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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 14 (9): 552-557, 2011
ISSN 1028-8880 / DOI: 10.3923/pjbs.2011.552.557
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Interpretation of Correlations Between Coagulation Factors FV, FVIII and vWF in Normal and Type 2 Diabetes Mellitus Patients

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Abstract: Increased risk of vascular diseases in hypercoagulable states is assumed to be the major cause for prevalence of vascular problems in type 2 diabetes mellitus patient. The present study was undertaken to explore the basis underlying the correlation between the aforesaid three coagulation factors with reference to the relevant mechanistic findings using statistical calculation. Our findings showed that the correlation between each pairs of coagulation factors, FV, FVIII and vWF could be easily interpreted using previously characterized interactions between these factors. Also, given the sequence similarities between FV and FVIII as well as the results of this study, it was indicated, for the first time, that FV may bind to vWF in the same way as FVIII binds. This finding seems to be of great importance to be evaluated through experimental techniques.

Key words: Type 2 diabetes mellitus, coagulation factor, statistical correlation, vascular problems, hypercoagulable states

INTRODUCTION

The human Factor V (FV) is a glycoprotein synthesized in megakaryocytes and hepatocytes as an inactive single-chain protein with 2196 amino acids (Asselta and Peyvandi, 2009; Van den Biggelaar *et al*., 2009a). There are three distinct types of domains in FV structure. The sub-domains of each type have sequence similarities. Type A domain includes three sub-domains, A1, A2 and A3, type C domain includes two sub-domains, C1 and C2 and a type B domain is without sub-domains (Suzuki *et al*., 2006; Hajifathali *et al*., 2005). The orders of these domains in FV sequence are as follow, NH2-A1-A2-B-A3-C1-C2-COOH (Suzuki *et al*., 2006). Like other glycoproteins during biosynthesis, FV is introduced to the lumen of Endoplasmic Reticulum (ER) and subsequently transported to Golgi apparatus for further translational modifications such as formation of disulfide bonds. Mature FV is secreted in blood stream as an inactive factor (Van den Biggelaar *et al*., 2009b). However, the activation of FV to FVa is taking place during blood coagulation process. FV is transported in plasma as a soluble protein which needs no carrier for transportation. The half life of the soluble FV is about 14 h (Ma *et al*., 2007). In case of activation the B domain of FV is deleted and the remaining sequence forms a heavy chain with A1 and A2 domains and a light chain with A3, C1 and C2 domains. These two chains are bound to each other by disulphide bonds in such a way to make active form of FVa with a heterodimeric structure (Suzuki *et al*., 2006).

The human factor VIII (FVIII), of glycoprotein structure, is most likely synthesized by hepatocytes and reticulocytes (Van den Biggelaar *et al*., 2009a). Factor VIII in its activated form, FVIII a, acts as a cofactor in prothrombinase and tenase complexes formation in the coagulation system. Newly synthesized FVIII is a single chain protein with 2332 residues. There is a close similarity between FVIII and FV structures. FVIII has the same domains as FV with similar order of NH2-A1-A2-B-A3-C1-C2-COOH. Moreover, there are intra domain similarities between Fv and FVIII; A and C domains of FVIII and FV show about 40% of amino acid homology (Asselta and Peyvandi, 2009; Van den Biggelaar *et al*., 2009b; Pipe, 2009; Fuentes-Prior *et al*., 2002). The C2 domain of both factors indicates phospholipid-binding capacity to membranes (Suzuki *et al*., 2006). Although, both factors contain one copper per protein molecule, the

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B domain of each factor has its unique properties with little similarity to each other. The B domain provides a cleaving site for proteolytic enzymes during activation process of these factors (Van den Biggelaar *et al*., 2009b; Fang *et al*., 2007). Unlike FV, the process of FVIII cleavage at the B domain and conversion to an active form of heterodimeric structure takes place prior to its secretion into the blood stream (Fang *et al*., 2007). Upon secretion, FVIII binds immediately and strongly to the VonWillebrand Factor (vWF) by non-covalent interactions. The binding of FVIII to vWF increases its short half life and prevents its degradation by proteolysis. Therefore, FVIII and vWF circulate in plasma as a non-covalent complex at a concentration of 0.01 mg dL^{-1} (about 10-20 fold lower than that of FV) (Van den Biggelaar *et al*., 2009b; Fuentes-Prior *et al*., 2002; Ni Ainle *et al*., 2009; Duffy *et al*., 2004). FVIII binds to vWF through its C2 domain while C1 domain acts as an enhancer for C2 domain to bind more strongly (Fang *et al*., 2007).

The vWF is a multimeric glycoprotein synthesized in megakaryocytes and vascular endothelial cells (Van den Biggelaar *et al*., 2009a, b; Duffy *et al*., 2004) and stored in Weible-Palade Bodies (WPBs) and secreted in case of stimulation (Rondaij *et al*., 2008). The vWF binds FVIII via its 272 residues of N-terminal, acting as a carrier for FVIII in plasma (Bowen *et al*., 2001). Co-expression of FVIII and vWF in endothelial cells results in their co-storage in WPBs and co-secretion to plasma as a complex (Van den Biggelaar *et al*., 2009a, b; Duffy *et al*., 2004).

LMAN1 (lectin mannose-binding protein 1) is a homo hexameric trans-membrane protein cycle between ER and Golgi complex, responsible for transportation of glycoprotein between ER and Golgi apparatus. There are miscellaneous reports which show correlations between concentrations of coagulation factors in different conditions. Since both FVIII and FV are transported by LMAN1 from ER to Golgi, any condition leading to deficiency in LMAN1 receptor biosynthesis can cause combined deficiency of FVIII and FV (Ni Ainle *et al*., 2009; Wigren *et al*., 2010).

In normal individuals the correlation between coagulation factors levels could be interpreted based on our knowledge of normal hemostasis. The main scope of the present work was to study the correlations between the three coagulation factors namely FV, FVIII and vWF in diabetics in contrast to normal control in order to elucidate the basis underlay this pattern.

MATERIALS AND METHODS

Participants: In the present study, 40 healthy subjects and 40 type 2 diabetic patients were chosen based on their medical history obtained from Grand Hospital of Dezfoul, Khuzestan Province, Iran. All diabetic patients with the age's range of 40 to 60 years were diagnosed from 2006 to 2011. Type 2 diabetic patients and healthy subjects were excluded in case they had history of: peripheral vascular disease, cardiovascular disease, coagulation disorders, neuropathy, retinopathy, nephropathy, insulin therapy, psychiatric illness, surgery, smoking and any manifest acute or chronic diseases. Only medication used by diabetics was anti-diabetic metformin pill. All participants gave written informed consent and all subjects were informed about the study and signed informed this forms. The Ethical Committee of our hospital approved the protocol of this study.

Sample preparation: Fasting blood samples of type 2 diabetes patients and healthy subjects were drawn in the morning after at least 8 h of fasting. The biochemical and hematological factors were analyzed in laboratory of Grand Hospital of Dezfoul (Dezfoul, Khuzestan Province, Iran). In the same day, specific activity of coagulation factors were evaluated in laboratory of Shafa Cancer Hospital of Ahwaz (Ahwaz, Khuzestan Province, Iran). The 2×9 mL of whole blood were collected into two plastic syringes (Monovette, Sarstedt, Nümbrecht, Germany) each containing 0.2 mL 0.106 M tri sodium citrate. The plasma was prepared using centrifuging of twice at 1500x g for 10 min each at 15-18°C and then was stored in polypropylene tubes at -70°C until measurement. The plasma sample with evidence of hemolysis was discarded. All samples were assayed in duplicated method.

Analytical method: Body Mass Index (BMI) was calculated as weight (kg) divided by height squared (m^2). Weight and height of all subjects were measured in light clothing and without shoes. Blood pressure was evaluated with a calibrated Omron M7 sphygmomanometer (HEM-780-E). The mean value of five measurements, made at intervals of 10 mins was used for analysis. The Fasting Plasma Glucose (FPG) of all participants was measured by the enzymatic colorimetric (GOD PAP) method. (Pars Azmun Co Kits, Karaj, Alborz Province, Iran) Duplicated glucose level of ≥ 6.1 mmol L^{-1} or ≥ 110 (mg dL^{-1}) was used as a criteria for diabetes mellitus. Also Total Cholesterol (TC), TG, Low Density Lipoprotein Cholesterol (LDL-C) and High Density Lipoprotein Cholesterol (HDL-C) were evaluated by colorimetric enzymatic technique (ParsAzmun Co Kits, Karaj, Alborz Province, Iran). Creatinine was measured using Jaffe reaction method (ParsAzmun Co Kits, Karaj, Alborz Province, Iran). Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) were assessed using Instrumentation Laboratory (IL) ACL 8000 coagulation analyzer (Beckman Coulter; Fullerton,

California) using PT-Fibrinogen HS PLUS kit and HemosIL APTT-SP Kit, respectively. The specific activity of coagulation co-factors of V, VIII were assessed in citrated plasma using HemosIL Factor V, VIII Deficient plasmas, respectively using IL ACL 8000 coagulation analyzer (Beckman Coulter). HemosIL von Willebrand Factor Antigen was used for determination of specific activity of vWF Antigen in human citrated plasma using ACL 8000 coagulation analyzer (Beckman Coulter). All parameters of PLT, MPW and PDW using SYSMAX KX-N21 Hematology Analyzer were measured. All experimental procedures involving human participants were conducted with due attention to the guidelines approved by the Research Ethical Committee of Chamran University.

Statistical analysis: The study results were analyzed statistically using the Statistical Package for the Social Science (SPSS-PC, version 15. SPSS, Inc., Chicago, IL.). All test statistics are asymptotically normally distributed. Independent two sample tests were used for determination of statistical differences in biochemical, demographical and hematological parameters between type 2 diabetic patients and healthy subjects. The differences between parameters were considered significant at $p<0.05$. The correlation between coagulation factors were analyzed with Pearson's test and significance level of 0.05. Missing data proportion was maximally 7.5% in all parameters which not affect the statistic results.

RESULTS AND DISCUSSION

The demographic and biochemical parameters for type 2 diabetic and control groups are shown in Table 1. As it is evident diabetic conditions affect whole the body metabolism and changed some parameters in regression direction. Significant increase in body index (28.65±0.90, in contrast to 25.42±0.37) and in systolic blood pressure (132.50±2.02 in contrast to 117.00±1.53) in addition to hyperglycemia and hypertriglyceridemia (166.46±13.65 in contrast to 106.66±15.44) gives bad prognosis for persistence of diabetic conditions and ultimately results in prevalence of long term complications. Table 2 and 3 show the Pearson test results for coagulation factors FV, FVII and vWF in normal and diabetic groups. Table 2 show that in normal individuals these factors are significantly inter-correlates with each other while this kind of correlation is completely missed in diabetic individuals (Table 3). The p-value for the correlation between FVIII and FV is 0.201, between FV and vWF is 0.064 and between FVIII and vWF is 0.387 showing no significant correlation between them. Coagulation factors FV, FVIII and vWF are very important factors playing critical roles in coagulation hemostasis (intrinsic or extrinsic pathways). The concentrations of these factors are influenced by diabetic conditions (Hameed *et al*., 2002; Camire and Bos, 2009; Behnam-Rassouli *et al*., 2010). Both decrement or increment in coagulation factors may leads to severe abnormalities of bleeding or thrombosis formation, respectively (Ma *et al*., 2007; Eikenboom *et al*., 2002; Asselta *et al*., 2006; Rezaeian *et al*., 2006). Table 2 and 3 summarize the Pearson tests results obtained for 40 type 2 diabetes mellitus patients and 40 normal controls.

As we previously reported (Mard-Soltani *et al*., 2011) the pair wise study of correlations between each two coagulation factors in diabetic patients in contrast to normal individuals is a helpful approach to interpret by the hemostasis complications concomitant with diabetic condition.

From statistical point of view the correlation values of two variables is a powerful technique used to study whether and how strongly these factors are interrelated. Based on normal distribution of our data, we used Pearson correlation test to study the correlation between coagulation factors. The obtained results are shown in Table 2 and 3.

In normal individuals (Table 2) vWF is significantly ($p = 0.003$) and positively correlates ($r = 0.712$) with FVIII, while it show significant ($p = 0.006$) but negatively ($r = 0.624$) correlation with FV. However FV and FVIII in turn show negative correlation ($r = 0.613$) with p-value of

Table 1: Demographic, biochemical and hematological characteristics of type 2 diabetic patients and healthy subjects

Parameters	Type 2 diabetics (n = 40)	Control (n = 40)	Significance
Gender (% male)	50	52	ns
Mean age (years)	54.11±1.6	51.67±2.12	ns
BMI (kg m^{-2})	28.65±0.90	25.42±0.37	p<0.01
Systolic blood pressure (mmHg)	132.50±2.02	117.00±1.53	p<0.001
FBG (mg dL^{-1})	166.46±13.65	106.66±15.44	p<0.01
TG (mg dL^{-1})	227.76±17.80	147.46±24.31	p<0.05
Total cholesterol (mg dL^{-1})	201.33±6.65	187.53±7.52	ns
HDL (mg dL^{-1})	59.96±1.91	58.40±4.31	ns
LDL (mg dL^{-1})	95.80±4.49	93.03±8.17	ns
Serum creatinine (μ mol L^{-1})	1.00±0.07	0.95±0.09	ns
PLT (10^{+3} μL^{-1})	261.76±10.56	255.06±18.30	ns
PDW (fl)	12.58±0.28	12.46±0.69	ns
MPV (fl)	9.78±0.16	9.49±0.30	ns

Table 2: Pearson correlation test for coagulation factors in normal groups

Factor	FVIII	vWF
FV		
Correlation	-0.613	-0.624
Significance	0.007	0.006
No.	40	40
FVIII		
Correlation	-	0.712
Significance	-	0.003
No.	-	40

Table 3: Pearson correlation test for coagulation factors in type 2 diabetes mellitus groups

Factor	FVIII	vWF
FV		
Correlation	0.206	0.303
Significance	0.201	0.064
No.	40	40
FVIII		
Correlation	-	0.144
Significance	-	0.387
No.	-	40

0.007. In type 2 diabetes mellitus (Table 3) the correlation between these three coagulation factors is omitted and their concentrations are changing in unrelated manner. There are very important questions to be answered before being able to interpret these correlations; the first question: what is the mechanistic basis for the statistical correlations between these three coagulation factors? Alternatively where is the correlation site in which the changes in concentrations of these factors are tight to each other? the second question: in type 2 diabetes mellitus what causes abolishment of correlations between these factors? We may reasonably hypothesize that the correlations between coagulation factors are attributed minimally to four loci or levels of interaction. The first level for correlation between two coagulation factors may be at the source of the protein biosynthesis. If the two proteins are synthesized by the same cell or tissue, their actual concentrations in the plasma will correlate to each other. The same pattern of biosynthesis by a given source causes the same changes in their concentrations. However, this kind of correlation is expected to be positive and the correlated parameters should change in the same direction. The second potential locus for correlation between two coagulation factors may be at transportation level by the same carrier in the plasma or between intracellular compartments (e.g., between ER and Golgi). The transport of these coagulation factors from ER to Golgi is carried out by LMAN1, through a non competitive mechanism. This transportation based on its mechanism similar to previous confirmative findings cause positive correlation between these cofactors (Eikenboom *et al*., 2002; Zheng *et al*., 2010). The third locus for positive or negative correlations between coagulation factors may be sought at biosynthesis level as both factors have the same gene enhancer or gene repressor or alternatively have an effectors with opposite effects. Finally, we postulate that the fourth locus for the correlation may occur at the presence of a third counterpart effector which correlates more strongly with either of the given two coagulation factors. In this case these two factors correlate indirectly to each other (lower concentration) via this counterpart effector. As mentioned earlier, in case of FV, FVIII and vWF, FV is synthesized by megakaryocytes and hepatocytes. This factor has a common source with FVIII (hepatocytes) while having a different source (megakaryocyte instead of reticulocytes). FV is also has a common source with vWF (megakaryocytes) while having a different source (hepatocytes instead of reticulocytes). By the same token, FVIII has a common source with vWF (reticulocytes) keeping a different source (hepatocytes instead of megakaryocytes). Therefore, the correlations between these three coagulation factors may not be attributed to their common sources. Since vWF binds FVIII in the plasma (Eikenboom *et al*., 2002) it would be acceptable to assume that in the normal group, the binding of FVIII to vWF be a suitable locus for the correlation between the two factors concentrations. As shown in Table 2, the correlation between FVIII and vWF is positive ($r = 0.712$) and significant ($p = 0.0032$). This correlation means that increase in the concentration of vWF increases the concentration of FVIII and vice versa. In diabetic patient this correlation was not significant ($p = 0.387$) and therefore their concentration changes could not be related to each other. There are reports showing that increased secretion of FVIII and vWF in diabetic patients leads to thrombotic abnormalities. In type 2 diabetes mellitus, the accelerated FVIII and vWF secretion by miscellaneous cells, with different rates of secretion, causes coagulation factors concentrations to exceed the levels determined by the correlation values of FVIII and vWF. Table 2 show that in normal group, FVIII and FV are correlated meaningfully ($p = 0.007$) with negative correlation ($r = 0.613$). This correlation means that the concentrations of FVIII and FV are linked to each other with a reverse relation. We hypothesize that a competitive binding of FVIII and FV to a certain carrier is a good explanation for this correlation. In blood coagulation cascade, both FVIII and FV bind to thrombin, though essentially to separate binding sites. Hence, the given correlation could not be exerted by this mechanism. As mentioned above, LMAN1 is the carrier for glycoprotein transport between ER and Golgi or their storage reservoirs. Therefore, intracellular binding of FVIII and FV to LMAN1 could be behind the statistical correlation between them. This claim may be fortified by that fact that many reports showed the cease

in LMAN1 biosynthesis leads to combined bleeding disorders due to FVIII/FV deficiency. The correlation between FVIII and FV in diabetic patient as depicted in Table 3 is disturbed, probably because of inconsistent increase in FVIII and FV concentrations in diabetic conditions (Table 3). Table 2 shows a significant ($p = 0.006$) correlation ($r = 0.624$) between vWF and FV. This means that the change in FV concentration has negative correlation with vWF concentration. There are three possible mechanisms for this specific correlation. The first mechanism is their co-biosynthesis in megakaryocytes, their intracellular co-transport by LMAN1 and their co-storage in WPBs which may follow a competitive behavior in certain stages. The second mechanism is the possible binding of FV to vWF. Since the C2 domain of FVIII which is responsible for FVIII binding to vWF show 50% sequence similarity with C2 domain of FV and the remaining amino acids are similar in their physicochemical properties. Therefore, it's not surprising to expect that FV like FVIII binds to vWF by its C2 domain. On the other hand, the correlation value of $r = 0.62$ for vWF and FVIII is resemble that of vWF and FV ($r = 0.61$). This indicates that the pattern of correlation of FV and FVIII is similar to the correlation pattern of FV and vWF. If this mechanism is considered to be correct, then the remaining activity of free fraction of FV (the measured fraction of FV in this study) should be decreased when vWF concentration is increased. Our results confirm this negative correlation between FV and vWF in normal individuals (Table 2). The third mechanism for the negative correlation between FV and vWF is the involvement of an intermediary factor (probably FVIII). This intermediate shows strong correlation with both of FV and vWF. In other word, FV and vWF correlate to each other via FVIII as an intermediary factor. In order to study the suspected effect of FVIII and vWF, we used regression method to achieve this goal. The only constrain was that FV and FVIII themselves correlate to each other, hence the problem of collinearity in using regression methods. Therefore, to solve this problem, we oblige to use stepwise mode of regression. Stepwise regression of vWF as dependent and FV and FVIII as independent variables show that FV has more significant effect ($p<0.001$) on vWF than FVIII. This means that at the presence of FV the effect of FVIII in prediction of vWF is negligible.

CONCLUSION

Based on our findings we can conclude that type 2 diabetes mellitus conditions increases coagulation factors concentrations (FV, FVIII and vWF) in a non uniform pattern and causes their concentrations to be unrelated. The uncontrolled unrelated increase in coagulation factors lead to hypercoagulable state with increased risk of vascular (micro and macro) problems. Our data also show that in normal individuals, there are negative correlations between FV with both FVIII and vWF which may be a negative control to prevent hypercoagulable state induction by these factors. Therefore, FV could be considered as a safety factor preventing increase in FVIII and vWF.

ACKNOWLEDGMENT

The financial support of Chamran University of Ahwaz and Payame Noor University, Tehran, Iran, are acknowledged.

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