Published in final edited form as:

J Perinat Med. 2008 ; 36(5): 377–387. doi:10.1515/JPM.2008.082.

PREMATURE LABOR: A STATE OF PLATELET ACTIVATION?

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Abstract

Objective—This study was undertaken to determine whether premature labor is associated with changes in the maternal plasma concentration of soluble ligand (sCD40L), a marker of platelet activation.

Methods—A cross-sectional study included patients in the following groups: 1) non-pregnant (n=21); 2) normal pregnancy (n=71); 3) normal pregnancy at term with (n=67) and without labor (n=88); 4) preterm labor (PTL) with intact membranes (n=136) that was divided into the following sub-groups: 4a) PTL who delivered at term (n=49); 4b) PTL without intra-amniotic infection and/or inflammation (IAI) who delivered preterm (n=54); and 4c) PTL with IAI who delivered preterm (n=33). sCD40L concentrations were measured by ELISA.

Results—The median maternal plasma sCD40L concentration was higher in pregnant than non-pregnant women (p=0.017). Patients with PTL had a higher median maternal plasma sCD40L concentration than women with normal pregnancies, regardless of the presence or absence of IAI and gestational age at delivery (p<0.001 for all comparisons). IAI was not associated with a higher median maternal plasma concentrations of sCD40L.

Conclusions—Normal pregnancy is a state in which there is a physiologic increase of sCD40L. PTL was associated with an increased median maternal plasma sCD40L concentration that could not be accounted for by IAI. Thus, our findings suggest that platelet activation occurs during an episode of preterm labor.

Keywords

labor; inflammation; prematurity; thrombin; coagulation; platelet; sCD40L

INTRODUCTION

Platelet activation and degranulation are key steps in the formation of a primary hemostatic plug and clot generation following blood vessel laceration[12, 15, 90, 92, 98, 122]. In addition to their classic hemostatic role, activated platelets participate in the process of acute and chronic inflammation[28, 94, 101, 103, 139], in which platelet degranulation as well as the direct contact of activated platelets with circulating monocytes, can generate an inflammatory response[28, 94, 101, 139].

CD40 ligand (CD40L), a member of the tumor necrosis factor superfamily[57], was originally identified on activated CD4⁺ T cells and has been associated with T cell regulation of B cell function[6, 45, 57, 79, 132]. In addition, CD40L is associated with platelet pro-inflammatory activity,[124] activation of the vascular endothelium[19, 52, 80, 115, 130, 135], as well as cyclooxygenase-2 (COX-2) expression and prostaglandin synthesis[97, 109].

Upon activation, platelets express CD40L on their membrane (from which it is subsequently cleaved by CD40), and the soluble form of CD40L (sCD40L) can be measured in the plasma[52, 53]. Activated platelets are the source of more than 90% of sCD40L in the plasma[52], and this cytokine has been proposed as a measurable marker of platelet activation[85, 107]. Circulating sCD40L has a role in platelet activation and arterial thrombi stabilization[4, 110]. High concentrations of sCD40L have been reported in chronic inflammatory diseases associated with platelet activation, such as cystic fibrosis[37], inflammatory bowel disease[24–26, 76, 89, 106], systemic sclerosis[73], and systemic lupus erythematosus [22, 44, 70, 131]. Moreover, elevated sCD40L plasma concentrations are associated with a higher risk for cardiovascular disease in asymptomatic patients[120] and for coronary artery restenosis after balloon angioplasty[23].

Platelet count and mean platelet volume do not change significantly with pregnancy[51, 129]. However, especially during the third trimester, a significant decrease in platelet count has been proposed to result from an increased consumption of platelets in the utero-placental unit[51]. Moreover, the inverse relationship between the mean platelet volume and the platelet count observed during normal pregnancy led to the proposal that pregnancy is a state of compensated thrombocytolysis[129]. There are inconsistent reports concerning the degree of platelet activation and the release of vasoactive products during normal pregnancy[8, 51, 60, 113, 114]. Women who develop obstetric complications, such as preeclampsia and fetal growth restriction, have a higher degree of platelet activation than both normal pregnant and non-pregnant women[1, 9, 11, 13, 38, 48, 50, 59, 62, 64, 65, 74, 75, 82, 86, 88, 96, 99, 102, 108, 116, 128, 137]; however, little information is available regarding the association between maternal platelet activation and preterm labor (PTL)[117].

Preterm labor is associated with an increased thrombin generation that is reflected by elevated maternal plasma concentrations of thrombin-antithrombin (TAT) complexes [18, 32]. Thrombin activates platelets[61, 67], thus, the increased thrombin generation[18, 32], along with the moderate maternal systemic inflammation observed in patients with PTL[43], may lead to subsequent platelet activation and an increased secretion of CD40L, which can further enhance this process.

The aims of this study were: 1) to determine maternal platelet activation through the marker sCD40L in patients with PTL; and 2) to investigate whether platelet activation is associated with the presence of intra-amniotic infection and/or inflammation (IAI) or a history of vaginal bleeding during pregnancy.

MATERIAL AND METHODS

Study groups and inclusion criteria

This cross-sectional study included patients in the following groups: 1) non-pregnant women (n=21); 2) normal pregnancy (n=71); 3) women with a normal pregnancy at term with (n=67) and without labor (n=88); 4) PTL (n=136) that were divided into the following sub-groups: 4a) women with PTL who delivered at term (n=49); 4b) patients with PTL without IAI who delivered preterm (n=54); and 4c) women with PTL and IAI who delivered preterm (n=33). Patients with multiple pregnancies or fetuses with congenital and/or chromosomal anomalies were excluded.

Samples and data were retrieved from our bank of biological samples and clinical databases. Many of these samples have been employed to study the biology of inflammation, hemostasis, angiogenesis regulation, and growth factor concentrations in non-pregnant women, normal pregnant women, and those with pregnancy complications. All women provided a written informed consent prior to the collection of maternal blood. The Institutional Review Boards of both Wayne State University and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD/NIH/DHHS) approved the collection and utilization of samples for research purposes.

Clinical definitions

Preterm labor was diagnosed by the presence of at least two regular uterine contractions every 10 minutes associated with cervical changes that required admission to the hospital before 37 weeks of gestation. Amniotic fluid collection was performed by trans-abdominal amniocentesis under ultrasonographic guidance in order to determine the microbiologic state of the amniotic cavity. In women with PTL amniotic fluid was transported to the laboratory in a capped plastic sterile syringe and cultured for aerobic and anaerobic bacteria, as well as for genital mycoplasmas. White blood cell (WBC) count, glucose concentration, and Gram stain for microorganisms were performed in amniotic fluid shortly after collection. Intra-amniotic infection was defined by the presence of positive amniotic fluid cultures for microorganisms and intra-amniotic inflammation by an amniotic fluid WBC count ≥ 100 cells/ml. The results of the amniotic fluid analyses were used for clinical management. A small for gestational age (SGA) neonate was defined as birthweight below the 10th percentile[3]. Placental histologic findings were classified according to a diagnostic schema proposed by Redline et al[112].

Blood samples collection

All blood samples were collected with a VacutainerR into 0.109M trisodium citrate anticoagulant solution (BD; San Jose, CA, USA). Samples were centrifuged at 1300g for 10 minutes at 4°C and stored at -70°C until assay.

Human sCD40L immunoassays

Maternal plasma sCD40L concentrations were determined by sensitive and specific immunoassays obtained from R&D Systems (Minneapolis, MN, USA). The assay was conducted according to the manufacturer's recommendations. The sensitivity of the sCD40L assay was 4.2 pg/ml. The intra-assay coefficient of variation is 5%, while the inter-assay coefficient of variation is 6.2%.

Statistical analysis

The Shapiro-Wilk and the Kolmogorov-Smirnov tests were used to test if the data was normally distributed. Soluble sCD40L plasma concentrations were not normally distributed;

therefore, Kruskal-Wallis and Mann-Whitney U tests were employed for comparisons of continuous variables, and Chi-square test was used to compare categorical variables. Spearman correlation was used to detect an association between the concentrations of sCD40L and gestational age at sample collection in women with a normal pregnancy. A p-value <0.05 was considered statistically significant. Analysis was performed with SPSS package, version 12 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics of women with normal pregnancies and PTL sub-groups are presented in Table 1. The rate of SGA in patients with PTL who delivered at term was significantly higher than in patients with PTL without IAI and in women with a normal pregnancy.

Changes in maternal plasma sCD40L concentrations during normal pregnancy and labor at term

Women with a normal pregnancy had a significantly higher median maternal plasma sCD40L concentration than non-pregnant women (normal pregnancy: median 369.5 pg/ml range 63.5–1848.7 vs. non-pregnant median 270.4 pg/ml range 94.2–568, $p=0.017$) (Figure 1). There was no correlation between maternal plasma sCD40L concentrations and gestational age at sample collection ($r=0.18$, $p=0.13$) (Figure 2).

Patients with normal pregnancy in labor at term had a significantly higher median maternal plasma sCD40L concentration than those at term not in labor (term in labor: median 384 pg/ml range 104–3784 vs. term no labor 338.5 pg/ml range 32.9–4125, $p=0.04$) (Figure 3).

Changes in the maternal plasma sCD40L concentration in patients with preterm labor

The median maternal plasma sCD40L concentrations were significantly different in patients in all PTL sub-groups (Kruskal Wallis, $p<0.001$) than in women with normal pregnancies (PTL without IAI: median 781.5 pg/ml range 107.1–3206.4 vs. normal pregnancy: median 369.5 pg/ml range 63.5–1848.7, $p<0.001$; PTL with IAI: median 988.2 pg/ml range 123.2–4422 vs. normal pregnancy: median 369.5 pg/ml range 63.5–1848.7, $p<0.001$; and PTL who delivered at term: median 686.7 pg/ml range 109.6–3102 vs. normal pregnancy: median 369.5 pg/ml range 63.5–1848.7, $p<0.001$) (Figure 4).

Amniocentesis was performed in 83.8% (114/136) of the patients presenting with PTL, of which 14% (16/114) had a positive amniotic fluid culture. All patients without amniocentesis were women with an episode of PTL who delivered at term. Among patients in the PTL sub-groups, there was no significant difference in the median maternal plasma concentration of sCD40L between those with (median 988.2 pg/mL range 123.2–4422) and without (median 781.5 pg/mL range 107.1–3206.4) IAI. Moreover, no significant differences in the median plasma concentration of sCD40L were detected between patients with PTL who delivered preterm (with or without IAI) and those with PTL who delivered at term (median 686.7 pg/mL range 109.6–3102) (Kruskal Wallis, $p=0.3$). There was no correlation between the admission-to-delivery interval and maternal plasma sCD40L concentrations ($r=-0.04$, $p=0.7$).

Data concerning episodes of vaginal bleeding during pregnancy was available in 88.2% (120/136) of the patients with PTL. Vaginal bleeding was not associated with significant differences in the median maternal plasma sCD40L concentration in the different PTL sub-groups (Kruskal-Wallis, $p=0.7$).

Placental histologic findings of chorioamnionitis, funisitis, maternal underperfusion, and fetal vascular thrombo-occlusion were not associated with significant changes in the median maternal plasma concentration of sCD40L in the different PTL subgroups (data not shown).

DISCUSSION

Principal findings of the study

1) The median maternal plasma sCD40L concentration is higher in pregnant than in non-pregnant women; 2) patients with preterm labor, regardless of the presence of IAI or gestational age at delivery, have a significantly higher median maternal plasma concentration of sCD40L than women with a normal pregnancies; 3) at term, the median maternal plasma sCD40L concentration is higher in women in labor than those not in labor.

The interaction between CD40-CD40 Ligand system, humoral immunity and inflammatory cells

CD40L (also known as CD154[27, 77], TRAP[7], and gp39[93]) is a 33kD transmembrane protein[6, 45, 79] that is expressed on platelets upon their activation[52, 53] as well as on CD4⁺ T cells[6, 45, 57, 79, 119, 132]. The structure of CD40L is homologous to that of tumor necrosis factor (TNF)- α [57]. Together, with its receptor CD40 (a 48kD transmembrane protein that is structurally homologous to the TNF receptor)[10, 57], CD40L exerts pro-inflammatory activity[6, 10, 19, 45, 52, 57, 79, 80, 115, 124, 130, 132, 135].

The CD40-CD40L system was first identified on activated T cells, where it plays an important role in B cell activation and isotype switching from IgM to IgG[6, 45, 57, 79, 132]. A mutation in the CD40L gene on the X chromosome was reported in X-linked immunodeficiency with hyper-IgM syndrome[5, 29, 36, 87, 91]. This syndrome is characterized by recurrent infections, increased susceptibility to neoplasms, and a high mortality rate[83]. Further support for the role of CD40L in T cell-dependent humoral immunity is derived from the results of the CD40L knockout mice model. These mice display a selective deficiency in humoral immunity with lower basal serum immunoglobulin isotype concentrations and undetectable IgE [81]. Moreover, these mice fail to mount a secondary antigen-specific response to immunization with a thymus-specific antigen, suggesting that CD40L is required for T cell-dependent antibody response[21, 105, 121, 136].

In addition to its expression on T cells, CD40 is expressed by monocytes macrophages and dendritic cells[14, 16, 47, 69, 133], and its interaction with CD40L leads to the synthesis of pro-inflammatory cytokines, such as interleukin (IL)-1[134], IL-6[111, 127], and TNF- α [134]. Furthermore, CD40L expression has been reported on eosinophils, mast cells, and basophils; the latter two cell types induce IgE production by B cells through the activation of the CD40 receptor by CD40L[39–42]. Collectively, these reports support the important role of CD40-CD40L system in humoral immunity, inflammation, and allergic reactions.

The role of the CD40-CD40L system in platelet activation and their pro-inflammatory effect

Platelets store CD40L and express it on their membranes upon activation[52, 54]. Shortly after its expression, CD40L is cleaved by CD40 and released into the plasma[53]. Platelets are the source of more than 90% of soluble CD40L[52], which is regarded as a marker for platelet activation[54, 85, 107].

The CD40L that is expressed upon the platelet membrane participates in many processes mediated by platelets, including the following: 1) pro-inflammatory response in vascular endothelial cells through their CD40 receptor leads to an increased endothelial cell

expression of adhesion molecules (E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1)[52]; as well as secretion of cytokines, such as monocyte chemotactic protein (MCP)-1, IL-18 and IL-6, through nuclear factor kappa B (NFκB) activation[52]; and 2) soluble CD40L propagates a procoagulant phenotype by inducing tissue factor expression and down-regulating thrombomodulin expression by human umbilical vein endothelial cells (HUVEC)[123] and weak induction of tissue factor expression on whole blood monocytes[84].

Soluble CD40L enhances platelet activation by increasing: 1) P-selectin expression[20, 63] and platelet degranulation[63]; 2) reactive oxygen and nitrogen species generation; [20] and 3) platelet aggregation and platelet-leukocyte conjugation[20]. CD40L appears to be an $\alpha_{IIb}\beta_3$ ligand that is necessary for the stability of arterial thrombi[4]. Indeed, CD40L^{-/-} mice had a lower platelet density in their thrombi, which was associated with a delayed vessel occlusion, frequent thrombi rupture, and embolization that were not observed in the wild type mice[4]. Moreover, the administration of recombinant sCD40L normalized clot formation in the CD40L^{-/-} mice [4]. Thus, the major source of plasma sCD40L is activated platelets. Soluble CD40L is a key player in the pro-inflammatory effect of activated platelets. Moreover, this chemokine sustains platelet activation and participates in their pro-thrombotic activity.

The changes in sCD40L during normal pregnancy and its possible role in parturition

The findings that median maternal plasma sCD40L concentration higher in pregnant than in non-pregnant women, as well as those in labor at term compared to those not in labor are novel. Pregnancy[58, 60] and labor[126] have previously been associated with platelet activation, and our results are in accord with these reports. However, a previous study[104] reported that non-pregnant women have a higher mean serum sCD40L concentration than women with a normal pregnancy. Differences in sample size, study population, and method of sCD40L measurement may explain the discrepancy between the studies.

The report that CD40 is expressed in the human reproductive tract[71] further supports the concept that platelets may play an active role in parturition. Indeed, the perivascular expression of CD40 receptors was reported in the endometrium, myometrium, and uterine cervix[71]. The latter also had strong CD40 expression in the basal but not in the surface epithelium[71]. The low basal CD40 expression reported in fibroblasts of the myometrium, endometrium, and uterine cervix increased after treatment with interferon γ (IFN γ)[71]. The treatment of these fibroblasts with CD40L and IFN γ induced a several-fold increase in their production of IL-6, IL-8 and MCP-1. This effect, however, was not observed with CD40L treatment alone[71].

Activated platelets induce COX-2 gene expression in endothelial cells through CD40L activity[97]. In addition, incubation of HUVEC cells with Jurkat D1.1 CD40L⁺ cells increased their COX-2 expression and prostaglandin E₂ (PGE₂) and PGI₂ secretion[31]; the latter was also increased by the exposure of HUVEC to recombinant soluble CD40L. Moreover, this study demonstrated that the CD40-CD40L-mediated induction of IL-6 in HUVEC is COX-2 dependent[31]. A similar effect on COX-2 activity and higher PGE₂ production by the CD40-CD40L system was also reported in human lung fibroblasts[140].

The induction of COX-2 expression in the reproductive tract by the CD40-CD40L system can be a mechanism by which activated platelets participate in term and preterm parturition. Indeed, cyclooxygenase is the rate-limiting step for prostaglandins and thromboxane A₂ production[100], and COX-2, an isoenzyme of cyclooxygenase, has been implicated in the mechanism of parturition through its effect on the synthesis of prostaglandins especially PGE₂[17, 30, 35, 46, 49, 55, 66, 72, 118, 125]. Thus, the induction of COX-2 expression by

the CD40-CD40L system in the non-pregnant uterus, cervix, and endometrium may be of importance during pregnancy, and labor since COX-2 expression may lead to the increased synthesis of prostaglandins and pro-inflammatory cytokines by fibroblasts in the reproductive tract, which in turn promotes uterine contractility and cervical ripening.

Platelet activation and pregnancy complications

There is a solid body of evidence supporting platelet activation in patients with preeclampsia and fetal growth restriction[1, 9, 11, 13, 38, 48, 50, 59, 62, 64, 65, 74, 75, 82, 86, 88, 96, 99, 102, 108, 116, 128, 137]. Indeed, elevated maternal plasma and platelet expression of markers for platelet activation, including P-selectin (CD62p)[48, 68, 74, 75, 86, 128, 137, 138], Annexin V[34, 50, 128], and sCD40L concentrations[2, 78, 95, 104], were reported in patients with these pregnancy complications. However, maternal platelet activation in women with preterm labor has not been studied extensively. It has been reported that patients with PTL had higher amniotic fluid concentrations of platelet activating factor than patients without labor at term[56], and that this chemokine was associated with lipopolysaccharide-induced PTL and delivery in mice[33]. In a study of platelet activation in the maternal circulation, patients with PTL had a higher mean platelet factor 4 and beta-thromboglobulin plasma concentrations than patients with term labor[117].

Our finding of a higher median maternal plasma sCD40L concentration in patients with preterm labor is novel and suggestive of increased maternal platelet activation in patients with PTL regardless of the presence of IAI or previous episodes of vaginal bleeding. This may result from the following underlying mechanisms: 1) increased thrombin generation in patients with PTL[18] may lead to platelet activation and, subsequently, to the shedding of sCD40L into the plasma. Of note, similar to our findings, the concentrations of TAT complexes were not significantly different in the presence of IAI or vaginal bleeding[18]. 2) In addition, the increased maternal leukocyte activation among patients with PTL, was not associated with the presence of IAI [43]. This suggests that maternal systemic inflammation may activate T cells which could bind platelets through the CD40 receptor and activate them, leading to a further enhancement of the inflammatory process and an increased release of CD40L from the activated platelets into the maternal plasma. The association between CD40L, increased COX-2 expression[31, 97], and prostaglandin generation[31] suggests that CD40L may participate in the process of labor. However, the lack of correlation between the admission-to-delivery interval and the maternal plasma concentrations of sCD40L, as well as the fact that patients with PTL and IAI had a significantly shorter admission to delivery interval than the other PTL sub-groups, suggest that this may not be the case. Nevertheless, sCD40L may have local activity in the endometrium, myometrium, and uterine cervix[71] that is not reflected in the maternal circulation.

Conclusions

Preterm labor is associated with an elevated maternal platelet activation that can be induced by increased thrombin generation or maternal leukocyte activation. Our findings suggest that platelet activation occurs in preterm labor and, therefore, there is a potential role for anti-platelet agents (dietary or pharmacologic) may play a role in the treatment or prevention of this condition.

Acknowledgments

This research was supported in part by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS.

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Figure 1.
Comparison of maternal plasma sCD40L concentrations between non-pregnant and pregnant women

Figure 2.
The correlation between gestational age at sample collection and maternal plasma sCD40L concentrations

Figure 3.
The comparison of the median maternal plasma sCD40L concentrations between patients with and without labor at term

Figure 4.

Comparison of median maternal plasma sCD40L concentration among patients with preterm labor who delivered preterm (with/without intraamniotic infection/inflammation) or at term and patients who delivered at term

Table 1

Demographic and clinical characteristics of the study population

	Normal pregnancy (n=71)	PTL without IAI (n=54)	PTL with IAI (n=33)	PTL delivered at term (n=49)
Maternal age (years)	24.0 (21.0,27.0)	22.0 (19.0,29.0)	23.0 (19.0,27.0)	20.0 (18.0,24.0) *
Gravidity [€]				
1	14(20.3)	8 (14.8)	12 (36.4)	11 (22.4)
2–5	45(65.2)	39 (72.2)	17 (51.5)	32 (65.3)
≥6	10(14.5)	7 (13)	4 (12.1)	6 (12.2)
Parity [§]				
1	39 (55.7)	30 (55.6)	24 (72.7)	31(63.3)
2–5	30(42.9)	21(38.8)	6 (18.2)	18(36.7)
≥6	1 (1.4)	3(5.6)	2 (6.1)	0
Ethnic origin [£]				
African-Americans	53 (77.9)	42 (79.2)	27(81.8)	42 (87.5)
Caucasian	11(16.1)	8 (15.1)	5 (15.2)	3 (6.2)
Hispanic	2 (3)	3 (5.7)	0	1 (2.1)
Asian	2(3)	0	1 (3)	2 (4.2)
Gestational age at blood collection (weeks)	31.6 (27.4,35.0)	29.5 * (25.1,32.2)	26.1 * (24.6,31.6)	31.4 (29.3,32.5)
Gestational age at delivery (weeks)	39.6 (38.4,40.4)	31.6 * (26.2,34.7)	27.9 * (25,33.5)	38.2 * (37.3,38.9)
Neonatal birthweight (g)	3330 (3050,3700)	1690 * (880,2335)	1040 * (642.5,1755)	2948 * (2710,3255)
Cesarean delivery *	22 (33.8)	20 (37.7)	6(18.2)	0 *
SGA	0	5 (9.4) *	5 (15.1) *	12 (24.5) *

Data are presented as median (25–75 interquartile range) or numbers (%)

* p<0.05 in comparison to normal pregnancy

[€] Normal pregnancy (n=69)[§] Normal pregnancy (n=70); PTL with IAI (n=32)[£] Normal pregnancy (n=68); PTL without IAI (n=53); PTL delivered at term (n=48)

PTL- preterm labor

IAI- intraamniotic infection/inflammation

SGA – small for gestational age