Diabetes In Press, published online February 28, 2007

Omentin Plasma Levels and Gene Expression are Decreased in Obesity

Received for publication 26 October 2006 and accepted in revised form 20 February 2007.

Celia M. de Souza Batista, [1,2] Rong-Ze Yang, [1] Mi-Jeong Lee, [1] Nicole M. Glynn, [1] Dao-Zhan Yu, [1] Jessica Pray, [1] Kelechi Ndubuizu, [3] Susheel Patil, [4] Alan Schwartz, [4] Mark Kligman, [5] Susan K. Fried, [1,6] Da-Wei Gong, [1,2] Alan R. Shuldiner, [1,2,6] Toni I. Pollin, [1] John C. McLenithan [1,2,6*]

[1]Division of Endocrinology, Diabetes and Nutrition, Department of Medicine; [2]Department of Physiology, University of Maryland School of Medicine, Baltimore; [3]Department of Biology, University of Maryland Baltimore County; [4]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore; [5]Division of General Surgery, Department of Surgery, University of Maryland School of Medicine, Baltimore; [6]Geriatric Research, Education and Clinical Center (GRECC), Baltimore Veterans Affairs Medical Center, Baltimore, Maryland

Key words:
omentin, obesity, insulin resistance, adipose tissue

* To whom correspondence should be addressed.
John C. McLenithan
660 West Redwood Street – Room 490
Baltimore, MD 21201
E-mail: jmcle001@umaryland.edu

Copyright American Diabetes Association, Inc., 2007

ABSTRACT

Central obesity and the accumulation of visceral fat are risk factors for the development of type 2 diabetes and cardiovascular disease. Omentin is a protein expressed and secreted from visceral but not subcutaneous adipose tissue that increases insulin sensitivity in human adipocytes. To determine the impact of obesity-dependent insulin resistance on the regulation of two omentin isoforms, gene expression and plasma levels were measured in lean, overweight and obese subjects. Omentin 1 was shown to be the major circulating isoform in human plasma. Lean subjects had significantly higher plasma omentin 1 levels than obese and overweight subjects. In addition, higher plasma omentin 1 levels were detected in women compared to men. Plasma omentin 1 levels were inversely correlated with BMI, waist circumference, leptin levels and insulin resistance as measured by HOMA; and positively correlated with adiponectin and HDL levels. Both omentin 1 and omentin 2 gene expression were decreased with obesity and were highly correlated with each other in visceral adipose tissue. In summary, decreased omentin levels are associated with increasing obesity and insulin resistance. Therefore, omentin levels may be predictive of the metabolic consequences or co-morbidities associated with obesity.

Obesity is a chronic pathological condition and a risk factor for type 2 diabetes and cardiovascular disease (CVD) (1-4). Several studies have shown that visceral obesity in particular is strongly associated with insulin resistance, hyperglycemia, dyslipidemia and hypertension. Subcutaneous fat deposition has also been associated with decreased risk of cardiovascular disease in some studies (5-9). In light of the divergent pathological consequences of differences in adipose tissue distribution, it is of great interest to resolve the molecular differences between visceral and subcutaneous adipose tissue depots. Although anatomical location and vascularization are clearly different (10), the molecular basis of differences in metabolism and secretory profile between visceral (omental) and subcutaneous adipose tissues and their impact on whole body physiology are still not totally understood.

Omentin is a newly identified secretory protein that is highly and selectively expressed in visceral adipose tissue relative to subcutaneous adipose tissue (11-13). Omentin has been identified in other tissues at lower expression levels and named intelectin (14), intestinal lactoferrin receptor (15) or endothelial lectin (16). It is expressed in intestinal Paneth cells (17), endothelial cells (16), as well as visceral adipose stromal-vascular cells (11). In vitro studies have shown that omentin increases insulin signal transduction by activating the protein kinase Akt/PKB, and enhancing insulin-stimulated glucose transport in isolated human adipocytes. Thus, omentin may play a paracrine or endocrine role in modulating insulin sensitivity.

A homolog of omentin has been identified that shares 83% amino acid identity with omentin/intelectin (16) and will be referred to as omentin 2. The two omentin genes, omentin 1 and omentin 2, are localized adjacent to each other in the 1q22-q23 chromosomal region (18) that has been previously linked to type 2 diabetes in several populations (19- 23).

Based on the preferential expression of omentin 1 in visceral fat, the presence of omentin in the circulation and its potential role as an insulin sensitizer, we evaluated the association of omentin levels with measures of obesity, insulin resistance and related features. We developed and validated a quantitative western blotting assay that allowed us to measure omentin levels in the plasma. We found that omentin 1 is the major circulating form of omentin and that its plasma levels and adipose tissue gene expression are decreased with obesity. In addition, plasma omentin 1 is positively correlated with plasma adiponectin and HDL and negatively correlated with HOMA, a measure of insulin resistance.

RESEARCH DESIGN AND METHODS

Subjects.

These studies involved healthy volunteers. Subjects that had malignant disease, diabetes, major renal, hepatic and/or thyroid dysfunction were excluded. None were on hormonal replacement therapy. The study protocols were approved by the Institutional Review Boards for Human Subjects Research at University of Maryland and Johns Hopkins University. Informed consent was obtained from all subjects.

Effect of obesity on omentin gene expression.

Human visceral (omental) adipose tissues were obtained from 20 subjects (M/F = 2/18) over a range of age (24 – 73 years) and BMI (21.5 – 66.6 kg/m^2) undergoing intra-abdominal surgery at the University of Maryland Medical Center or The Johns Hopkins Bayview Medical Center. After excision, tissue was immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction and quantitative RT-PCR analysis. In a subgroup of these subjects (n = 14, M/F = 2/12) over a range of age (24 – 50 years) and BMI (38.1 – 66.6 kg/m^2), blood samples were collected after overnight fasting on visit prior to the day of the surgery. Plasma was separated and stored at -80°C for subsequent quantification of omentin levels.

Effect of obesity on human plasma omentin levels.

A subset of 94 healthy subjects (54 women and 40 men) from the previously described Amish Family Diabetes Study (AFDS) (24) were selected as 47 sib pairs that best fit the criterion of age- (within 5 years) and sex-matched BMI discordance (> 3.0 kg/m^2 difference). The BMI-discordant sib pair design was chosen to maximize the power to detect association with BMI. One subject did not fall within the detection limits of the assay and two subjects were considered outliers (> 3 SD from the mean). Consequently, these three subjects were eliminated from all analyses. Therefore, 44 pairs were used in the sib-pairs analysis. For additional analyses, the participants (n = 91, 52 women and 39 men) were divided into three groups: (a) lean (n = 39; BMI < 25 kg/m^2), (b) overweight (n = 30; 25 kg/m^2 ≤ BMI < 30 kg/m^2), and (c) obese (n = 22; BMI ≥ 30.0 kg/m^2).

Identification of omentin 1 and omentin 2 in human plasma by two-dimensional gel electrophoresis.

Human plasma samples, purified recombinant omentin 1 (11) and omentin 2 were separated by two-dimensional gel electrophoresis followed by western blotting with anti-omentin monoclonal antibodies. Omentin 2 cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, California) and transfected into HEK-293A cells using Lipofectamine Plus (Invitrogen, Carlsbad, California). Stably transfected cells were selected with 300 μg/mL G418 (Invitrogen, Carlsbad, California). Omentin 2 expressing cells were cultured in 10% FBS/DMEM until 80% confluent and then switched to serum-free media for 5 days. Conditioned media containing omentin 2 was then harvested and concentrated using Centricon centrifugal concentrators (Millipore, Billerica, Massachusetts). Plasma samples, omentin 1 and/or omentin 2 standards were diluted in rehydration buffer (Bio-Rad, Hercules, California). First-dimension isoelectric focusing was conducted using immobilized pH gradient strips (pH 3-10, 11 cm, Bio-Rad, Hercules, California) in a Bio-Rad IEF Cell. After focusing, strips were equilibrated, subjected to 10% SDS-PAGE. Proteins were then transferred to Immobilon-PVDF membrane (Millipore, Billerica, Massachusetts), blocked with Starting Block Solution (Pierce, Rockford, Illinois) plus 0.1% Tween 20 (Pierce, Rockford, Illinois) and incubated with 3G1B3, a human omentin-specific monoclonal primary antibody (11), followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (KPL, Gaithersburg, Maryland). Immunoreactive spots were visualized by chemiluminescent detection with the Femto-West kit (Pierce, Rockford, Illinois) on a Fluorchem 8000 chemilumenescent/fluorescent imager (Alpha Innotech, San Leandro, California) (Figure 1).

Quantification of human plasma omentin levels.

The quantification of human plasma omentin levels was performed in three steps. Initially, purified omentin was quantitated against a BSA standard curve using 10% SDS-PAGE. After electrophoresis, protein bands were visualized with Sypro Ruby fluorescent protein stain (Bio-Rad, Hercules, California). A standard curve was constructed using the log transformed BSA concentrations versus the band intensities and purified omentin 1 was quantitated by extrapolation from this curve. In the next step, a subset of plasma samples were analyzed by quantitative western blotting (described above) using the previously quantified purified omentin 1 as a standard. A standard curve was constructed using the purified omentin concentrations versus the optical densities and "standard plasma samples" were quantitated by extrapolation from this curve (Figure 2). In the last step, "standard plasma samples" were then used to quantify the rest of the samples by quantitative western blotting. A standard curve was constructed using the "standard plasma" concentrations versus the optical densities and plasma samples were quantitated by extrapolation from this curve. Fluorescent or

immunoreactive band intensities were visualized and quantified on a Fluorchem 8000 chemilumenescent/fluorescent imager (Alpha Innotech, San Leandro, California). Duplicate measurements differing by >10% were rejected and the samples retested.

Quantification of human plasma adiponectin levels.

Total adiponectin levels were determined in fasting plasma samples by a commercial radioimmunoassay kit (Linco Research, Saint Louis, Missouri). The samples were analyzed in duplicate and measured on an automated Packard Cobra-II Auto Gamma counter (Perkin-Elmer, Wellesley, Massachusetts). The duplicate measurements with counts differing by >10% were rejected and the samples retested.

Quantitative Real-Time PCR analysis.

Omentin 1 and omentin 2 mRNA levels were measured by real time quantitative RT-PCR (QRT-PCR) in a LightCycler 480 (Roche Applied Science, Indianapolis, Indiana). Total RNA was extracted from omental adipose tissue samples with Trizol (Invitrogen, Carlsbad, California). Total RNA was reverse transcribed using the Roche Transcriptor cDNA kit. cDNA (50 ng) was quantified using LightCycler 480 Probes Master kit (Roche Applied Science, Indianapolis, Indiana) and Taqman probe/primer sets (omentin 1 - Hs00214137_m1; omentin 2 - Hs00365614_m1; Applied Biosystems, Foster City, California). 18S mRNA (18S RNA - Hs999999_sl) was used as an internal control for normalization. Each sample was run in duplicate. Duplicates exhibiting standard deviations of more than 0.25 CT were repeated. Copy numbers of the specific mRNAs were obtained using standard curves generated with omentin 1 (11) and omentin 2 (pcDNA3) containing plasmids. Normalized gene expression values were obtained using LightCycler Relative Quantification software (LightCycler 480 - Roche Applied Science, Indianapolis, Indiana).

Statistical Analysis.

In order to adjust for family structure, variance components analysis as implemented in SOLAR, which allows the inclusion of a kinship matrix to allow for residual intra-pair correlations between relatives, was used for all analyses involving the association of circulating omentin levels with other obesity and metabolic syndrome-related traits in Amish subjects (25). Prior to analysis, data were tested for normality of distribution by the Shapiro-Wilk test. Consequently, HOMA, leptin, insulin and triglyceride levels were natural log transformed to obtain a normal distribution. Spearman rank correlations were used to estimate relationships between omentin gene expression and other quantitative variables in the non-Amish (unrelated) subjects using SAS 9.1 software package (SAS, Cary, North Carolina).

RESULTS

Human plasma samples contain detectable omentin 1 levels but not omentin 2 by to two-dimensional western blotting.

Since two highly homologous isoforms of omentin/intelectin exist (16,18) and may contribute to total circulating immunologically detectable omentin levels, two-dimensional (2D) western blots were used to determine specificity of the 3G1B3 monoclonal antibody (11) as well as the relative abundance of omentin1/2 in plasma. Two-dimensional (2D) gel electrophoresis of omentin 1 purified from conditioned media (11) followed by western blotting resolved three spots with pI ≅ 5.6 and an apparent molecular weight of 38 kDa (Figure 1A). All immunoreactive species, most likely resulting from post-translational modification, were confirmed to be omentin 1 by MALDI-TOF mass spectrometry analysis (data not shown). A similar pattern was observed when human plasma samples were loaded (Figure 1B). When omentin 2 conditioned media was analyzed alone, only one spot with a slightly lower molecular weight and a pI of 8.2 was observed (data not shown). When omentin 2 conditioned media was added

to human plasma, three spots with pI ≅ 5.6 plus one spot with pI ≅ 8.2 were observed in a similar molecular weight range (Figure 1C). The experimentally determined pIs were identical to the predicted pI values for omentin 1 and 2 based on amino acid sequence (26). These data suggest that the 3G1B antibody recognizes both omentin 1 and 2, and that omentin 1 is present in human plasma, but omentin 2 is either absent or below the limit of detection of the quantitative western blot.

Human plasma omentin 1 levels negatively correlate with obesity and insulin resistance markers.

Metabolic and demographic characteristics of the AFDS subjects adjusted for family structure are presented in Table 1. Plasma omentin 1 levels, determined by quantitative western blotting, were significantly higher in the leaner members of the BMI-discordant sib pairs than in their heavier siblings (paired t-test, n = 44 sib-pairs, p = 0.002). After regrouping subjects into established BMI categories, a variance component analysis, adjusted for age, sex and family structure, was performed to study the differences between lean and overweight, and lean and obese subjects. Plasma omentin 1 levels (± SE, standard error) were shown to be significantly higher in lean (0.37 ± 0.02 μg/mL, n = 39) than in overweight (0.31 ± 0.02 μg/mL, n = 30, p = 0.0009) and obese groups (0.31 ± 0.02 μg/mL, n = 22, p = 0.009). In a sex-stratified analysis in which adiposity level was coded on a linear scale (0 = lean, 1 = overweight and 2 = obese), in both females and males, increasing levels of adiposity were associated with decreased plasma omentin 1 levels (p = 0.006 and p = 0.03, respectively, Figure 3). A sex difference in circulating omentin 1 levels was observed when comparing all females to all males, adjusted for BMI category (0.36 ± 0.03 μg/mL vs. 0.31 ± 0.02 μg/mL, p = 0.03) as well as when comparing lean females with lean males (0.41 ± 0.03 μg/mL vs. 0.33 ± 0.02 μg/mL, p = 0.03, Figure 3). Significant correlations based on variance components analysis adjusted for sex, age and family structure were found between plasma omentin 1 levels and BMI (r = –0.34, p = 0.0007), waist circumference (r = –0.35, p = 0.0005), Ln HOMA Index (r = –0.33, p = 0.002), Ln leptin (r = –0.41, p = <0.0001), Ln fasting insulin (r = –0.31, p = 0.003), adiponectin (r = 0.29, p = 0.005), and HDL levels (r = 0.31, p = 0.003) (Figure 4 and Table 2). The correlation coefficients were calculated by taking the square root of the percent reduction in variance in omentin when adding in the specified predictor variable in a model already containing age and sex. When the variance components analysis was adjusted for BMI or waist circumference as well as sex, age, and family structure, only leptin retained a significant although reduced correlation with omentin 1 levels (r = –0.25, p = 0.02 and r = –0.24, p = 0.02, respectively) (Table 2).

Omentin gene expression correlates negatively with body mass index.

Omentin 1 and omentin 2 mRNA levels (copy numbers) were measured in visceral fat from surgical subjects by real time QRT-PCR using standard curves generated with omentin 1 (11) and omentin 2 (pcDNA3) containing plasmids. Using Spearman regression analysis adjusted for sex and age, significant negative correlations between BMI and omentin 1 (n = 20, r_s = –0.62, p = 0.006) and omentin 2 mRNA levels (n = 20, r_s = –0.73, p = 0.0006) were found (Figure 5A and B). Additionally, when comparing omentin 1 and omentin 2 gene expression, a highly significant positive correlation was observed (n = 20, r_s = 0.76, p = 0.0002, Figure 5C). Although omentin 1 and 2 gene expression are highly correlated in visceral adipose tissue, the mRNA level of omentin 1 is approximately 50 times greater than omentin 2. In light of the small number of male subjects (2 of 20), the same analysis adjusted for age was performed only with females and revealed essentially the same results. Both omentin 1 and 2 gene expressions correlated negatively with BMI (n =18, r_s =