Effect of a monoclonal antibody against human relaxin-2 on cancer cell growth inhibition

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Received: 15 July 2016 / Accepted: 10 August 2016 / Published online: 19 August 2016
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Abstract  Relaxin-2 has gained much attention because of its importance in cancer cell proliferation, invasion, and angiogenesis. However, the potential of relaxin-2 as a target for immunotherapy has never been directly validated before. In the present study, we produced a human relaxin-2-specific antibody by immunizing mice using a specific liposome complex containing relaxin-2 epitope peptide and CpG-DNA. We isolated a hybridoma clone, 1B11F12, producing the anti-relaxin-2 monoclonal antibody. We confirmed with immunoprecipitation analysis that the antibody specifically recognizes native human relaxin-2. Treatment with the antibody suppressed the growth of some cancer cell lines. Therefore, we suggest that relaxin-2 could be a reasonable target of cancer therapeutics, and the monoclonal antibody could be used to treat cancer in humans.

Keywords  Cancer · Monoclonal antibody · Relaxin-2

Introduction

The relaxin family members including relaxin-1, relaxin-2, and relaxin-3 are synthesized as preprohormones like insulin (De Meyts 2004). The relaxins undergo post-translational modification characterized by proteolytic cleavage and two interchain disulfide bonds forming active heterodimers (Nair et al. 2012). Relaxin-2, mainly synthesized in the ovary and the prostate, is secreted into the blood (Shabanpoor et al. 2009). The expression of angiogenic factors (VEGF and bFGF), growth promoting genes [insulin-like growth factors (IGFs) and IGF-binding proteins], and matrix degrading enzymes (MMPs and TIMPs) is induced by relaxin (Ohleth et al. 1997; Unemori et al. 2000). These results suggest that relaxin-2 can contribute to the growth, angiogenesis, and metastasis of cancer.

In fact, relaxin-2 is upregulated in cancer tissues. Hence, relaxin-2 has been proposed as a target to treat cancer (Feng et al. 2007; Nair et al. 2012). Several lines of evidence have shown the relationship between relaxin expression and prostate cancer progression (Thompson et al. 2006). Prostate cancer cell lines LNCaP and PC3 Relaxin-2 did express relaxin-2. In a xenograft mouse model, overexpression of relaxin-2 markedly enhanced prostate cancer growth (Feng et al. 2009; Silvertown et al. 2006).

Relaxin-2 is also implicated in breast cancer progression (Silvertown et al. 2003). Circulating relaxin-2 was increased in breast cancer patients. Relaxin-2 is upregulated in malignant and benign mammary tissues. Further, circulating relaxin-2 was dramatically increased in patients with metastatic breast cancer patients (Tashima et al. 1994; Binder et al. 2004). Relaxin-2 promoted growth of...
transplanted breast cancer cell lines (Bani et al. 1999; Radestock et al. 2008). Furthermore, relaxin-2 enhanced growth of spontaneously developed breast cancers and the metastatic brain colonization in mice overexpressing erbB2 (Binder et al. 2014). In addition, overexpression of relaxin-2 was observed in patients with osteosarcoma, and relaxin-2 was implicated in the cell proliferation, migration, and invasion of osteosarcoma (Huang et al. 2014; Ren et al. 2015).

CpG-DNA represents synthetic CpG oligonucleotides with immunomodulatory activity (Klinman et al. 2004). CpG-DNA has been extensively studied as a vaccine adjuvant (Kim et al. 2011). Previously, we developed a method for screening potent B cell epitopes of antigens and efficiently generating antibodies in mice by immunization using a specific liposome composition containing B cell epitope peptide and CpG-DNA (Park et al. 2014; Kim et al. 2015). We found that production of peptide-specific antibodies with the aid of CpG-DNA was significantly increased by a specific liposome complex. In the present study, we produced a relaxin-2-specific monoclonal antibody and showed that the relaxin-2-specific antibody has functional effects on relaxin-2-induced cancer cell growth.

Materials and methods

Oligodeoxynucleotides

We developed natural CpG-DNA with immunomodulatory activity (MB-ODN 4531(O)) (Lee et al. 2006). The specific natural CpG-DNA was synthesized by Samchully Pharm (Seoul, Korea).

Cell culture

We obtained the following cell lines from ATCC (Manassas, USA): human pancreatic cell lines (AsPc-1, CaPan-1, Mia-PaCa-2 and PANC-1), human breast cancer cell lines (MCF-7 and MDA-MB-231), and a human prostate cancer cell line PC3. We also obtained the following cell lines from the Korean Cell Line Bank (Seoul, Korea): human hepatocellular carcinoma (HCC) cell lines (Hep3B, Huh-7, SNU-398, SNU-423, SNU-739 and SNU-761), human colon cancer cell lines (HCT-116, HT-29 and LoVo), a human breast cancer cell line T47D, and a human prostate cancer cell line LNCaP. RPMI1640 medium (Life Technologies, Grand Island, NY, USA) was used for Huh-7, SNU-398, SNU-423, SNU-739, SNU-761, HCT-116, HT-29, LoVo, AsPc-1, CaPan-1, PC3, and LNCaP cells, and DMEM medium was used for Mia-PaCa-2 and PANC-1 cells. EMEM with 0.01 mg/ml of human recombinant insulin was required for culture for MCF-7 cells. MDA-MB-231 cells were cultured in Leibovitz’s L-15 medium (Life Technologies) at 37 °C in an incubator without CO2.

Reverse transcription PCR analysis

We identified the expressions of relaxin-1, relaxin-2, and relaxin family peptide receptor (RXFP1) in cancer cell lines by performing a RT-PCR. RT-PCR was performed according to the previously described method (Kim et al. 2014). Total RNAs were isolated using a PureLink™ RNA isolation Kit (Life Technologies). cDNAs were synthesized at 42 °C for 1 h using Reverse Transcription System (Life Technologies). Then, a standard PCR reaction was conducted. The primer sequences used were as follows: human relaxin-1, 5'-CCATTTGCGGCATGAGCAC-3' and 5'-GAATGGTTGCCTCTCAGATAG-3'; human relaxin-2, 5'-TCTGTTTGCCTCTCAGATAG-3'; human relaxin-2, 5'-TCTGTtTACTCTGAAACCAATTtTT-3' and 5'-CATGGCAACATTATTAGCtAA-3'; and human RXFP1, 5'-GGGCGGAGGATGTGTC-3' and 5'-CACCCGCGCTGAGCAG-3'.

Recombinant relaxin-2 expression

To express the human pro-relaxin-2 as histidine (His)-tagged proteins, total mRNA was obtained from MCF-7 cell, and PCR reaction was performed to amplify human pro-relaxin-2 cDNA with the following primer set: 5'-AAAGCTTATGCCTCGCCTGTTTTTTTTC-3' and 5'-CTCGAGCTTGAAGAGATCCTT-3'. The amplified cDNA fragments were then ligated into the expression vector pET-22b containing the C-terminus His tag. Expression of the recombinant pro-relaxin-2 protein was performed in Escherichia coli Rosetta with 1 mM IPTG. The bacterial cells in a lysis buffer were destroyed by sonication. After centrifugation, the soluble and insoluble fractions were used for Western blot analysis.

Selection of relaxin-2 B cell epitopes

The candidate B cell epitope peptide of relaxin-2 proteins was selected using a computer tool (http://tools.iedb.org/main/bcell) as described previously (Kim et al. 2015). The epitope peptide sequence of the RLN2-5 peptide was 161RQLYSALANKCCHVG175 (hRLN2-5). The peptides were synthesized and isolated to a purity greater than 90 % by Pepton (Daejeon, Korea).

Immunization

We obtained approval of the Institutional Animal Care and Use Committee of Hallym University (Hallym 2015–2056) for animal experiments. BALB/c (H-2b) mice were obtained from Nara Biotech, Inc., and maintained in.
specific-pathogen-free conditions. As described previously (Kim et al. 2011), the mice were boosted in intraperitoneal cavity using a specific liposome composition including 50 µg of RLN2-5 peptide and 50 µg of CpG-DNA 3 times at 10 day intervals.

**ELISA assay**

The RLN2-5 peptide was used for coating in 96-well immunoplates (Nalgen Nunc International, Penfield, NY, USA). The RLN2-5 peptide-specific total IgG amounts were measured by ELISA as previously described (Park et al. 2015). To measure the isotype of the sera antibodies, we used HRP-conjugated anti-mouse IgG antibody, anti-mouse IgG1 antibody, anti-mouse IgG2a antibody, anti-mouse IgG2b, or anti-mouse IgG3 antibody (BD Biosciences, San Diego, CA, USA).

**Production of monoclonal antibody against relaxin-2**

BALB/c mice were immunized as described above. The spleen cells from the immunized mice were used for fusion in accordance with the standard hybridoma technique. The hybridoma clones in HAT medium and HT medium were selected with a standard limiting dilution protocol. To obtain the ascites, the selected hybridoma clone was i.p. injected to BALB/c mouse. Anti-relaxin-2 monoclonal antibody in the ascites fluid was purified with protein A column. The isotype of antibody was determined using an isotyping kit (Southern Biotechnology Associates Inc., Birmingham, AL, USA) as previously described (Shang et al. 2009).

**Reactivity of monoclonal antibody against relaxin-2**

We performed ELISA assay to identify the binding affinity of the monoclonal antibody against relaxin-2. 96-well immunoplates were coated with hRLN2-5 peptide. The anti-relaxin-2 monoclonal antibody was applied to the wells with serial 1:5 dilutions in PBST starting from 1 µM. The amount of bound antibodies was analyzed by colorimetric assay using a developing kit from KPL (Gaithersburg, MD, USA). The results were analyzed with the SigmaPlot program to calculate the binding affinity.

**Western blot and immunoprecipitation analysis**

To analyze the specificity of the anti-relaxin-2 monoclonal antibody, Western blot and immunoprecipitation assays were performed. Briefly, the insoluble fraction of E. coli cell lysate including recombinant pro-relaxin-2 protein and recombinant human relaxin-2 peptide (rhRLN2, Peprotech, Seoul, Korea) were resolved on SDS-PAGE, and antibody reactivity was determined by Western blot with rat anti-human relaxin-2 monoclonal antibody (R&D systems, Minneapolis, MN, USA) or anti-relaxin-2 monoclonal antibody (1B11F12). For immunoprecipitation, 10 µg of rhRLN2 peptide was incubated with normal mouse IgG, rat anti-human relaxin-2 monoclonal antibody, or 1B11F12 monoclonal antibody. After incubation, samples were mixed with protein A agarose and washed with PBST, and then, Western blot analysis was performed.

**Detection of cell proliferation**

To identify the effect of relaxin-2 on cancer cell growth, 96-well culture plates (Nalgen Nunc International) were plated with 2 × 10³ cells/well of breast (MCF-7, MDA-MB231, and T47D) and prostate (PC3 and LNCaP) cancer cell lines, and cultured in an incubator at 37 °C. The cells were treated with rhRLN2 at a concentration of 500 ng/ml and serial 1:5 dilutions. The cells were cultured for 3 days in an incubator at 37 °C. The growth of the cells was identified using cell counting kit-8. To measure the effect of monoclonal antibody against relaxin-2 on rhRLN2 peptide-dependent growth of the cell, rhRLN2 peptide was pre-incubated with anti-relaxin-2 monoclonal antibody at 4 °C for 1 h. The incubated samples were then applied to breast cancer cells and prostate cancer cells, and the cell proliferation assay was performed.

**Results**

**Expression of recombinant relaxin-2 precursor protein**

To identify the specificity of anti-relaxin-2 monoclonal antibody, full-length cDNA of relaxin-2 was obtained from the mRNA of MCF-7 cells and cloned into the plasmid vector (pET-22b) for the expression of relaxin-2 precursor in E. coli. Expression of recombinant protein with a C-terminal poly histidine affinity tag was induced by IPTG treatment. Total cell lysates, soluble fraction, and insoluble fraction were analyzed by SDS-PAGE (Fig. 1A). Recombinant relaxin-2 precursor was found in the insoluble fraction, which was detected with an anti-His antibody (Fig. 1B).

**Production of anti-relaxin-2 antibody**

We reported the protocol to prepare epitope-based peptide vaccines and to produce monoclonal antibodies (Kim et al. 2015). In the present study, we applied the method to produce a monoclonal antibody against human relaxin-2. First, we synthesized the candidate B cell epitope of the
human relaxin-2 and made a peptide epitope (RLN2-5 peptide)-CpG-DNA-liposome complex. Then, we immunized BALB/c mice using the specific liposome complex. The immunized mouse sera were collected and analyzed for the antibody titers. The ELISA results clearly showed a significant increase of human relaxin-2 peptide epitope-specific IgG (Fig. 2A). The mouse with the highest IgG titer was selected and injected with the same antigen complex. After 10 days, mouse splenocytes were collected and used to generate hybridoma cells. The cell culture supernatants of the hybridoma cells were analyzed by indirect ELISA to isolate clones highly producing peptide epitope-specific antibodies. Among the two hybridoma cell lines obtained (1B11F12, 2G9F11), the 1B11F12 clone was selected to generate ascites. The anti-relaxin-2 monoclonal antibody was purified from the ascites, analyzed by SDS-PAGE (Fig. 2B). The monoclonal antibody isotype was identified as an IgG2a (Fig. 2C).

**Characteristics of anti-relaxin-2 monoclonal antibody**

We analyzed the reactivity of anti-relaxin-2 monoclonal antibody against the human relaxin-2 peptide epitope, RNL2-5. We quantitatively determined the binding affinity of the anti-relaxin-2 monoclonal antibody using ELISA. The anti-relaxin-2 monoclonal antibody reacted with human relaxin-2 peptide with an equilibrium dissociation constant \( (K_d) \) of \( \sim 250 \) pM (Fig. 2D).

To identify whether the monoclonal antibody 1B11F12 clone could recognize the relaxin-2 precursor protein and mature relaxin-2 specifically, we performed Western blot analysis. The insoluble fraction of the bacterial cell lysate
that included the recombinant relaxin-2 precursor protein and synthetic mature relaxin-2 were resolved by SDS-PAGE and analyzed by Western blotting. Anti-relaxin-2 monoclonal antibody, which is commercially available, can detect recombinant relaxin-2 precursor protein and synthetic mature relaxin-2 (Fig. 3A). The anti-relaxin-2 monoclonal antibody 1B11F12 clone can recognize recombinant relaxin-2 precursor protein; however, it has no reactivity with the synthetic mature relaxin-2 (Fig. 3B). To identify whether the monoclonal antibody could detect native synthetic mature relaxin-2, we performed immunoprecipitation analysis. As shown in Fig. 3C, the anti-relaxin-2 monoclonal antibody 1B11F12 clone could detect native synthetic mature relaxin-2. These results show that the anti-relaxin-2 monoclonal antibody can recognize the recombinant relaxin-2 precursor protein by Western blot analysis and native synthetic mature relaxin-2 by immunoprecipitation.

**Detection of relaxin-2 expression**

To check the expression of relaxin-2 and its receptor hRXFP1 in various cancer cell lines, we conducted RT-PCR. Expression of relaxin-2 was weakly detected in MCF7 cell as shown in Fig. 4. Expression of relaxin-1 and relaxin-2 was most strongly detected in LNCaP cells. However, expression of hRXFP1 was widely seen in most of the cells. Therefore, the results suggest that relaxin-2/hRXFP1 signaling could widely work in many cancer cell lines as shown in Fig. 4. Considering that a higher expression of relaxin-1 and relaxin-2 along with its receptor expression was observed in LNCaP cells, a strong autocrine effect may contribute to the properties of the LNCaP cells.

**Cell growth inhibition by anti-relaxin-2 monoclonal antibody**

Relaxin-2 was reported to be involved in the progression of breast and prostate cancers. Therefore, to identify the effect of the anti-relaxin-2 antibody in various cancer cell lines, we treated the cells with recombinant relaxin-2 along with normal IgG or anti-relaxin-2 monoclonal antibody. As shown in Fig. 5, treatment with relaxin-2 alone enhanced the growth of MCF-7 and LNCaP cells, and the growth was reduced when the anti-relaxin-2 antibody was present. The effect of the anti-relaxin-2 antibody was not prominent in MDA-MB-231, T47D, and PC3 cells. Therefore, we can conclude that interruption of relaxin-2 signaling with the monoclonal antibody can inhibit the tumor growth at least in some breast and prostate cancer cells.

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**Fig. 3** Specificity of anti-relaxin-2 monoclonal antibody. (A), (B) Insoluble fraction of E. coli lysate including recombinant relaxin-2 precursor protein and synthetic rhRLN2 peptide were separated by SDS-PAGE. Western blot analysis was performed using commercially available rat anti-human relaxin-2 monoclonal antibody (A) and anti-relaxin-2 monoclonal antibody prepared from the 1B11F12 hybridoma clone (B), respectively. (C) rhRLN2 peptide was incubated with normal mouse IgG, rat anti-human relaxin-2 monoclonal antibody or 1B11F12 monoclonal antibody. The immunocomplex was captured with protein A bead, and then subjected to Western blot analysis with rat anti-human relaxin-2 antibody. Black left pointing triangle: Relaxin-2 precursor, white left pointing triangle: mature Relaxin-2, HC heavy chain, LC light chain.
Figure 4
Expression of human relaxin-2 mRNA in cancer cells. To analyze the expression of relaxin-1, relaxin-2, and relaxin family peptide receptor (RXFP1) genes, we performed RT-PCR analyses in HCC cells (A), pancreatic cancer cells (B), colon cancer cells (C), breast cancer cells (D), and prostate cancer cells (E). The amount of β-actin mRNA was used as a control.

Figure 5
Inhibition of cancer cell growth by anti-relaxin-2 monoclonal antibody. rhRLN2 peptide was pre-incubated with anti-relaxin-2 monoclonal antibody at 4 °C for 1 h, and the incubated samples were applied to breast cancer cells (MCF-7, MDA-MB-231, and T47D) and prostate cancer cells (PC3 and LNCaP). The growth of cancer cells was measured by cell proliferation assay. ***p < 0.001

Discussion
Relaxin-2 is involved in growth of cancer tissues through induction of cell proliferation, migration, and invasion. Therefore, relaxin-2-mediated signaling is postulated to serve as a target to prevent prostate cancer, breast cancer, and osteosarcoma. Suppression of relaxin and relaxin receptor in prostate cancer cells with short interfering RNAs (siRNA) significantly induced apoptosis and reduced growth and metastasis (Feng et al. 2007, 2010). A relaxin analogue with lysine residues instead of arginine (R13 and R17 K) in the B-chain was produced from the lentiviral vector or chemical synthesized and confirmed to have antagonistic effect on the relaxin-induced progression of prostate cancer cells (Silvertown et al. 2006). Another antagonist, targeting relaxin receptor (the LDL-A module of RXFP1), inhibited the downstream signaling of the relaxin in prostate cancer cell line PC3 (Feng et al. 2009). Therefore, accumulating data support that strategies to block or neutralize the autocrine or paracrine effect of relaxin could be a rational approach to treat relaxin-2 related cancers. However, simpler and efficacious antagonists have to be developed to make this strategy effective.

Although diverse studies have been done to develop cancer therapeutics targeting relaxin-2, its immunotherapy was never directly validated before. Therefore, we produced anti-relaxin-2 monoclonal antibody as an approach to develop potential therapeutics. Previously, we developed a new technology to produce peptide-specific antibodies without carriers, and in this study, we produced an efficacious monoclonal antibody specific to human relaxin-2. The anti-relaxin-2 monoclonal antibody 1B11F12 clone recognized only recombinant relaxin-2 precursor protein but not the native synthetic mature relaxin-2 by Western blot analysis (Fig. 3B). However, the 1B11F12 clone did...
recognize the native synthetic mature relaxin-2 based on immunoprecipitation analysis (Fig. 3C). Therefore, we speculate that our peptide-based antibody production protocol can induce the production of an antibody recognizing the conformational epitope.

In the present study, we showed the expression of relaxins and the relaxin receptor in various cancer cell lines. Relaxin-2 expression was observed in the MCF-7 cell line and LNCaP cell line (Fig. 4). Furthermore, we validated the application of anti-relaxin-2 monoclonal antibody as a therapeutic to prevent cancer cell growth (Fig. 5). To confirm the inhibition of cancer cell growth by anti-relaxin-2 monoclonal antibody in vitro, we applied anti-relaxin-2 monoclonal antibodies to relaxin-2-treated cancer cell cultures. The growth of MCF-7 and LNCaP were suppressed by the anti-relaxin-2 monoclonal antibody but not by the control IgG (Fig. 5). Therefore, our results prove that the anti-relaxin-2 monoclonal antibody has a suppressive effect on the growth of some cancer cells. Further, we suggest that the relaxin-2-specific monoclonal antibody can be potentially used as therapeutics for breast and prostate cancers.

Acknowledgments This work was supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning in the Republic of Korea (NRF-2015R1A2A2A01007209, 2013R1A2A2A03067981, 2014M3C1A3051473, 2013M3A9A9050126, 2009-0093812).

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