Association between angiopoietin-like protein 2 and lectin-like oxidized low-density lipoprotein receptor-1 ligand containing apolipoprotein B in patients with type 2 diabetes

Tatsuhiko Suzuki: Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Koshigaya, Saitama, Japan

Email address: tthkszk1025@yahoo.co.jp

Kohzo Takebayashi: Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Koshigaya, Saitama, Japan

Email address: takebaya@dokkyomed.ac.jp

Kenji Hara: Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Koshigaya, Saitama, Japan

Email address: haraken@dokkyomed.ac.jp

Takafumi Tsuchiya: Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Koshigaya, Saitama, Japan

Email address: ttenmon@dokkyomed.ac.jp

Toshihiko Inukai: Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Koshigaya, Saitama, Japan

Email address: t-inukai@dokkyomed.ac.jp

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Abstract

Objective: This study was designed to evaluate the association of serum levels of angiopoietin-like protein 2 (ANGPTL2) with circulating inflammatory markers and oxidized and modified low density lipoprotein cholesterol (LDL) evaluated by lectin-like oxidized LDL receptor 1 containing apolipoprotein B (LAB) in type 2 diabetic patients.

Methods: The study included 70 type 2 diabetic patients hospitalized for glycemic control and 9 control subjects.

Results: The serum levels of ANGPTL2 were significantly elevated in the type 2 diabetic patients compared with that in the healthy controls. There was a significant positive correlation between ANGPTL2 and either high sensitivity C reactive protein, fibrinogen, or LAB levels, and a significant negative correlation between ANGPTL2 and estimated glomerular filtration rate (eGFR) levels.

Conclusions: The results suggest that serum ANGPTL2 levels have a close positive association with inflammatory markers, especially fibrinogen and oxidized and modified LDL as evaluated by LAB, and that serum ANGPTL2 levels are influenced by renal function as reflected by the eGFR.

Introduction

Angiopoietin-like proteins (ANGPTLs) are glycosylated proteins with an N-terminal coiled domain and a C-terminal fibrinogen-like domain except for ANGPTL8. These are structurally similar to angiopoietins. Currently 8 members (ANGPTL1-8) of this family have been identified.[1-3]. Although ANGPTLs cannot bind to the angiopoietin receptors, such as Tie2 or to the related protein Tie1[1,2], they can regulate angiogenesis similar to that by the angiopoietins.[2,3] In addition, some of the ANGPTLs, including ANGPTL3, 4, 6, and 8, also contribute to lipid, glucose, and/or energy metabolisms.[1-7]

ANGPTL2 is abundantly expressed in visceral adipose tissues.[2,3,8] Apart from its angiogenic activity, it causes inflammation of adipose-tissue in obesity resulting in insulin resistance in humans.[2,3,8] Furthermore, ANGPTL2 can also cause vascular inflammation,[8] endothelial dysfunction,[9] and increased oxidative stress[10], and it may be associated with tumor metastasis.[11] Although the main sources of circulating ANGPTL2 in humans appear to be adipocytes, endothelial cells, and infiltrated macrophages in visceral fat tissues,[2,3] it can also be secreted by the heart,[1,12] and by both endothelial cells[13] and macrophages[14] in various other tissues. Interestingly, recent reports demonstrated that circulating ANGPTL2 levels are associated with the new onset of type 2 diabetes,[15] and with both cardiovascular events and mortality in type 2 diabetic patients.[16] In type 2 diabetic patients, the association of circulating ANGPTL2 levels with high sensitivity C reactive protein (hsCRP) as a circulating inflammation-marker has been previously reported.[8,17] However, the association between ANGPTL2 levels with other inflammatory markers, such as fibrinogen, has not yet been investigated. The association between ANGPTL2 and fibrinogen levels may be particularly interesting because ANGPTLs, including ANGPTL2, have a fibrinogen-like domain at the C

terminus as described above.[1-3] Furthermore, although it is evident that low density lipoprotein cholesterol (LDL-C) is the most important established marker for cardiovascular risk in type 2 diabetes (18), recently the importance of oxidation of LDL for the progression of atherosclerosis has been recognized[19]. Oxidized LDL, but not LDL, binds to the lectin-like oxidized LDL receptor 1 (LOX-1, an oxidized and modified LDL receptor) in vascular endothelial cells[20] and can play an important role in the formation of an atheroma.[19] LOX-1 ligands containing apolipoprotein B (LAB)[21] are a marker of the atherogenicity of oxidized and modified LDL more specifically than one antigenic determinant of oxidized LDL.[22, 23] Since ANGPTL2 can enhance both inflammation and oxidative stress,[8,10] we hypothesized that there was a potential relationship between ANGPTL2 and LAB.

We investigated the association between circulating ANGPTL2 levels and inflammatory markers, such as hsCRP and fibrinogen, circulating soluble (s) LOX-1, and circulating LAB in type 2 diabetic patients. We also evaluated the association of ANGPTL2 with the various markers related to type 2 diabetes. We hypothesized that circulating ANGPTL2 levels would be positively associated with hsCRP and fibrinogen, and that these would also be positively associated with LAB levels rather than with sLOX-1 levels.

Methods

Patients

This study was registered in UMIN000025767. A total of 70 hospitalized type 2 diabetic patients were prospectively and consecutively enrolled from February 2017 and to August 2017.

The detailed key inclusion and exclusion criteria of the type 2 diabetic patients in the study has been previously described in the UMIN clinical registered system[24]. The characteristics of the 70 patients and 9 healthy subjects on enrollment are shown in Table 1.

Methods

Blood tests on the type 2 diabetic patients were performed at 9:00 a.m. after an overnight fast for at least 10 hr on the day after hospitalization. The control subjects also had the blood tests performed under similar conditions, although they were not hospitalized. Until analysis, samples for analyses of ANGPTL2, LOX-1, and LAB were preserved frozen at -80°C. At the time of sampling, measurements of body weight (BW) and blood pressure were also performed.

Measurement of serum ANGPTL2 levels

Fasting serum ANGPTL2 levels were measured in duplicate using a human ANGPTL2 enzyme-linked immunosorbent (ELISA) assay kit (sandwich ELISA) (Human ANGPTL2 assay Kit-IBL, code no. 27745, Immuno-Biological Laboratories Co., Ltd, Gumma, Japan). The intra-assay and inter-assay CV were 3.9-5.9% and 6.3-10.5%, respectively[25], based on the manufacturer's information.

Measurement of serum sLOX-1 and LAB levels

The measurement of the plasma levels of sLOX-1 was performed using a sandwich chemiluminescence enzyme immunoassay (CLEIA) with two different monoclonal antibodies against the extracellular domain of LOX-1 (B017M and a chicken monoclonal anti-human LOX-1 antibody HUC3-48). The plasma levels of LAB were measured by a sandwich CLEIA using recombinant sLOX-1 and a monoclonal antibody

against the extracellular domain of ApoB (a chicken monoclonal anti-human ApoB antibody HUC20).

Measurement of the intimal medial complex thickness of the carotid artery (IMT)

The IMT was evaluated in the carotid artery on the right side using echography (TOSHIBA aplico i900 and aplicoXV, TOSHIBA, Tokyo, Japan). The maximum point in 3 suitable consecutive points with 1 cm intervals was estimated as the IMT in each patient.

Measurements of fasting plasma glucose, HbA1c, and insulin levels

Fasting plasma glucose (FPG) was measured just after blood collection using an automated glucose oxidase analysis system (Glucose Auto Stat GA1160®; Arkray, Kyoto, Japan). HbA1c was evaluated by an enzymatic assay using protease in the first order reaction and both fructosylpeptide oxidase (FPOX) and peroxidase (POD) in the second order reaction (Nordia®N HbA1c, Sekisui Medical Inc., Tokyo, Japan). Serum insulin levels (immune-reactive insulin) were evaluated by a CLEIA using the lumipulse Presto Insulin Kit® (Fujirebio, Tokyo, Japan).

Homeostasis model assessment-insulin resistance (HOMA-IR.)

HOMA-IR was used as an indicator of insulin resistance and was calculated as follows: HOMA-IR = FPG (mg/dL) × immunoreactive insulin (μU/mL)/405.[26]

Estimated glomerular filtration rate (eGFR)

The estimated glomerular filtration rate (eGFR) was calculated as follows: for men, eGFR (ml/min/1.73 m^2) =194×creatinine (mg/dL)$^{-1.094}$×age (year)$^{-0.287}$, and for women, eGFR (ml/min/1.73 m^2) =194×creatinine (mg/dL)$^{-1.094}$×age (year)$^{-0.287}$ ×0.739.[27]

Measurement of serum hsCRP and plasma fibrinogen

HsCRP was measured by the latex agglutination method using a kit, CRP-LatexX2

Seiken (Denka Seiken Co., Ltd, Tokyo, Japan), and plasma fibrinogen was measured based on the thrombin time method using a fibrinogen kit Coagpia® (Sekisui Medical Co., Ltd, Tokyo, Japan).

Evaluation of diabetic retinopathy

Diabetic retinopathy, including no diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), and proliferative diabetic retinopathy (PDR), was evaluated **based on the Davis' criteria[28)]** by ophthalmologists in our hospital.

Ethical considerations. All subjects gave written informed consent for inclusion in this study, and the Local Ethics Committee at our hospital approved this study. This study was performed according to the guidelines of the Declaration of Helsinki.

Statistical methods. **We expected that a correlation coefficient is greater than 0.35 between ANGPTL2 and other important biomarkers when these correlations are statistically significant. The required sample sizes would respectively be 85 and 62 for mother correlation coefficients of 0.3 and 0.35, with 0.05 of significance levels (two-sides) and 0.8 of power.** The normality of the data in each variable was confirmed by an χ^2 goodness of fit test and/or by a Kolmogorov-Smirnov test. Serum ANGPTL2 levels had a normal distribution. Among the variables included in Table 2, body mass index (BMI), diastolic blood pressure (DBP), LDL-C, fibrinogen, and CAVI-index had normal distributions. Therefore, the correlation of ANGPTL2 with these variables was confirmed using Pearson's correlation. The remaining variables had skewed distributions. Among the variables with a skewed distribution, FPG, HbA1c, TG, HDL-C, Insulin, HOMA-R, hsCRP, eGFR, and LAB had normal distributions after $\log_{10}$-transformation. Therefore, the association of ANGPTL2 with these variables was confirmed using the data after $\log_{10}$-transformation by Pearson's correlation. All of the

remaining variables did not have a normal distribution even after log_{10}-transformation. Thus, the correlation between ANGPTL2 and these variables was confirmed using Spearman's correlation as a non-parametric coefficient. In multiple regression analysis, we used 2 types of model groups, i.e., an inflammatory-related group and a metabolic-related group. Except for ANGPTL2, BMI, LDL-C, and fibrinogen, these variables were log_{10}-transformed. Comparisons between type 2 diabetic patients and healthy subjects for BMI were performed using an unpaired *t* test. Comparisons between these groups for age, FPG, HbA1c, and eGFR were performed by the Mann-Whitney U test because of the skewed distribution of these variables. Comparisons for ANGPTL2 between the two groups were performed using an unpaired *t*-test. For comparison of ANGPTL2 among the 3 groups, the homogeneity was confirmed using by the Bartlett test. A parametric comparison was done using one-way analysis of variance (ANOVA) after the confirmation of equal variance. After the confirmation of the significance by the ANOVA, a *post hoc* Holm test was conducted. All statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd, Tokyo, Japan). A *P* value of less than 0.05 was considered as statistical significance (two-sided).

Results

The serum ANGPTL2 levels were significantly elevated in type 2 diabetic patients (n =70) compared with that in the healthy controls (n =9) [3.47 ± 1.10 (range with 1.65-6.91) vs. 2.51± 0.42 ng/mL (range with 1.99-3.04)] (P <0.0001) (Fig. 1A).

There were no gender-related differences. ANGPTL2 levels for men and for women were respectively 3.42 ± 1.07 and 3.57 ± 1.13 ng/mL (P =0.6050). When the patients

were classified into 2 groups with (n =20) and without statins (n =50), the serum ANGPTL2 levels were respectively 3.16 ± 0.67 vs. 3.60 ± 1.21 ng/mL with a trend toward lower values in the ANGPTL2 levels in the group treated with statins vs. the group not treated with statins (P =0.0551). There were no significant differences in the ANGPTL2 levels in patients with (n =35) and without insulin therapy (n =35) (3.45 ± 1.08 vs. 3.50± 1.12 ng/mL, P =0.8697). In addition, no difference was noted between patients having (n =29) and not having (n =41) a current smoking habit (P =0.8398). Furthermore, when patients were divided into 3 groups based on the degree of diabetic retinopathy [NDR (n =52), SDR (n =5), PDR (n =13)], the serum ANGPTL2 levels were 3.45 ± 1.14, 3.61 ± 1.22, and 3.51 ± 0.93 ng/mL, respectively. There were no significant differences in the ANGPTL2 levels among these groups [NDR vs. SDR (P =1.0000), NDR vs. PDR (P =1.0000), and SDR vs. PDR (P =1.0000)].

The data obtained from the correlation of the ANGPTL2 levels with multiple variables and the data obtained from the multiple regression analysis for the ANGPTL2 levels as the dependent variable are summarized in Tables 2 and 3. A significant positive correlation between ANGPTL2 and hsCRP, fibrinogen, and LAB levels (P =0.0457, P =0.0001, and P =0.0147) and a significant negative correlation between ANGPTL2 levels and the eGFR (P =0.0063) were found. For patients not on insulin therapy whose serum insulin levels were measured (n =30), there was a significant positive correlation between ANGPTL2 and HOMA-IR levels (P =0.0419). In multiple regression analysis, ANGPTL2 had a significant positive association with fibrinogen in all models (models 2-4), including fibrinogen in the inflammatory-related model group, while ANGPTL2 did not have a significant association with hsCRP, excluding model 1 in this model group. ANGPTL2 also had a significant positive association with LAB in the models,

including LAB (models 3 and 4) in this model group. In the metabolic-related model, ANGPTL2 did not have significant associations with HbA1c, BMI, TG, and LDL-C, excluding 1 model with HbA1c (Model 1). ANGPTL2 showed either a significant negative association or a trend toward a negative association with eGFR in all models in both the inflammatory-related and metabolic-related models. **We also investigated the correlation between ANGPTL2 and either fibrinogen or LAB in subgroups classified based on the duration of diabetes [group 1: 0-5 years (n =33), group 2: more than 5 to 10 years (n =16), group 3: more than 10-to 40 years (n =21)]. The number of patients treated with insulin was 12 (36%), 8 (50%), and 15 (71%), respectively in these groups. There was a significant correlation between ANGPTL2 and fibrinogen in group 1 (R =0.4493, P =0.0087) and in group 3 (R =0.6709, P =0.0017), but not in group 2 (R =0.3568, P =0.1749). ANGPTL2 was correlated with LAB only in group 3 (R =0.4507, P =0.0403), while no significant correlation was observed in group 1 (R =0.3347, P =0.0569) and in group 2 (R =0.1158, P =0.6695). There was a significant positive correlation between the duration of diabetes and the urinary albumin excretion (UAE)(R =0.4952, P <0.0001) and the CAVI index (R =0.3513, P =0.0038).**

Discussion

In this study, serum ANGPTL2 levels were positively associated with hsCRP as an inflammatory marker, and these data are in agreement with previous studies.[8,17] These results appear plausible given the fact that ANGPTL2 can cause inflammation in both adipose tissues and systemic vascular endothelial cells.[2,3,8] However, this association was

lost in a multiple regression analysis except for 1 model in this study, which suggests that the association may be **independent**. On the other hand, ANGPTL2 was also positively correlated with fibrinogen, and the association was confirmed by multiple regression analysis. Therefore, the association between ANGPTL2 and fibrinogen is more **dependent** than that shown between ANGPTL2 and hsCRP. Although fibrinogen is an important factor associated with the coagulation system, it along with hsCRP are also representative inflammatory markers.[29,30] We also previously reported the close positive correlation between these markers.[31] Furthermore, a recent report suggests that baseline and long-term fibrinogen levels are associated with the risk of sudden cardiovascular death.[32] Interestingly, it is reported that fibrinogen acts as a ligand for integrins (adhesion factors), including α5β1, which are abundantly expressed in monocytes, macrophages, and endothelial cells. Furthermore, this interaction promotes leukocyte adhesion and the secretion of cytokines resulting in inflammation. [29,30] On the other hand, it is likely that ANGPTL2 causes inflammation by activating the Ras-related C3 botulinus toxin substrate 1 (Rac1)/nuclear factor kappa-B (NFκB) pathway via α5β1 intergrin in vascular endothelial cells.[8] This appears to be initiated by the binding of the C terminal fibrinogen-like domain of ANGPTL2 to α5β1 intergrin. In contrast, ANGPTL2 is highly expressed in both vascular endothelial cells and infiltrated macrophages in atherosclerotic vessels

in humans, which may contribute to the elevation of ANGPTL2 in the circulation.[9] Taken together, we speculate that circulating fibrinogen as well as circulating ANGPTL2 itself can also promote ANGPTL2 expression in vascular endothelial cells by a similar mechanism, resulting in the elevation of circulating ANGPTL2. It is possible that systemic vascular inflammation induced by ANGPTL2 contributes to the up-regulation of fibrinogen expression in the liver probably by cytokines produced in inflammatory cells, because it is reported that recombinant ANGPTL2 can promote the expression of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin 6 (IL6).[14] These mechanisms may partially explain the potentially close association between ANGPTL2 and fibrinogen found in this study. Another possible reason for this close association between ANGPTL2 and fibrinogen levels may be based on the issue of potential cross-reactivity of the antibodies in the ANGPTL2 kit used in this study with fibrinogen because ANGPTL2 has a C-terminal fibrinogen-like domain. However, both antibodies used in the sandwich ELISA have epitopes located in the coiled domain. On the other hand, the fibrinogen-like domain does not possess epitopes for these antibodies, and therefore these antibodies do not react with the fibrinogen-like domain in the ANGPTL family. Thus, it is unlikely that the antibodies used in this kit can cross-react with fibrinogen.

In this study, we found for the first time a significant positive association between serum ANGPLTL2 levels and serum LAB levels which reflects oxidized and modified LDL.[21] The association was also confirmed by multiple regression analysis. Although it is evident that LDL-C is an important risk factor for cardiovascular events in type 2 diabetic patients,[18] the role of oxidized and modified LDL for the formation of atheroma was recently confirmed.[19] Oxidized and modified LDL, but not LDL, can bind to LOX-1 in vascular endothelial cells[20] and cause local inflammation and a consequent atheroma.[19] In fact, circulating LAB was positively associated with the CAVI-index (a marker of arterial stiffness) in men.[33] Furthermore, it is reported that circulating LAB, but not sLOX-1, was associated with the risk of cardiovascular disease and ischemic stroke.[34] The reason for the potential positive association between ANGPTL2 and LAB confirmed in this study is not fully apparent. However, the LAB levels are associated with lipid sedimentation,[35] inflammation,[36] and foam cell formation.[37] Therefore, we speculate that LAB by this mechanism may be able to promote the expression of ANGPTL2 although this has not been directly demonstrated to date. Furthermore, because ANGPTL2 can induce oxidative stress,[10] circulating ANGPTL2 or that expressed in endothelial cells appears to be partially involved in the oxidization of LDL. Based on these hypotheses for the mechanisms involved, it might be important to evaluate whether the possible association between ANGPTL2 and oxidized and modified LDL as reflected by the LAB levels can be a risk factor for cardiovascular disease in future studies with a larger number of patients.

Interestingly, the correlation between ANGPTL2 and either fibrinogen or LAB was stronger in the subgroup with the longest duration of diabetes, compared with those in shorter duration of diabetes subgroups. As the progression of diabetes,

diabetic patients may have different complications and may use different medications which probably affect the levels of biomarkers. In fact, diabetic duration was positively correlated with the UAE and the CAVI-index, which generally respectively reflects diabetic nephropathy, and the degrees of arteriosclerosis, and the number of patients on insulin therapy was the highest in the subgroup with the longest duration of diabetes. This may have partially influenced the results.

In this study, there was the negative correlation between ANGPTL2 levels and the eGFR. In addition, the trend toward a positive correlation between ANGPTL2 and the UAE was also noted. These findings, which support the results in a previous study,[38] suggest the close association between ANGPTL2 and the progression of diabetic nephropathy. One of the possible reasons for these results is likely due to the possible decrease of clearance in the kidney of ANGPTL2. However, the expression of ANGPTL2 in glomeruli in diabetic patients is up-regulated.[39] In addition, ANGPTL2 may promote fibrosis in the kidney due to the increased TGFβ via α5β1 intergrin/extracellular signal-regulated kinase.[40] Therefore, the association between ANGPTL2 and renal dysfunction observed in this study may be partially independent of the clearance of ANGPTL2 in the kidney.

In the current study, the **ANGPTL2 levels had a significant positive association with HbA1c in a multiple regression model with eGFR and HbA1c as independent variables. However, because the ANGPTL2 levels did not have a significant association with the HbA1c levels in both the correlation analysis and other models of multiple regression analyses, it does not appear that the ANGPLT2 levels were strongly influenced by the HbA1c levels.** The result was basically in agreement with

previous reports[16,38] although one study showed a significant positive association between ANGPTL2 and HbA1c levels.[17] On the other hand, ANGPTL2 levels positively correlated with HOMA-IR, which reflects insulin resistance, in patients who did not receive insulin therapy. The result agrees with those in previous reports,[8,17] and it appears to be a reasonable finding because ANGPTL2 is produced mainly in visceral adipose tissues and can cause inflammation, resulting in systemic increased insulin resistance.[8]

In the current study, ANGPTL2 levels in type 2 diabetic patients were significantly higher compared with those in non-diabetic control subjects in agreement with a previous report.[8] It is difficult to explain the detailed reason for this result because ANGPTL2 did not **basically** have a significant association with glycemic control, BMI, and age. In addition, there were no differences between BMI, age, and eGFR between these groups. Plausible explanations might include the potential difference in insulin resistance and low-grade inflammation between these groups. However, we did not measure serum insulin levels and inflammatory markers in the healthy subjects.

There are some limitations of this study. This study was a cross-sectional observation. The number of diabetic patients was relatively small. **This may have been especially important in the correlation between ANGPTL2 and either eGFR or LAB because the correlation coefficients were relatively small although these were statistically significant. Furthermore, the number of control subjects was very small compared with that of the diabetic patients, and this prevented us from investigating the correlation between ANGPTL2 and various markers in control subjects, resulting in the difficulty of comparing the difference in these correlations between diabetic patients and control subjects.** In addition, the patients

took multiple different drugs for diabetes, which may have potentially influenced the circulating ANGPTL2 levels. **Finally, cardiovascular events were not studied in this study so it could not be concluded that ANGPTL2 is a risk factor.**

In conclusion, ANGPTL2 levels in type 2 diabetic patients were significantly higher compared with those in non-diabetic control subjects. ANGPTL2 had a significant positive association with inflammatory markers evaluated by hsCRP, fibrinogen, and LAB, which reflects oxidized and modified LDL. There was a significant negative association between the serum ANGPTL2 levels and eGFR. On the other hand, ANGPTL2 were not **basically** influenced by glycemic control based on HbA1c or FPG, and by BMI. It is important that the potentially close association between ANGPTL2 and either LAB or fibrinogen be evaluated in a more detailed analysis for its clinical significance.

Interest of conflict: Authors have nothing to declare

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Figure Legends

Fig. 1:

Serum levels of ANGPTL2 in non-diabetic healthy subjects (n =9) and in type 2 diabetic patients (n =70)

Fig. 2

The correlation between ANGPTL2 and either hsCRP (A), fibrinogen (B), LAB (C), and eGFR (D) in patients with type 2 diabetes

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