasmid pRsetA-DDR2-DR, and then subcloned into vector pFBHTa. After *Eco*RI and *Sal*I double digestion, positive clones produced two expected segments of 5.2 and 1.1 kb, respectively, indicating a correct insertion (Fig. 1A). Later DH10Bac competent cells were transformed by pFBHTa-DR, and white single colonies were selected from culture plates and used to amplify and extract Bacmid DNA. Templated by Bacmid DNA, PCR identification was assayed. The results showed that a 1.2 kb DNA segment was amplified, and confirmed positive clone of DR by DNA sequencing (Fig. 1B). sf9 cells were transfected by Bacmid

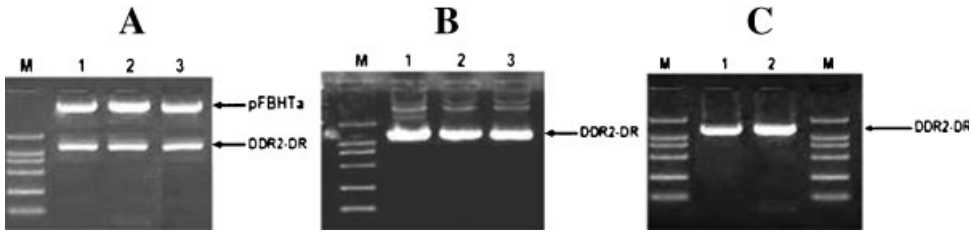

Fig. 1. Construction and characterization of recombinant transfer plasmid and baculovirus. **A**: Restriction enzyme digestion analysis of recombinant plasmid. M: DNA marker DL2000; **Lanes 1–3**: pFBHTa-DDR2-DR. **B**: Agarose gel electrophoresis of PCR products of Bacmid. M: DNA marker DL2000; Lanes 1–3: PCR product of chimeric gene DDR2-DR (1.2 kb). **C**: Agarose gel electrophoresis of PCR products of chimeric gene in recombinant baculovirus. M: DNA marker DL2000; Lanes 1–2: PCR product of chimeric gene DDR2-DR (1.2 kb).

clone, and recombinant baculovirus containing DR were obtained. These viruses produced the same DNA segments identified by PCR, indicating that recombinant baculovirus Bac-DR had a correct DR insertion (Fig. 1C).

Recombinant Baculovirus Expression Product Detected by IFA

Baculovirus-infected sf9 cells were stained at 96 h post-infection by anti-His mAb, and specific green fluoresce was observed in the cytoplasma, demonstrating that DR had been expressed in sf9 cells (Fig. 2A).

Characterization of Baculovirus Expression Product by Western Blotting

Baculovirus-infected sf9 cells were subject to SDS–PAGE and Western blotting. SDS–PAGE results exhibited that there appeared a new band at about 46 kDa, consistent with the expected DR (Fig. 2C upper). By thin film scanning, the expressed protein in supernatant was about 12% of the total expressed proteins. Western blotting results revealed that His-DR was indeed expressed in insect cells (Fig. 2C bottom).

Purification of His-DR Protein

Ni-NTA agarose chromatographic column via metal chelate was utilized to purify DR. Native soluble His-DR was obtained from supernatants of the recombinant Bac-DR-infected sf9 cells. DR has 378 amino acid residues plus 36 of $6 \times$ His tag part. The molecular weight of $6 \times$ His-DR fused protein was expected at about 46 kDa, consistent with the protein we purified. After thin film scanning, the target protein was about 92% of purity (Fig. 2D). Through protein quantification, the productivity of DR was 350 μg/L (data not presented).

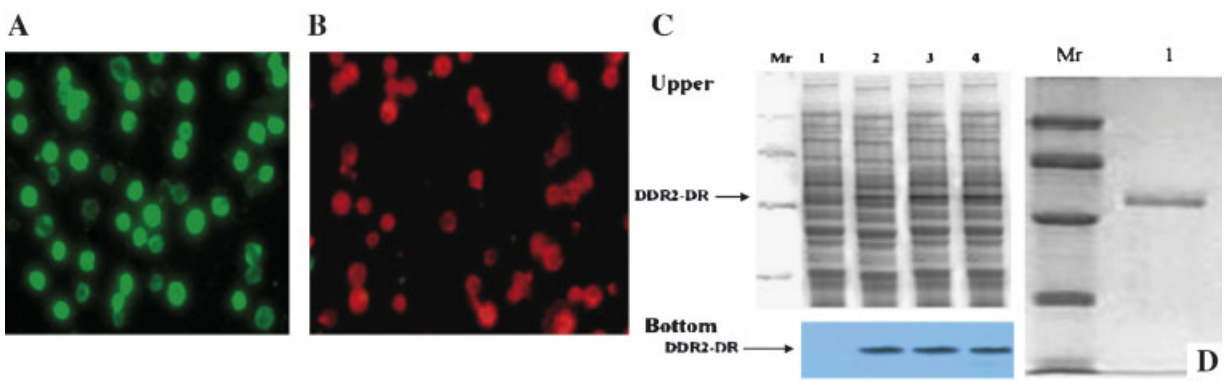

Fig. 2. Bac-DR expression in infected sf9 cells detected by indirect immunofluorescent assay and Western blotting. **A**: Bac-DR infected sf9 cells detected by anti-His monoclonal antibody. **B**: Non-Bac-DR infected sf9 cells detected by anti-His monoclonal antibody. **C**: Bac-DR expression in sf9 cells detected by Western blotting. Upper: Bac-DR expression identified by SDS–PAGE. Mr: protein marker (from top to bottom: 97, 66, 43, 31 kDa); Lane1: Bac infected sf9 cells; **Lanes 2–4**: Bac-DR infected sf9 cells; Bottom: Bac-DR expression identified by Western Blotting. **D**: Purification of 6ÂiÃ His-DR fusion protein by Ni-NTA affinity chromatography. Mr: protein marker (from top to bottom: 97, 66, 43, 31 kDa). Line 1: 6ÂiÃ His-DR fusion protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Competitive Inhibition by DR of Type II Collagens and DDR2 Binding

To confirm the blocking activity of DR to the binding of collagen and DDR2, we tested the function of the fused DR with control of purified His proteins (0.1 mg/L) and RA synoviocytes expressing DDR2. The concentration of collagen II was 0.025 mg/L, and DR was diluted in 1:10; 1:100; 1:200; 1:400; 1:800; l:1,600; l:3,200; 1:6,400; 1:12,800; 1:25,600. Due to the insolubility of collagen II, the reaction was taken place in PBS solution (Fig. 3A). For collagen group, the color of testing wells was the darkest after adding purified His proteins, demonstrating that purified His proteins did not block the binding of collagen and DDR2 on cell surfaces. For DR groups, ranging from 0.35 mg/L to 1:800, no color was displayed, suggesting that DR completely blocked the binding of collagen and DDR2. In addition, the color of testing wells (1:1,600 to 1:3,200–1:25,600) was increasingly darkened, demonstrating that DR competitively inhibited the binding of collagen and DDR2 on cell surfaces. Figure 3B is the diagram of ELISA A_{410}.

Cellular Expression of MMP-1 mRNA Detected by RT-PCR

MMP-1 mRNA levels were detected by RT-PCR in two cell lines out of four that we obtained. For synoviocytes, in groups without collagen (Group I), only basal levels of mRNA expression were observed; in groups with simple collagen (Group II), MMP-1 mRNA increased significantly; in groups with purified His protein plus collagen (Group III), there was no significant difference between MMP-1 mRNA expression compared with Group II, that is to say, MMP-1 mRNA expressions were not inhibited; in groups with DR plus collagens (Group IV), mRNAs were greatly inhibited, suggesting DR competitively inhibited the binding of collagens and DDR2 on cell surfaces (Fig. 4B). For NIH3T3 cells, the results from the

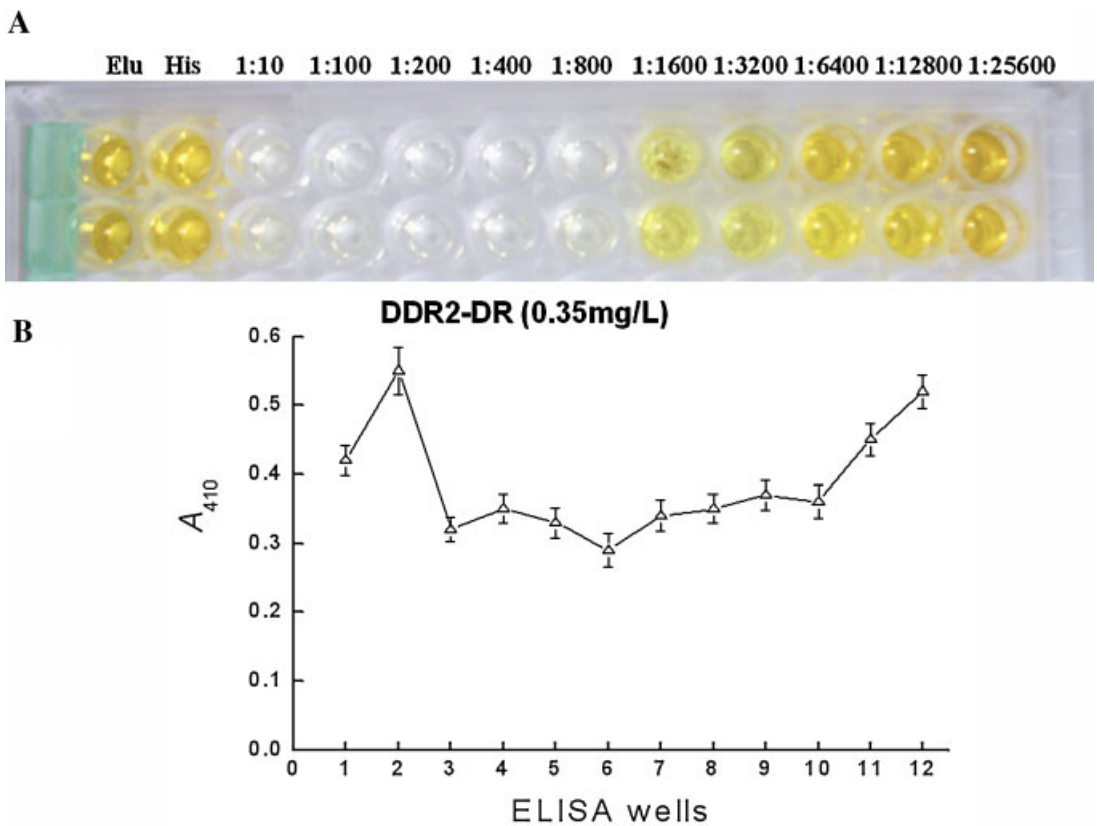

Fig. 3. ELISA analysis of DR on interaction between collagen II and DDR2 on surface of RA synovial fibroblasts. **A**: The staining in the wells of His was the strongest in color. There is no staining in wells of DR from 1:10 to 1:800. Staining color became to appear increasingly stronger from 1:1,600 to 1:25,600. **B**: The diagram of ELISA A410. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

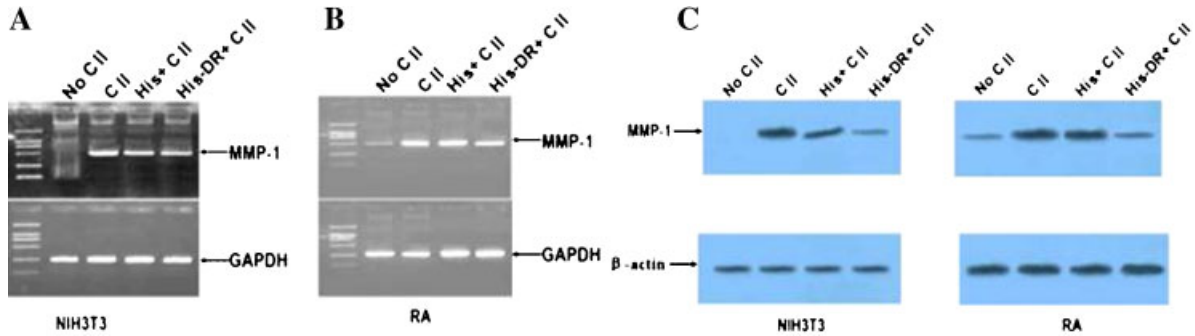

Fig. 4. MMP-1 mRNA and protein expression in cells detected by RT-PCR and Western blotting. **A**: NIH3T3 cells without collagen stimulation: no basal MMP-1 mRNA expression was observed. The results from the other three groups were similar to that of in synoviocytes groups. **B**: RA synoviocytes without collagen stimulation (Group I): only a basal mRNA expression of MMP-1 could be observed; Simple collagen stimulation (Group II): MMP-1 mRNA expression significantly increased; Purified His protein plus collagen stimulation (Group III): MMP-1 mRNA expression was not influenced; Purified His-DR protein plus collagen stimulation (Group IV): MMP-1 mRNA expression was greatly inhibited. **C**: MMP-1 protein expression in NIH3T3 cell culture supernatants detected by Western blotting in testing groups except NIH3T3 cells without collagens. For Group II and Group IV, MMP-1 expression significantly increased in the two cells compared with controls; for Group IV, MMP-1 expression in the two cells decreased to some extents compared with Group II; D: MMP-1 protein expression in RA synovial cell culture supernatants detected by Western blotting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

last three groups were similar to that of synoviocytes groups (Fig. 4A). In groups without collagen, no basal MMP-1 RNA expression was observed, constant with previous study [Wang et al., 2002].

MMP-1 Expression in Cell Culture Supernatants Detected by Western Blotting

Activated MMP-1 expressions were detected in cell culture supernatants by Western blotting except group of NIH3T3 without collagens. In Groups II and IV, MMP-1 expression in the two cells increased enormously, especially compared with controls, whereas in Group IV MMP-1 expression decreased to some extent, especially compared with Group II (Fig. 4C,D).

Activity of MMP-2 and MMP-9 Detected by Gelatinase Zymography

Gelatinase zymography was an assay that gelatin-decomposing enzyme was initially separated by non-degenerative SDS–PAGE, then gelatin decomposition reaction was taking place on the gel, and the activity of enzyme was quantified according to the position and quantity of gelatin decomposed [Rosenfeld, 1999]. As 1 mg/ml gelatin was added into the gel, the gel incubated at 37°C long enough to let the gelatin to be decomposed. After Coomassie brilliant blue staining, the position of enzyme located was achromatic due to the absence of gelatin, while other positions were stained blue as gelatin existed. Our results demonstrated that for RA synoviocytes and NIH3T3 cells, the catalytic activity of MMP-2 in cell culture supernatants from Group II clearly increased with varied extents compared with controls, whereas in Group IV MMP-2 activity decreased compared with Group II. However, catalytic activity of MMP-9 appeared not to be influenced in all testing groups (Fig. 5).

DISCUSSION

DDRs is a group of receptors of PTKs, which are named DDRs after containing a DR structure resembling discoidin agglutinin in its ECD. DDR2 is a special kind of PTK, and most kinds of PTKs are involved in over-proliferation or apoptosis of cells. DDR2 up-regulated MMP-1 too, and the ligand of DDR2 was fibrillic collagen II, which exists mainly in ECM. DDR2 might be related to the clearing of collagen in matrix by up-regulating the production of MMP-1. The process might be enhanced in the growth of some tumors, because collagen clearance is beneficial for metastasis of tumor cells [Wang et al., 2002].

In addition, our in situ hybridization results demonstrated [Wang et al., 2001] that in lung and ovary cancers, DDR2 was highly expressed in interstitial cells around the cancer cells, suggesting DDR2's important role in tumor development as malignant cells need to break through normal tissue barriers by lysis of ECM. Human matrix-degrading diseases, like pulmonary fibrosis, hepatocirrhosis, and rheumatoid

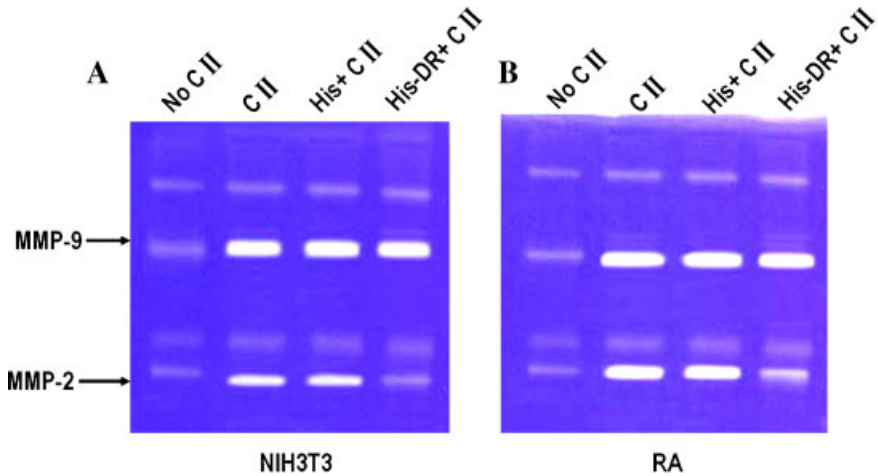

Fig. 5. Activity of MMP-2 and MMP-9 detected by gelatinase zymography. For RA synoviocytes and NIH3T3 cells, catalytic activity of MMP-2 with simple collagen stimulation increased to varied extents especially compared with controls. In Group IV, catalytic activity of MMP-2 decreased somehow compared with Group II, but catalytic activity of MMP-9 in all testing groups appeared unchanged. **A**: NIH3T3 cells; (**B**) RA synoviocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

arthritis, may all exist DDR2 expression and signal transduction, presuming one possible important function of DDR2 was to control the levels of ECM via regulating the synthesis and degradation of collagens. If an antagonist of DDR2 is found out, it may be helpful to prevent the metastasis of tumors or reduce cartilage destruction in rheumatoid arthritis.

To investigate further physiological function of DDR2, it is necessary to get DDR2 expressed. So we chose DDR2 ECD containing all 378 amino acid residues as the blocker. In previous studies, we constructed vectors containing GST and a 6 × His tag, which were all expressed in inclusion bodies. Besides, to improve purification of the expressed DR, we tried baculovirus expression system.

In this study, we applied an efficient and novel site-specific transposition system, Bac-To-Bac® Baculovirus Expression System (Invitrogen), obtained recombinant baculovirus Bac-DR of DDR2 ECD, and got it purified and characterized. The system uses baculovirus shuttle vector propagated in *E. coli* to generate a recombinant baculovirus [Luckow et al., 1993], providing several advantages over the traditional method using homologous recombination, such as shorter time to generate recombinants, simpler procedure of virus plaque purification, and simultaneous isolation of multiple recombinant baculovirus. The system takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA. Our target gene, *DR*, was first cloned into pFastBac™ vector, controlled by strong *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter for high-level expression in insect cells, and transformed into host DH10Bac™ *E. coli* cells containing a baculovirus shuttle vector (bacmid) with a mini-*att*Tn7 target site and a helper plasmid. When transformed into DH10Bac™ cells, transposition reaction occurs between the mini-Tn7 element on the pFastBac™ vector and the mini-*att*Tn7 target site on the bacmid to generate a recombinant bacmid in the presence of transposition proteins supplied by the helper plasmid pMON7124 (13.2 kb, coding the transposase), provides the Tn7 transposition function *in trans*, and confers resistance to tetracycline. Once the transposition is performed, the bacmid DNA is isolated and transfected into insect cells to generate a recombinant baculovirus. After the baculoviral stock was amplified and titered, this stock would be used to infect insect cells for large-scale expression of the protein of interest, while N-terminal 6×His tag for purification of fusion protein using metal-chelating resin and a TEV protease cleavage for removal of His tag were utilized. In our tests, the results illustrated that DR was expressed in insect cells with purity of about 92% after chromatographic purification. Competitive inhibition binding test result displayed

that DR at 0.5 μg/L was capable of completely blocking the binding of collagen type II and native DDR2 on cell surfaces.

To further confirm the competitive inhibition of DR between collagens and DDR2 on cell surfaces and the induced changes of MMP-1, MMP-2, and MMP-9 expression within the cells, we used RT-PCR and Western blotting to detect MMP expression stimulated by collagen with or without DR in synoviocytes and NIH3T3 cells respectively. The results showed that DDR2 activation increased after collagen stimulation, thus the induced MMP-1 expression also increased at both mRNA and protein levels, whereas the fused DR inhibited such increases to varied extent but not completely. The reason might be: ① the affinity of DR to collagen is not strong enough to stop the binding, and ② MMP-1 expression is controlled by other mechanisms within the cells. Whether total DDR2 blocking would completely inhibit MMP-1 expression is still worthy of investigating. Gelatinase zymography results also showed that MMP-2 expression in cell culture supernatants varied greatly in synoviocytes and NIH3T3 cells stimulated by collagen II with or without DR. MMP-2 expression increased after collagen stimulation, but decreased to different extents after collagen stimulation with DR inhibition. Thus we had to suppose that the activation or inhibition of DDR2 may increase or decrease MMP-2 expression and activity respectively. Also, we could see that DR partially inhibited MMP-1 and MMP-2 expression in synoviocytes and NIH3T3 cells stimulated by collagen, clearly indicating that: ① expression of MMP-1 and MMP-2 was intermediated by DDR2 in synoviocytes and NIH3T3 cells, and ② soluble DR could somehow inhibit such MMP expressions as an antagonist.

DDR1 and DDR2 are both composed of an N-terminal 150-amino acid discoidin homology (DS) domain [Baumgartner et al., 1998], followed by a sequence of ~220 amino acids unique to DDRs, a transmembrane domain, a large cytosolic juxtamembrane domain, and a C-terminal catalytic tyrosine kinase domain. The functionally important collagen binding site is contained within the DS domain, and the isolated ECDs bind directly to collagen with high affinity, and such binding requires these domains to be dimerized [Leitinger, 2003]. Based upon the crystal structures of the C2 domains of blood coagulation factors V and VIII, the only three-dimensional structures of the DS domain available indicated that the C2 domain structures consist of a β-sandwich core with three prominent loops (L1, L2, and L4) protruding like spikes. Sequence alignment of several DS domains indicated a high degree of conservative amino acids forming the β-sandwich core and several variable regions, three of which correspond to those spikes seen in the factor V structures, and these three adjacent loops in DDR2 DS domain are involved in collagen binding [Leitinger, 2003; Leitinger et al., 2004]. However, our results did not offer any supporting evidence on DDR2 dimerization and the three adjacent surface loops. The reason may be due to the conformational differences of ECD and whole DDR2 molecules. The expressed DR alone containing the DS still acted as an active and strong antagonist of DDR2, blocking DDR2 binding to natural DDR2 receptors on NIH3T3 and synovial cell surfaces. Our test results are in agreement with the notion that the ECD is necessary and sufficient for collagen binding [Curat et al., 2001; Abdulhussein et al., 2004; Leitinger et al., 2004].

As mentioned above in introduction, several of our colleagues had proposed a hypothesis about the role of collagen II in the pathogenesis of cartilage destruction in rheumatoid arthritis patients based upon those observations so far [Wang et al., 2001; Wang et al., 2002a; Wang et al., 2002b]. The hypothesis is about a possible vicious cycle between DDR2 and collagen II in RA joints. In the present study, our results demonstrated that the cycle quite possibly exists, because the expressed DR did block the binding of collagen to DDR2 on testing cell surfaces and also suppress expression of MMP-1 and MMP-2 activity, which is in agreement with the previous report [Vogel et al., 1997b], suggesting an important role for this receptor in regulating collagen matrix degradation and reorganization. If the vicious cycle is thus broken by exogenous DR, blocking the interaction of collagen II and DDR2 could potentially become a strategy to treat joint cartilage destruction in RA patients. These data is also consistent with those obtained from yeast cells of *Pichia pastori* in our previous study [Zhang et al., 2006]. At present, we are performing experiments to see whether the expressed DR works so in a mouse model.

During endochondral ossification, collagen X is deposited in the hypertrophic zone of the growth plate, and is capable of interacting

directly with chondrocytes, primarily via integrin $\alpha 2\beta 1$ [Luckman et al., 2003]. In another study in chondrocytic cell line MC615, MMP-13 expression, playing an active role in remodeling cartilage in fetal development, osteoarthritic cartilage and long bone development, was induced in cultured type I collagen gel, but not type II collagen; and via type I collagen, it is integrin $\alpha 1\beta 1$ that mediates the MMP-13-inducing cellular signal transduction [Ronzière et al., 2005]. In addition, MMP-13 was stimulated by fibronectin fragment in human chondrocytes through $\alpha 5\beta 1$ integrin receptor and activation of ERK, JNK, and p38 mitogen activated protein (MAP) kinase [Forsyth et al., 2002]. Recently, it has been shown that this fibronectin-induced signaling involves activation of protein kinase C (PKC) [Loeser et al., 2003]. Besides, MAP kinase activation has been linked to MMP-13 expression in response to interleukin-1β in chondrocytes [Mengshol et al., 2000; Mengshol et al., 2001; Liacini et al., 2002]. In addition, experiment results from another research group in our department displayed that MMP-13 also participate in degrading collagen and cartilage in RA pathogenesis, and has been shown to be more potent than MMP-1 in such situations. DDR2 up-regulated MMP-13 expression and activity by promoting its transcription, and such interaction could be inhibited by DR fragment (personal communication with Dr. Zhou Jie. *Paper to be published*). Even so, the role of integrin in cartilage maturation or pathology is still of little knowledge and worthy of investigation.

To summarize, our studies indicated that the fused DR ECD expressed in baculovirus system blocked DDR2 functioning, and inhibited MMP-1 and MMP-2 secretion from NIH3T3 cells and RA synoviocytes stimulated by collagen II. DR thus could be utilized in selecting antagonists with high affinity for potential pharmaceutical and research applications such as DDR2 roles in tumor metastasis and cartilage destruction in RA, DDR2 signal transduction pathway(s), experimental treatment of tumors or RA, and preparation of antibodies against DDR2.

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