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Prevention of Reg I-induced β-cell apoptosis by IL-6/dexamethasone through activation of *HGF* gene regulation

Kei Nakagawa [a,b], Shin Takasawa [a,c,*], Koji Nata [a,d], Akiyo Yamauchi [c], Asako Itaya-Hironaka [c], Hiroyo Ota [c], Kiyomi Yoshimoto [c], Sumiyo Sakuramoto-Tsuchida [c], Tomoko Miyaoka [c], Maiko Takeda [c], Michiaki Unno [b], Hiroshi Okamoto [a,e]

[a] *Department of Advanced Biological Sciences for Regeneration (Kotobiken Medical Laboratories), Tohoku University Graduate School of Medicine, Sendai 980-8575, Miyagi, Japan*
[b] *Department of Surgery, Tohoku University Graduate School of Medicine, Sendai 980-8575, Miyagi, Japan*
[c] *Department of Biochemistry, Nara Medical University, Kashihara 634-8521, Nara, Japan*
[d] *Department of Medical Biochemistry, Iwate Medical University, School of Pharmacy, Yahaba 920-0293, Iwate, Japan*
[e] *Tohoku University, Sendai 980-8577, Miyagi, Japan*

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ABSTRACT

Reg (regenerating gene) product, Reg protein, is induced in pancreatic β-cells and acts as autocrine/paracrine growth factor for regeneration via the cell surface Reg receptor. However, high concentrations of Reg I protein induced β-cell apoptosis. In the present study, we found that hepatocyte growth factor (HGF) attenuated the β-cell apoptosis induced by the high concentrations of Reg I protein and that the combined stimulation of interleukin-6 (IL-6) and dexamethasone (Dx) induced the accumulation of *HGF* mRNA as well as *Reg I* mRNA in β-cells. The accumulation of the *HGF* mRNA was caused by the activation of the *HGF* promoter. Deletion analysis revealed that the region of −96 to −92 of the *HGF* gene was responsible for the promoter activation by IL-6 + Dx. The promoters contain a consensus transcription factor binding sequence for signal transducer and activator of transcription (STAT). Site-directed mutations of STAT-binding motif in the region markedly attenuated the *HGF* promoter activity. Chromatin immunoprecipitation assay showed that STAT3 is located at the active *HGF* promoter in response to IL-6 + Dx stimulation. These results strongly suggest that the combined stimulation of IL-6 and glucocorticoids induces the activation of both *Reg* and *HGF* genes and that the anti-apoptotic effects of HGF against the Reg I-induced apoptosis may help β-cell regeneration by Reg I protein.

1. Introduction

A limited capacity for regeneration in adult β cells is thought to be a predisposing factor for the development of diabetes [1–3]. Thus, strategies for introducing the replication and growth of the β cells mass are important for the prevention and/or treatment of diabetes [2]. We established a model for islet regeneration in 90% depancreatized rats by the administration of poly(ADP-ribose) polymerase (PARP) inhibitors such as nicotinamide (NA) and 3-aminobenzamide (3AB) [4]. By screening a regenerating islet-derived cDNA library, we isolated a novel gene, *Reg* (regenerating gene), specifically expressed in regenerating islets [5] and found that both rat and human Reg protein (rat Reg I and human REG Iα [6,7]) induced the proliferation of β cells to ameliorate the diabetes of 90% depancreatized rats [8] and of non-obese diabetes (NOD) mice [9,10]. Recently, we revealed that the *Reg I* gene transcription was activated by the combined addition of interleukin-6 (IL-6) and dexamethasone (Dx) in β cells and that PARP inhibitors such as NA and 3AB further enhanced the expression [11]. We also found that high concentrations of Reg I protein (300–1000 nM) induced β cell apoptosis via Reg receptor [12]. Thus, we assumed that the Reg–Reg receptor system could regulate both the proliferation and apoptosis of pancreatic β cells to maintain the insulin-producing cell mass by controlling the concentrations of Reg I protein [2].

Accumulating evidence indicates that hepatocyte growth factor (HGF) is an anti-apoptotic as well as a mitogenic and insulinotropic factor for pancreatic β cells [13–16]: HGF was reported to protect insulin-producing rat RINm5F β cells against free fatty acid-induced apoptosis

Abbreviations: 3AB, 3-aminobenzamide; ChIP, Chromatin immunoprecipitation; Dx, dexamethasone; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA, transcription factor; HGF, hepatocyte growth factor; HSF-2, heart shock factor 2; IL-6, interleukin-6; NA, nicotinamide; NOD mouse, non-obese diabetic mouse; Nrf-2, nuclear respiratory factor 2; PARP, poly(ADP-ribose) polymerase; Reg, regenerating gene; RT-PCR, reverse transcription polymerase chain reaction; STAT, signal transducer and activator of transcription; WST-1, 4[-3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

* Corresponding author at: Department of Biochemistry, Nara Medical University, 840 Shijo-cho, Kashihara 634-8521, Nara, Japan. Tel.: +81 744 22 3051x2227; fax: +81 744 24 9525.
E-mail address: shintksw@naramed-u.ac.jp (S. Takasawa).

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by counteracting oxidative stress [13]. From the findings, we conceived a possibility that HGF inhibits the high concentration of Reg I protein-induced apoptosis. In the present study, we tested the possibility and found that HGF inhibited the pancreatic β cell apoptosis induced by the high concentrations of Reg I protein and that the combined stimulation of IL-6 and Dx induced the *HGF* transcription via signal transducer and activator of transcription (STAT) binding sequence in pancreatic β cells.

2. Materials and methods

2.1. Cell culture

RINm5F cells, a rat β cell line, were maintained as described [11,12,17–19]. For the stimulation experiments, cells were treated with 20 ng/ml human IL-6 (Genzyme, Cambridge, MA), 100 nM Dx (Sigma-Aldrich, St. Louis, MO), 10 mM NA, 1 mM 3AB, 25 ng/ml recombinant human HGF (Genzyme), 1000 nM rat Reg I protein or combinations thereof [11]. Pancreatic islets were isolated by collagenase digestion method [20,21] from male Wistar rats (200–300 g), transferred to 6-well culture dishes in groups of 200 islets, and stimulated by the combined addition of IL-6 + Dx. 1.1B4 cells, a human pancreatic β cell line, were purchased from European Collection of Cell Culture (Salisbury, UK) and were maintained as described [22].

2.2. Measurement of apoptosis

Rat Reg I protein was expressed in *Pichia pastoris* and purified to homogeneity using cation exchange column chromatography as described [11,12,17]. RINm5F cells were cultured in RPMI 1640 with 1% FCS in the presence of 1000 nM rat Reg I protein with other stimulants for 24 h. Apoptosis was detected by the TUNEL method using an apoptosis screening kit (Wako Pure Chemical, Osaka, Japan) as described [12,19].

2.3. Measurement of viable cell numbers by tetrazolium salt cleavage

After a 24-h incubation of the RINm5F cells (5×10^4 cells/well) in RPMI 1640 with 1% FCS in the presence of stimulants, a solution containing 4[-3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, F. Hoffmann-La Roche Ltd, Basel, Switzerland) was added to the medium, and the cells were incubated for another 30 min as described [11,12,23].

2.4. Induction of HGF messenger RNA

After a 24-h incubation with various stimulants, cells were harvested, and total RNA was prepared as described [19]. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using primers corresponding to nucleotides 1556–1575 and 2208–2227 of rat *HGF* mRNA [24], and 135–155 and 951–971 for rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA as described [18]. Real-time RT-PCR was performed as described [19,25]. In brief, the cDNA was synthesized from total RNA as template using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) for template of real-time PCR and real-time PCR was performed using Fast SYBR® Green Master Mix (Applied Biosystems) and Thermal Cycler Dice® Real Time System (Takara Bio Inc., Otsu, Japan). PCR primers corresponding to nucleotides 2145–2167 and 2235–2254 of human *HGF* mRNA (NM_000601), and 420–437 and 492–509 for human *β-actin* mRNA (NM_001101) were synthesized by NGRL (Sendai, Japan). Target cDNAs were cloned into pBluescript SK(-) plasmid (Stratagene, La Jolla, CA) and sequential 10-fold dilutions from 10^2–10^7 copies/μL were prepared. The serial dilutions were run to verify the specificity and to test the sensitivity of the SYBR Green-based real-time RT-PCR. *HGF* mRNA value was normalized to that of *β-actin* mRNA, which was used to account for differences in the efficiency of reverse transcription between samples.

2.5. Measurement of HGF protein in culture medium

RINm5F cells were cultured in RPMI 1640 with 1% FCS in the presence of stimulants for 24 h. The concentration of HGF protein in the medium was measured by using a rat HGF EIA kit (Institute of Immunology, Tokyo, Japan) according to the instructions of the supplier.

2.6. Construction of reporter plasmid and luciferase assay

A 1395-bp genomic fragment containing the 5′-flanking region of the rat *HGF* gene (−1336 to +59) [26] was inserted into pGL3-Basic vector (Promega, Madison, WI). Unidirectional deletions were made using a Deletion Kit for Kilo-Sequencing (Takara Bio Inc.). Mutants of potential binding sites for Stat and GATA factors (STAT-M1, STAT-M2, GATA-M) were constructed on pGL3 vector containing the *HGF* promoter by PCR. Promoter plasmid was transfected into RINm5F cells by using DMRIE-C (Invitrogen Corp., Carlsbad, CA) as described [11,18]. In brief, RINm5F cells were seeded at 1×10^5 cells per well in a 24-well dish in RPMI1640 supplemented with 10% FCS. After 24 h, the medium of each well was replaced with fresh medium containing stimulants and incubated further for 24 h. Cells were harvested in 1 ml of ice-cold PBS, washed twice with PBS, and extracts were prepared in extraction buffer (0.1 M potassium phosphate, pH 6.8/1 mM DTT). To monitor transfection efficiency, pCMV-SPORT- βgal plasmid (Invitrogen Corp.) was co-transfected in all experiments at a 1:15 dilution. Luciferase activity was measured using a PicaGene Luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the β-galactosidase activity as described [11,18].

2.7. Chromatin immunoprecipitation (ChIP) assays

After 24 h incubation with stimulants, RINm5F and 1.1B4 cells were processed for the ChIP assay as per the protocol described previously [18] using a ChIP-IT kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. The antibody used for the ChIP was STAT3 antibody (SantaCruz). The sequences for rat and human *HGF* gene promoters were as follows: sense primers, 5′-AGCTGGGATCT GTTGCTTGT-3′ (for rat) and 5′-GGGATCTGTTTGGTGCTGTT-3′ (for human) and antisense primers, 5′-ATGCCGGGCTGAAAGAATCC-3′ (for rat) and 5′-AGTTTGGTCACCCACATGGT-3′ (for human). The PCR products were analyzed on 2.5% agarose gel electrophoresis and documented.

3. Results

3.1. HGF attenuates the apoptosis induced by the high concentrations of Reg I protein

We previously reported that high concentrations of Reg I protein (300–1000 nM) induced the apoptosis of RINm5F β cells [12]. More higher serum concentrations of Reg family protein (over 10 μM) were reported in pathological conditions such as pancreatitis [27]. As shown in Fig. 1A, a single addition of IL-6, Dx, NA, nor 3AB alone did not inhibit the apoptosis induced by 1000 nM Reg I protein. On the other hand, the combined addition of IL-6 and Dx significantly inhibited the apoptosis. The addition of PARP inhibitors, NA or 3AB, to IL-6 + Dx was ineffective. Moreover, the addition of 2.5 and 25 ng/ml HGF showed an anti-apoptotic activity against the Reg I-induced apoptosis as well as the combined addition of IL-6 + Dx. In the presence of 1000 nM Reg I protein, the viable cell numbers were increased by the addition of IL-6 + Dx as well as by the addition of HGF (Fig. 1B). The combined addition of IL-6 + Dx and HGF did not increase significantly more WST-1 cleavage than the addition of IL-6 + Dx or HGF alone. These results