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Reg IV Protein is Expressed in Normal Rat Tissue

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ABSTRACT

The Reg IV gene has been documented in the colon, small intestine, stomach and pancreas of the human. Expression of the Reg IV in different cell types has been associated with regeneration, cell growth and cell survival, cell adhesion and resistance to apoptosis. It is unknown whether the Reg IV protein is present in the normal rat tissue. The aim of this study was to reveal the expression of the Reg IV protein in the rat spleen and colon. Western blot analysis using antibody specific for Reg IV protein were performed on rat spleen and colon extracts. Low level of Reg IV expression was found in all examined colon samples. The expression of Reg IV protein in spleen tissue was significantly higher than in the colon. Reg IV protein was immunohistochemically stained in a few epithelial cells in the basal portion of colon crypts and in a large spleen cells which were scattered in the red pulp. Our results demonstrate for the first time the presence of the Reg IV protein expression in the healthy spleen and colon tissue of the rat. Other members of the Reg family, Reg I and Reg III proteins have been shown to act as a growth factors in gastrointestinal tract, but without further experiments we can only assume the potential role of the Reg IV protein in spleen and colon cell growth.

Key words: *spleen, Reg IV protein, rat, colon, Western blot*

Introduction

In 1988, Terazono et al.[1] first isolated a gene expressed in regenerating pancreatic β cells which he named regenerating gene (Reg). Several reg and reg-related genes have been isolated from human, rat and mouse tissue and they constitute a multigene family, the Reg gene family. Based on the primary structure of the proteins encoded by Reg family genes, the members of the Reg family were grouped into three subclasses, type I, II and III[2]. Reg I was originally isolated from a rat regenerating islet-derived complementary DNA (cDNA) library. The sequence of the rat Reg I gene encodes a 165 amino acid protein, with a 21 amino acid signal peptide[1]. Activation of Reg family proteins is induced by proinflammatory citokines such as interleukin-6 and different growth factors such as hepatocite growth factor (HGF) and epidermal growth factor (EGF) which are involved in regeneration process of many organs[3,4]. Reg I protein acts as a growth factor for pancreatic β cells in an autocrine/paracrine manner[2]. Except in the pancreatic tissue, Reg I protein expression has also been found in the gastrointestinal tissues and kidney[5]. Reg II has been found only in mice. The expression of Reg III is stimulated following motor neuron injury and acts as a Schwann cell mitogen during nerve regeneration[6]. Reg III is also expressed in hepatocellular carcinomas and during acute pancreatitis, suggesting a significant role as a growth factor in alimentary tract and pancreatic acinar cells and neuronal cells[7]. Recently identified Reg IV gene and discovered that expression of Reg IV messenger RNA (mRNA) in human tissues was higher in the colon, small intestine, stomach and pancreas[8]. Reg IV is also expressed in colorectal adenocarcinoma[9], pancreatic cancer[10], gastric adenocarcinoma[11], prostate adenocarcinoma[12] and in inflammatory bowel disease[13]. It is unknown whether the Reg IV protein is expressed in various types of rat tissues. There is a single study investigating Reg IV gene expression in injured motor neurons after axotomy of the rat hypoglossal nerve[14]. The aim of this study was to investigate

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whether Reg IV protein is expressed in two structurally and functionally different rat organs. The spleen is parenchymal organ and one of the centers of activity of the reticuloendothelial system which is a part of the immune system. The colon is a part of digestive system responsible for nutrient absorption. Moreover, the purpose of this investigation was to quantify the level of Reg IV expression in these two organs.

Materials and Methods

Rat tissue specimens

Six healthy male Wistar rats weighing 250–300 g were obtained from Institute for Medical Research in Zagreb. The animals were housed in a pathogen-free environment and allowed food and water ad libitum. The animals received human care in compliance with the local ethic committee. The animals were killed by an overdose of diethyl ether anesthesia. Fragment of the spleen and colon samples were excised and immediately frozen in liquid nitrogen and stored at –80 °C until extraction of protein.

Western blot analysis

Tissues were homogenized in lysis buffer with 50 mmol/L of Tris-HCl buffer (pH 8.0) containing 100 μg/mL of the protease inhibitor PMSF, and then a 2 μL protease inhibitor coctail (Sigma-Aldrich Corporation, Missouri, USA) was added. The tissue were centrifuged at 2,000 × g for 30 min at 4 °C, and the supernatant containing total proteins was used in further tests. Fifty micrograms of protein estimated by the Bradford method was submitted to 15% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 5% skim milk in TBS containing 0.05% Tween-20 for 2 hours, and then incubated for 16 hours with 1:200 dilution of goat anti-Reg IV polyclonal antibody (Santa Cruz Biotechnology, Inc., California, USA) at a temperature of 4 °C. After washing four times in TBS, the blots were reacted with 1:2000 dilution of horseradish peroxidase-conjugated donkey antigoat Ig (Santa Cruz Biotechnology, Inc., California, USA) at room temperature for 2 hours. After washing four times in TBS, the signals were visualized using enhanced chemiluminescence reagents (ECL; Amersham, Buckinghamshire, UK). The PVDF membrane exposition started 2 minutes after the detection reagent was added and lasted for 10 minutes. The signal intensity of the protein product was quantified by densitometry (Kodak Image Station 440, LabImage, Halle, Germany).

Immunohistochemistry for Reg IV

The cryostat sections were prepared on glass slides with a 5 μm thickness. For Immunohistochemistry, sections were fixed in cold acetone at 4 °C for 10 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. After incubation in normal blocking serum for 30 min, sections were incubated with primary goat anti-Reg IV polyclonal antibody (Santa Cruz Biotechnology, Inc., California, USA) overnight at 4 °C in a humified environment. After washing in PBS-Tween 20 for 3 × 5 min, the sections were incubated with secondary antibody at room temperature for 30 min (Streptavidin/HRP; DAKO, Denmark) according to the manufacturer's instructions. Antibodies were visualized with 3,3'-diaminobenzidine tetra hydrochloride.

Statistics

Results were expressed as the X ± SEM. Statistical analysis was performed using Student's t-test. Differences were considered significant when p were less than 0.05.

Results

We studied the expression of the Reg IV protein in the rat spleen and colon which are the organs with different structure and function. Western blot analysis with anti-Reg IV antibody detected Reg IV as a 20 kilodalton (kDa) protein in the spleen and colon extracts (Figure 1). The expression of Reg IV protein in spleen tissue was higher than in the colon tissue ($p<0.001$, Figure 2). Results of Western blots were quantified by densitometric analysis and results of this analysis demonstrated a 1.5-fold higher expression of Reg IV in the spleen. Colon showed low expression level in all examined extracts.

In normal colonic mucosa, Reg IV was expressed in a few epithelial cells in the basal portion of Lieberkühn crypts (Figure 3). We found that Reg IV positive cells were mainly present in the lower part of the crypts in colonic mucosa. Expression of Reg IV was not detected in

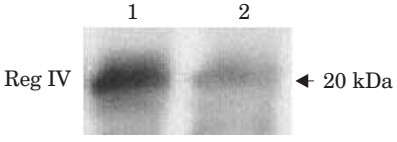

Fig. 1. Western blot with Reg IV antibody showing a band with the 20 kDa expected size, respectively. Lane 1 = spleen; lane 2 = colon.

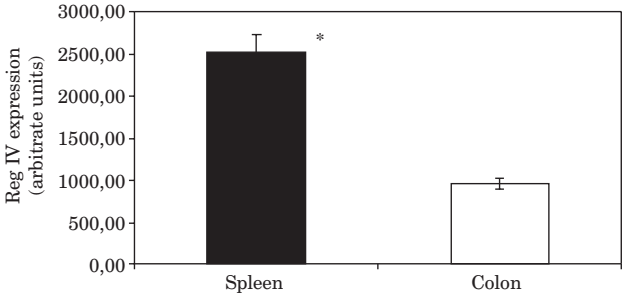

*Fig. 2. Results of Western blots were quantified by densitometric analysis and represented as the X ± SEM, black bar: spleen; white bar: colon. * significance at $p < 0.05$ comparing spleen versus colon values.*

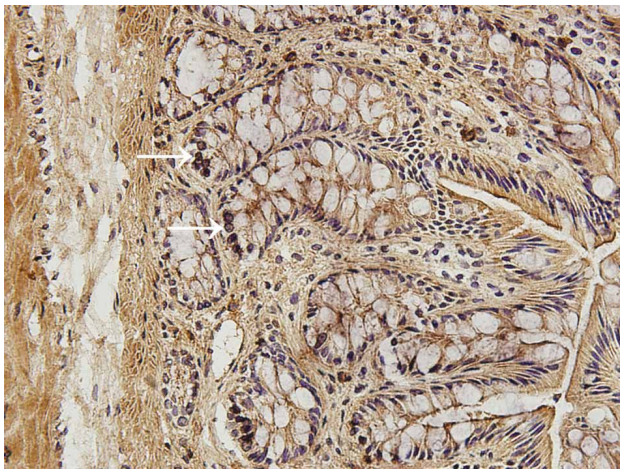

Fig. 3. Result of immunohistochemical staining for Reg IV protein in rat colon. Histological section was stained with anti-Reg IV polyclonal antibody. Reg IV immunoreactive signal was detected in a few epithelial cells in the basal portion of crypts (arrows). Original magnification: ×40.

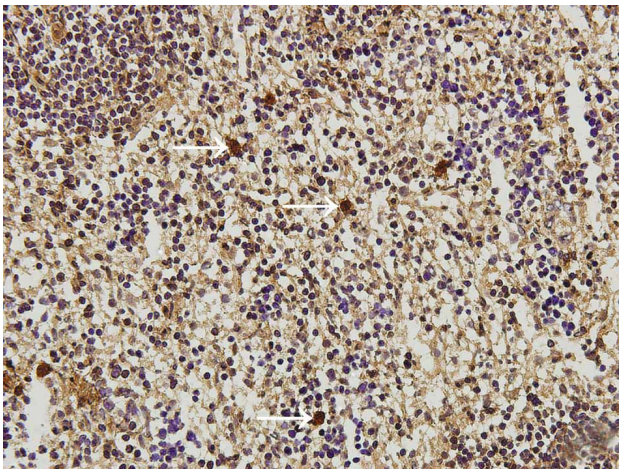

Fig. 4. Result of immunohistochemical staining for Reg IV protein in rat spleen. Histological section was stained with anti-Reg IV polyclonal antibody. Reg IV immunoreactive signal was detected in a large spleen cells in the red pulp (arrows). Original magnification: ×40.

stromal cells (Figure 3). High magnification showed that immunoreactive Reg IV protein was stained in a fine granular pattern in the cytoplasm of epithelial cells (data not shown).

In normal spleen tissue, Reg IV was expressed in a few cells which were scattered in the red pulp. Reg IV immunoreactivity was observed in the certain large spleen cells with a large nucleus (Figure 4). High magnification showed that Reg IV immunoreactivity was detected in the cytoplasm of large spleen cells (data not shown).

Discussion

Our study demonstrated that Reg IV protein was expressed in the spleen and colon tissue of the rat. We showed histologically that in the normal colon Reg IV protein is expressed in a few epithelial cells in the basal portion of Lieberkühn crypts. This finding is compatible with the observation by Nanakin et al.[4] that showed that these epithelial cells showing neuroendocrine features and suggests that Reg IV expression may be associated with proliferative behavior of epithelial cells in the colon mucosa. Interestingly, in the colon epithelium, Reg Iα is expressed in only a few small cells in the basal portion of a colonic crypt where putative stem cells reside[15]. These data strongly suggest that Reg proteins may play a role in the self renewal of colonic mucosa under certain physiological conditions. Recently, using the polymerase chain reaction technique, Namikawa et al.[14] investigated the expression of the Reg family members, including the Reg IV, in response to rat hypoglossal nerve injury and revealed that there is no expression of the Reg IV mRNA in either the normal or injured hypoglossal nerve. Reg IV mRNA has been found to be expressed in the human gastrointestinal tract, including colon, small intestine, stomach and pancreas[6]. Although some normal human tissues express Reg IV, levels of Reg IV expression are much lower in normal than in tumor tissues. Overexpression of Reg IV mRNA was detected in colorectal adenocarcinoma and adenoma[9,4], in inflammatory and metaplastic gastrointestinal mucosa[13,16], in gastric cancer[17], in prostate cancer[12] and pancreatic cancer[10]. Our results showed that the Reg IV protein expression is higher in the rat spleen than in the colon. The weak expression of the Reg IV protein in the colon allows us to speculate that the level of Reg IV is different in various normal rat tissues. The presence of Reg IV protein in culture medium stimulated cell proliferation in dose dependent manner, which indicates that Reg IV could promote cell proliferation in an autocrine/paracrine manner. Bishnupuri et al.[18] have found that Reg IV is a novel mediator which increase activation of the epidermal growth factor receptor (EGFR)/phosphoinositide 3-kinase/Akt signaling pathway. EGFR activation are associated with poor prognosis from a numerous of malignancies and are linked to increased invasiveness of the carcinomas and resistance to apoptotic cell death. The presence of the Reg IV in the rat spleen and colon is the first demonstration of such a high level of Reg IV protein expression in rat normal tissues. It has been demonstrated Reg IV protein expression in the human colon. Immunohistochemical analysis performed on the colon tissue showed only weak or no expression of Reg IV in epithelial cells but strong expression of Reg IV was detected in neuroendocrine cells[11]. Even though, the Reg IV is expressed by cells of the intestinal epithelium which comprises the proliferating cells, Reg IV mRNA is also abundant in the non dividing cells of the intestinal mucosa. Their expression has been associated with proliferation, cell survival and resistance to apoptosis[11]. In the present study, we have shown that Reg IV protein is expressed in a large spleen cells which were scattered in the red pulp. Expression of the Reg IV protein in the rat spleen raises question about its function in that organ.

As it is well known the spleen is acting as a part of the immune system. The white pulp of the spleen is made up mainly of lymphocytes while the red pulp contains mainly red blood cells and macrophages. Partial removal of the spleen tissue is followed by rapid regeneration of the tissue[19]. Faustman's team[20] have found that the spleen might be a source of adult stem cells that could regenerate the pancreatic β-cells. Lonyai et al.[21] showed that adult human spleen contain a reservoir of multilineage adult stem cells that expressed the developmental transcription factor Hox11. Hox11 is a controller of key steps in embryonic development of the spleen and contribute to development of hindbrain, pancreas, salivary glands and other organs and tissues. Although, their role in humans is not known, Hox11 stem cells from the mice spleen which is infused into diseased NOD mice differentiate into the pancreatic β-cells[21]. Because all this facts we can only assume the potential role of the Reg IV protein in spleen cell growth. Also, other members of the Reg family, Reg I and Reg III proteins have been shown to act as growth factors in gastrointestinal tract. Reg IV may be a potential candidate marker of stem cells in the spleen. We would like to emphasize the necessity for further investigations to establish the localization and the biological role of the Reg IV in the spleen cells and also in cells of different rat organs. We can only assume that biological role of Reg IV and other Reg genes are similar. For example, some Reg genes are involved in regeneration process of many organs and are highly expressed in various pathological processes. It would be very interesting to investigate the expression of Reg IV during skeletal muscle regeneration and muscular atrophy[22,23] since we know that skeletal muscle has ability to be repaired upon injury and to induce a proliferation of progenitor satellite cells, the process which is mediated by nitric oxide (NO) release[24].

Conclusion

Our results demonstrate for the first time the Reg IV protein expression in the rat spleen and colon tissue. Expression of Reg IV protein in spleen tissue is significantly higher than in the colon tissue which suggests the different protein level in various organs. We believe that Reg IV has potential role in growth and proliferation of the spleen and colon cells like the other members of the Reg family which acts as a growth factors in the gastrointestinal tract.

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EKSPRESIJA REG IV PROTEINA U NORMALNOM TKIVU ŠTAKORA

S A Ž E T A K

Reg IV gen je dokazan u humanom tkivu kolona, tankog crijeva, želuca i gušterače. Ispoljavanje Reg IV gena u različitim tipovima stanica je povezano sa procesom stanične regeneracije, rastom i preživljavanjem stanica, procesima stanične adhezije te otpornošću na apoptozu. Do sada nije istraženo ispoljavanje Reg IV proteina u normalnim tkivima štakora. Cilj ovog istraživanja je odrediti ispoljavanje Reg IV proteina u tkivu slezene i kolona kod štakora. Upotrebom Western blot analize i imunohistokemijske metode dokazali smo Reg IV protein u ekstraktima tkiva slezene i kolona koristeći poliklonalno protutijelo specifično za Reg IV protein. Rezultati Western blot analize su kvantificirani denzitometrijskom metodom i pokazuju višu razinu ispoljavanja Reg IV proteina u tkivu slezene u odnosu na tkivo kolona. Imunohistokemijska metoda je pokazala ekspresiju Reg IV proteina u nekoliko epitelnih stanica smještenih na dnu kripti kolona te ekspresiju u velikim stanicama slezene koje su raštrkane u crvenoj pulpi slezene. Naši rezultati su po prvi puta pokazali prisutnost Reg IV proteina u normalnoj slezeni i kolonu štakora. Iako Reg IV protein pripada skupini Reg obitelji za koje se zna da djeluju kao faktori rasta u organima gastrointestinalnog trakta, daljnja istraživanja su potrebna da bi se ustanovila biološka uloga Reg IV proteina u stanicama slezene.