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INSL5 may be a unique marker of colorectal endocrine cells and neuroendocrine tumors

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ABSTRACT

Insulin-like peptide 5 (INSL5) is a member of the insulin superfamily, and is a potent agonist for RXFP4. We have shown that INSL5 is expressed in enteroendocrine cells (EECs) along the colorectum with a gradient increase toward the rectum. RXFP4 is ubiquitously expressed along the digestive tract. INSL5-positive EECs have little immunoreactivity to chromogranin A (CgA) and might be a unique marker of colorectal EECs. CgA-positive EECs were distributed normally along the colorectum in INSL5 null mice, suggesting that INSL5 is not required for the development of CgA-positive EECs. Exogenous INSL5 did not affect the proliferation of human colon cancer cell lines, and chemically-induced colitis in INSL5 null mice did not show any significant changes in inflammation or mucosal healing compared to wild-type mice. In contrast, all of the rectal neuroendocrine tumors examined co-expressed INSL5 and RXFP4. INSL5 may be a unique marker of colorectal EECs, and INSL5–RXFP4 signaling might play a role in an autocrine/paracrine fashion in the colorectal epithelium and rectal neuroendocrine tumors.

1. Introduction

The human relaxin/insulin superfamily consists of insulin, insulin-like growth factor 1 and 2 (IGF1 and IGF2), relaxins-1 (H1 relaxin), -2 (H2 relaxin), insulin-like peptide 3–6 (INSL3–6), and relaxin-3/INSL7. Except for IGF1 and IGF2, which are single chain peptides, all members of the family consist of two chains (an A-chain and a B-chain) that are linked by two disulfide bonds with a third intramolecular disulfide within the A-chain, which is a signature structure of insulin-like molecules [1,2]. Relaxins have been shown to be multifunctional peptides involved in numerous physiological processes, including uterine relaxation, reproductive tissue growth, collagen remodeling in females, wound healing, cardiac protection and allergic responses [2].

INSL5 was first identified through database searches of expressed sequence tags for the presence of the B-chain Cys motif, which is conserved within the relaxin/insulin superfamily [3]. Human INSL5 mRNA has been detected in peripheral tissues, including the rectum, colon and uterus [3]. A quantitative RT-PCR study revealed the presence of INSL5 mRNA in a variety of human tissues, including the pituitary and at lower levels, the brain [4]. In mice, the highest expression of INSL5 mRNA is found in the colon [3] and kidneys [5]. INSL5 is also expressed in a population of cells in the mouse hypothalamus and pituitary, and it increases the internal [Ca^{2+}] by a mechanism that involves both Ca^{2+} influx and Ca^{2+} release from intracellular stores [6]. The high concentration of immunoreactive INSL5 in the hypothalamic–pituitary axis suggests that it has a neuroendocrine function in mice [6].

The G-protein-coupled relaxin family peptide receptors 4 (RXFP4), also known as G protein-coupled receptor 142 or GPR100, was discovered by searching the human genomic database using the RXFP3 sequence. Receptor–ligand binding assays revealed that INSL5 is the ligand for RXFP4, whereas relaxin-3/INSL7 binds to RXFP3 [4]. The Northern blot analyses conducted with RNA revealed RXFP4 expression in the heart, skeletal muscle, kidneys, liver and placenta [7]. Similar results were obtained by RT-PCR, with mRNA also detected in the human colon, thyroid, salivary glands, prostate, thymus, testes and brain [4,8]. Intriguingly, the genes for both INSL5 and its receptor are dysfunctional in the rat and dog genomes [4].

In the gastrointestinal mucosa, several types of enteroendocrine cells (EECs) are dispersed throughout the epithelial layer of the digestive tract. Unlike other endocrine systems, EECs have high turnover rates, with a lifespan on the order of 4–6 days [9]. EECs

Abbreviations: Ab, antibody; CgA, chromogranin A; DSS, dextran sulfate sodium; DMEM, Dulbecco's modified Eagle's medium; EEC, enteroendocrine cell; IGF, insulin-like growth factor; INSL5, insulin-like peptide 5; LDCV, large dense core vesicles; mTOR, mammalian target of rapamycin; NET, neuroendocrine tumor; NSE, neuron-specific enolase; PCR, polymerase chain reaction; RXFP, relaxin family peptide receptor; SLMV, synaptic-like microvesicles; WT, wild-type.

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produce and secrete multiple regulatory molecules, which play important roles in food intake, gut motility, intestinal transit, the absorption of nutrients, energy homeostasis, mucosal immunity and repair [9,10]. They act as sensors of luminal contents, either in a classical endocrine fashion, or by a paracrine effect on proximate cells.

There are at least 15 subtypes of EECs, based on the ultrastructural characteristics of their secretary granules and the diverse number of hormones produced [11]. As with extragastrointestinal endocrine systems, the co-expression, and presumably co-secretion, of more than one mediator is widely observed [9]. EECs present two regulated pathways of secretion characterized by large dense core vesicles (LDCV) and synaptic-like microvesicles (SLMV). EECs are recognized by the expression of several general markers, including the LDCV marker, chromogranin A (CgA), and the SLMV marker, synaptophysin, in addition to the cytosolic marker, neuron-specific enolase (NSE) [11]. The expression of different hormones identifies specific cell types.

Neuroendocrine tumors (NETs, also known as carcinoid tumors) are rare and slow-growing tumors derived from EEC populations, representing approximately 0.5% of all malignancies [12]. NETs consist of a spectrum of malignancies that can arise from neuroendocrine cells throughout the body [13]. Yao et al., reported that the incidence and prevalence of NETs has increased substantially over the past three decades at all primary sites and for all disease stages using the Surveillance, Epidemiology, and End Results (SEER) program (USA) [13].

In spite of numerous studies on INSL5, its biological function(s) remain largely elusive. Recently, Burnicka-Turek et al., has reported that INSL5-deficient mice displayed alterations in glucose homeostasis and impaired fertility [14]. However, the intense expression of INSL5 in the colorectum and its cognate receptor, RXFP4, strongly suggests that it has an autocrine/paracrine function in the peripheral digestive tract. The aim of this study was to examine the role of the INSL5–RXFP4 ligand–receptor system in the colorectum.

2. Materials and methods

2.1. Materials

The antibodies (Abs) used in the studies are listed in Supplementary Table 1.

2.2. Cell culture

Human colon cancer cell lines, $CaCO_2$ (RBRC-RCB0988) and LoVo (RBRC-RCB1639) [15] were purchased from RIKEN Cell Bank (Tsukuba, Japan). The COLO320DM (JCRB0225) cell line, derived from an untreated human colon carcinoid tumor [16], was purchased from the Health Science Research Resources Bank (Osaka, Japan). $CaCO_2$ cells and COLO320DM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% and 10% fetal bovine serum (FBS), respectively, at 37 °C in a humidified environment of 95% air and 5% CO_2. LoVo cells were cultured in HamF12 medium containing 10% FBS. All media contained 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Animal models

C57BL/6 INSL5 knock-out mice (*Insl5*$^{-/-}$ mice, targeted disruption of the gene) were obtained from the Jackson Laboratory (ME, USA). All mice were maintained in a specific pathogen-free (SPF) animal facility at Akita University. Eight- to sixteen-week-old mice were used in the study. The primer sequences used for genotyping are listed in Supplementary Table 2. All experiments using mice were approved by the Institutional Animal Care and Use Committee of Akita University.

2.4. Induction of dextran sulfate sodium (DSS)-induced colitis and grading of histologiccal changes

DSS [molecular weight, 36–50 kDa; MP Biomedicals (Solon, OH)] was added to the drinking water at a final concentration of 3% (wt./vol.) for 5 days (days 1–5) [17]. On day eight, the mice were sacrificed, and colorectal samples were extracted. The degree of inflammation on microscopic cross sections of the colorectum was graded semiquantitatively based on a scoring system that considers architectural derangement, goblet cell depletion, edema/ulceration and the degree of inflammatory cell infiltration [18]. All criteria were graded on a 0–3 scale (0, absent; 1, mild; 2, moderate; 3, severe). The four scores were added together to give a total score for each section of the colorectum.

2.5. Human tissues

All NET tissue samples (rectum ($n = 12$), stomach ($n = 3$) and duodenum ($n = 1$)) were collected from the Department of Gastroenterology, Akita University Hospital, by endoscopic resection for clinical indications. This study was reviewed and approved by the ethics committee of Akita University, Faculty of Medicine, and The Institute of Medical Science, The University of Tokyo. All patients gave their written consent for the use of their tissue specimens.

2.6. Northern blot analysis

Human MTN Blots and Human Digestive System 12-Lane MTN Blots were purchased from Clontech (Palo Atlo, CA). The blots were hybridized with a ^{32}P-labeled *Insl*5 and *Rxfp*4 cDNA probes. The blots were rehybridized with a ^{32}P-labeled β-*actin* cDNA fragment to confirm the equal loading of RNA.

2.7. Conventional reverse-transcription polymerase chain reaction (RT-PCR)

The total RNA was obtained using an RNeasy Mini kit (QIAGEN, Valencia, CA). First-strand complementary DNA was synthesized using the Superscript™ First-stranded Synthesis System for Reverse-Transcription Polymerase Chain Reaction (Invitrogen, Carlsbad, CA). The PCR primers and the conditions used in the study are listed in Supplementary Table 2.

2.8. Immunohistochemistry

After deparaffinization, heat-induced epitope retrieval and the quenching the endogenous peroxidase, the samples were immunostained sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary Abs and secondary Abs. Specific immunostaining was developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (DAKO).

For the co-localization of human INSL5 and CgA, NETs samples accompanying normal colorectal areas were used. Immunostaining proceeded similarly using a Cy3-conjugated anti-rabbit IgG (for INSL5) and an Alexa Fluor 488 anti-mouse IgG (for CgA) as the secondary Abs. As a negative control, the primary Ab was replaced with a species-specific IgG isotype control at the same concentration (DAKO).