Published in final edited form as:

Cancer Epidemiol. 2011 October ; 35(5): 485–489. doi:10.1016/j.canep.2010.10.007.

Plasma Levels of Resistin-like Molecule Beta (RELM β) in Humans

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Abstract

Background—Resistin-like molecules (RELM) are expressed in many tissues and among those, RELM β is most abundantly expressed in the colon. Based on animal studies, RELM β is induced by high fat diets, obesity, and intestinal microflora and may play a role in insulin resistance and intestinal inflammation. In this present study, we evaluated whether RELM β could be measured in human plasma and the influence of selected host and behavioral factors on RELM β levels, including known risk factors for colorectal cancer.

Methods—The subjects for this pilot study were derived from healthy controls who participated in a population-based case-control study of colorectal cancer in Metropolitan Detroit. The subjects were 45–80 years of age without history of cancer or colorectal resection.

Results—RELM β was present in human plasma, with levels in the range of 0.08–0.26 ng/ml. Lower RELM β levels were found in subjects with non-Caucasian race, lower pack-years of smoking, and higher physical activity index scores. Other variables such as dietary intakes, gender, obesity, use of non-steroidal anti-inflammatory agents and history of polyps were not associated with RELM β levels.

Conclusions—The direct association of RELM β with smoking and inverse association with physical activity, both of which are risk factors for colon cancer, indicates that RELM β may be involved in mediating the effects of these two lifestyle factors on risk of colon cancer.

Keywords

Resistin-like molecule beta; Resistin; Colorectal cancer; Smoking; Physical activity

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Conflict of interest

We declare no conflict of interest.

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1. Introduction

Resistin is a small, cysteine-rich protein hormone secreted from adipose tissue and named for its ability to induce insulin resistance. Structurally related “resistin-like molecules” (RELM) (RELM- α , β , γ /FIZZ1-3) exist and are expressed in a tissue-specific manner [1]. Among those, RELM β is most abundantly expressed in proximal and distal colon, and at lower level in cecum and ileum, but not in adipose tissue [1]. It is secreted by goblet cells into the intestinal lumen and detected at high levels in the stool, and can be detected in the serum of experimental animals [2–4]. RELM β expression is induced by intestinal microbial colonization, and RELM β plays a key role in epithelial barrier function and integrity [5–8]. RELM β has been shown to affect insulin signaling and metabolism, and its expression in animals depends on nutritional status, i.e., it is induced by a high fat diet and obesity [2–4,7,9]. Like resistin, RELM β increases insulin resistance via activation of the MAPK/ERK and JNK pathways, which may also enhance cellular proliferation and promotes inflammatory responses [3,4,10].

In animal models of cancer, enhanced RELM β expression was detected in human colon cancer, gastric cancer and premalignant lesions by immunohistochemistry [11–14]. RELM β was virtually absent in normal gastric mucosa, whereas 65% of gastric tumors exhibit RELM β [12]. In tumors, RELM β staining was positively correlated with differentiation, and negatively correlated with infiltration. Long-term survival was better for gastric cancer patients with positive versus negative RELM β staining [12]. In normal human colonic mucosa, RELM β was present at low levels, whereas >80% of colon tumors strongly expressed RELM β [11]. Positive RELM β staining in tumors was again associated with favorable prognostic features. As with gastric tumors, the postoperative survival time of patients was significantly longer for those with positive versus negative RELM β tumors [11].

These clinical studies, together with the molecular effects of RELM β , indicate potential roles of RELM β in gastrointestinal diseases as well as conditions associated with obesity such as insulin resistance. It is not clear, however, if the effects of RELM β are favorable or not, and it may depend on the background diet, microbial colonization of the gut and/or presence of disease states. Unfortunately, the vast majority of the data are from rodents, and to our knowledge there have been no reports as to circulating levels of RELM β in human subjects. Data on RELM β in humans is limited to tissue expression [11,13,15]. It has been proposed that plasma RELM β may be a useful biomarker of colorectal cancer risk [4,10], and plasma RELM β concentrations reflect secretion in the gut in animals [2]. To foster further investigations of RELM β as a potential biomarker, this proof-of-principle study aimed to demonstrate that RELM β could be measured in plasma samples from healthy subjects and to evaluate their relationships to selected known risk factors for colorectal cancer.

2. Materials and methods

2.1. Subjects

The subjects for this pilot study were derived from population controls who participated in a population-based case-control study of colorectal cancer in Metropolitan Detroit and provided a peripheral blood sample through a home phlebotomy service. These subjects were 45–80 years of age without history of cancer or colorectal resection and they signed informed consent to participate. The study was approved by the Institutional Review Board of Wayne State University. Details concerning the eligibility, recruitment and characteristics of the study subject enrolled in the parent study were described elsewhere [16]. Dietary intake was assessed by a validated semi-quantitative food frequency Block 98.2 questionnaire

(FFQ, Block Dietary Data Systems, Berkeley, CA) [17,18]. Other risk factors were obtained from a study-specific structured questionnaire. Physical activity index was calculated as the weighted sum of time the subject spent in the following 5 levels of activities per day; sleeping/lying down (weight 1.0), sedentary (1.1), light (1.5), moderate (2.4) and heavy/strenuous (5.0) as described previously [19]. Non-fasting blood was collected using EDTA as an anticoagulant and samples were refrigerated overnight. Refrigerated samples were centrifuged to separate plasma and plasma aliquots were then stored at -80°C until RELM β assay was performed. Samples for this pilot study were selected from the original pool of 1092 subjects described above, unless the following exclusion criteria were met, 1) plasma samples that have been already used, 2) self-reported history of diabetes or anti-diabetic drug use; or 3) missing important covariates (i.e., dietary intake and body mass index). The resulting 874 subjects were then stratified by obesity (BMI <30 vs. ≥ 30) and gender, and 44 subjects of each stratum were randomly selected (N=176). We did not include colorectal cancer samples because the cases were ascertained through a population based-cancer registry and thus their blood samples were collected after their surgical resection.

2.2 RELM β Assay

RELM β levels in plasma samples were assayed in duplicate in 96-well plates using a Human RELM Beta “Super X” ELISA kit from Antigenix America (Huntington Station, NY, #RHF774CKX2) according to manufacturer’s instructions using four plates assayed on the same day with the same reagents. This assay employs a highly specific polyclonal anti-RELM β capture antibody that is affinity purified against purified recombinant RELM β antigen. For analysis, 100 μL plasma was added to each well, plates were processed according to manufacturer instructions. Absorbance of wells was measured on a multichannel plate reader at 420 nm with correction at 595 nm.

2.3. Statistical Analysis

Four outliers were rejected based on the Grubbs-Smirnov test ($p < 0.05$) after natural log-transformation, and thus the rests of analyses were based on 172 samples. With the sample size of 172 this study had 80% statistical power to detect a correlation coefficient between RELM β and a continuous epidemiologic covariate as low as 0.21 with $\alpha = 0.05$ (two-tailed).

Due to the skewed distribution even after the log transformation, most of the analyses were based on non-parametric tests. Wilcoxon rank sum test was used to assess differences in the distributions of the measurements by gender, age group and obesity status. Spearman correlation analyses were used to test the correlation between the plasma measurements and selected risk factors for colorectal cancer. In addition, odds ratios (OR) and 95% confidence intervals (CI) per quartile increase in plasma measurements associated with the selected risk factors were estimated by ordinal logistic regression models. The assumption of proportional odds in these models was confirmed by score test. These personal characteristics were generally divided at the median, unless otherwise specified, and the lowest level was used as a reference category to calculate the ORs. The analyses were also performed stratifying the subject by obesity status. The interactions between obesity status and these dichotomized personal characteristics were tested by including their multiplicative interaction terms. All statistical analyses were performed using SAS version 9 (SAS Institute Inc., Cary, NC). Statistical significance was defined as $P < 0.05$.

3. Results

Standard curves for the ELISA were obtained using authentic RELM β standard over the range of 0–20 ng/mL. The limit of quantitation for the assay was about 0.1 ng/ml and

variability between replicate readings averaged 10% for the serum samples. Unlike what could have been expected from animal studies, there were no significant differences in plasma RELM β levels by obesity or gender in any age group, nor were there any significant trends with age for all subjects combined (Table 1). We then explored the effects of colon cancer risk factors on RELM β levels in all subjects combined. As shown in Table 2, risk factors with significant ($P<0.05$) correlation to RELM β levels were non-Caucasian race, pack-years of cigarette smoking, and physical activity index. Lower RELM β levels were found in persons of non-Caucasian race. There were 37 African Americans and 7 other minorities (2 Asians, 2 Hispanic and 3 others) in this group, and removing the other minorities weakened the relationship with non-Caucasian race modestly (from $p<0.001$ to $p=0.01$). None of the other factors, including dietary factors, were significantly correlated with RELM β levels.

The three factors significantly correlated with RELM β levels were then examined in more detail for their relationships with RELM β levels. Table 3 shows calculated ORs and 95% CIs for an increase in plasma RELM β level by one quartile according to the selected subject characteristics together with the median values for each group. In this analysis, physical activity was not quite significantly associated with RELM β while race and smoking were still significant. When these analyses were stratified by obesity status, high physical activity tended to decrease the odds ratio for having higher RELM β levels only in non-obese subjects, although the p -value for interaction by obesity status was not significant (OR=0.45 for non-obese subjects and OR=0.94 for obese subjects, $p=0.177$, data not shown). The impact of smoking on increasing RELM β levels was stronger in obese than non-obese subjects, but again the interaction by obesity status was not significant ($p=0.445$).

4. Discussion

This study is the first to report blood levels of RELM β in human subjects. Half of the subjects had RELM β plasma levels ranging from 0.087–0.167 ng/mL. In rodents, circulating RELM β levels have typically been measured using immunoblotting techniques, and are reported as fold-changes from control, with little data on actual concentrations [2], but one RIA kit reports that mean serum level of 0.143 ng/mL [27]. By comparison, typical serum levels of resistin in humans are 5–20 ng/mL [20–26] and the corresponding rodent levels are 5–15 ng/mL [28].

The results of the present study should be interpreted very cautiously due to several limitations. Cross-reactivity of ELISA assays is always a concern. Previous studies with rodent anti-RELM antibodies, however, have shown very little cross-reactivity between RELM α , β , and γ [2]. Many serum samples were also near the limit of quantitation (0.1 ng/mL), but the reproducibility of the assay for the same sample was generally good. Another important potential limitation of this study is the manner in which the blood samples were collected. Samples were collected from non-fasting subjects at their homes during various times of the day. Circadian rhythm has been shown to affect levels of other adipokines, cytokines, and hormones [29–31] and overnight fast is known to affect circulating RELM α levels [32]. Degradation during transport and overnight storage may also be a concern. Null association with obesity in this study is possibly due to several factors common in obese populations, including high prevalence of comorbidities (e.g., metabolic syndrome) and larger measurement errors in energy intake (overreporting) and physical activity (underreporting) by up to 50% [33–36]. Besides, FFQs measures usual dietary patterns, but do not estimate intakes as precisely as food records [18,36]. Recent intakes may differ from usual intakes.

One important factor that could not be explored in the present study is the role of intestinal microflora. RELM β is known to be induced by intestinal bacteria [7,8,37,38]. Broad associations have been made between colonic bacterial ecology and colorectal cancer risk factors such as diet, obesity, smoking, and age [39–42]. However, it is unknown how human intestinal microflora profiles correlate with RELM β expression. This and other uncontrolled factors discussed above should be studied in other or newly collected samples.

Gender and age were not associated with plasma RELM β , but Caucasians had higher plasma RELM β levels than other racial groups, which may indicate a genetic component in RELM β regulation. The findings of the present study in healthy subjects also indicates that, unlike what could be expected from animal studies, obesity and high fat diets may not be major predictors of plasma RELM β levels. The physical activity effect was modest, but increased physical activity was significantly associated with lower RELM β . Physical activity is known to be protective against colon cancer as well as other chronic diseases such as cardiovascular disease and diabetes [43–46]. On the other hand, RELM β levels were strongly positively associated with cigarette smoking in this study. Smoking increases risk of colon cancer modestly and is also a risk factor for formation of aberrant crypt foci in the colon [47–50]. Interestingly, both physical activity and smoking affect bowel movements, and this could modulate intestinal bacterial composition. Although plasma levels may not directly predict tissue levels, the hormonal and cytokine signaling effects of RELM β should be studied further in mediating the effects of smoking on increased colon cancer risk. In conclusion, the present study demonstrates that RELM β can be measured in human plasma samples and further investigation for associations with colon cancer risk is warranted.

Acknowledgments

The authors thank Ms. Danijela Popadic for excellent technical assistance in sample processing management. This research was supported by NIH grant R01-CA93817 and Cancer Center Support grants CA-22453 and CA46592.

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Table 1

Medians and quartile ranges of plasma RELMβ levels (ng/mL) by age, gender and obesity status.

Group		Age groups				Total	P-values ^a
		45–50	50–59	60–69	70–80		
Female	No. of subjects	8	37	22	19	86	
	Median	0.139	0.127	0.115	0.104	0.118	
	Quartile Range	0.124–0.162	0.078–0.157	0.088–0.177	0.075–0.146	0.085–0.159	
Male	No. of subjects	17	35	21	13	86	
	Median	0.116	0.126	0.155	0.095	0.125	
	Quartile Range	0.097–0.194	0.091–0.175	0.088–0.254	0.078–0.152	0.088–0.188	0.333
Non-obese	No. of subjects	13	35	16	21	85	
	Median	0.116	0.118	0.146	0.100	0.115	
	Quartile Range	0.097–.194	0.076–0.163	0.107–0.258	0.073–0.146	0.082–0.163	
Obese ^b	No. of subjects	12	37	27	11	87	
	Median	0.126	0.131	0.124	0.118	0.126	
	Quartile Range	0.114–.156	0.090–.167	0.085–0.218	0.095–0.157	0.088–0.175	0.374
All subjects	No. of subjects	25	72	43	32	172	
	Median	0.125	0.126	0.126	0.104	0.123	
	Quartile Range	0.108–0.188	0.086–0.166	0.088–0.231	0.077–0.150	0.087–0.168	0.254

^aP-values are for differences by gender, obesity and across age groups based on the Wilcoxon rank sum test.

^bObesity was defined as a BMI ≥ 30 kg/m².

Table 2

Percentage of subjects with the indicated characteristic in each RELMβ quartile (Q1–Q4).

Characteristics	RLMβ (ng/mL)				P-values for Spearman correlation
	Q1 ^a <0.087	Q2 0.087–0.122	Q3 0.123–0.167	Q4 >0.168	
Proportions					
No college education	37.21%	26.19%	29.60%	30.23%	0.111
Non-Caucasian ^b	37.21%	30.95%	22.73%	11.63%	<0.001
Current smoker	23.26%	21.43%	18.60%	30.23%	0.433
Daily alcohol use	13.95%	16.67%	23.64%	30.23%	0.126
Regular NSAID use ^c	30.23%	23.81%	31.82%	30.23%	0.987
History of Polyp	13.95%	21.43%	20.45%	27.91%	0.919
Colorectal cancer in family	13.95%	16.67%	6.82%	18.60%	0.624
Medians					
Pack-years of cigarette smoking	3.50	2.60	9.00	20.45	0.006
Body mass index	28.46	28.27	31.07	30.01	0.480
Total energy intake (kcal/day)	1976	2282	2180	2109	0.673
% calories from fat	38.56	39.60	38.52	36.96	0.457
Red meat (servings/week)	8.56	10.29	8.69	9.20	0.721
Dietary fiber (g/day)	17.96	17.14	18.95	17.65	0.249
Total calcium (mg/day)	943	879	911	861	0.719
Height (m)	1.70	1.70	1.69	1.75	0.582
Physical activity index	30.80	30.64	29.49	28.87	0.031

^aN for quartiles Q1–Q4 are 43,42,44 and 43, respectively, except 41, 42, 34, and 42 for pack-years and 43,42,43 and 43 for physical activity.

^bNSAID: non-steroidal anti-inflammatory drugs, regular use ≥ 3 times per week for 6 months or longer.

^cNon-Caucasians (N=44) include 37 African Americans, 2 Hispanic and 2 Asian and 3 others.

Table 3

Odds ratios (OR) and 95% confidence intervals (CI) for an increase in plasma RELM β levels by one quartile according to selected subject characteristics together with median values for each group.

Characteristics	No	OR	(95% CI)	Median (ng/ml)	Quartile Range	P-values
Caucasian	135	1.00		0.135	0.094-0.194	0.001
Non-Caucasian ^a	44	0.41	(0.22-0.77)	0.101	0.078-0.135	
Physical activity < 30 ^b	85	1.00		0.137	0.091-0.189	0.096
Physical activity > 30	86	0.65	(0.38-1.12)	0.111	0.084-0.156	
Smoking <7.5 pack yrs	84	1.00		0.109	0.082-0.156	0.009
Smoking $\geq$ 7.5 pack yrs	84	2.02	(1.16-3.49)	0.141	0.097-0.208	

^aMedian (range) in African American subjects only (n=37) was 0.108 ng/ml (0.078-0.146)

^bPhysical activity index scores from a questionnaire are shown, as described in methods.