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Developmental Changes in Soluble CD40 Ligand

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Abstract

Objectives—To determine if soluble CD40 ligand (sCD40L; formally CD154) levels vary with age and to identify age-dependent ranges in healthy pediatric and adult populations.

Study design—sCD40L was measured in 25 neonates, 74 children (3 months –15 years) and 20 adults using an enzyme-linked immunosorbent assay. For age group comparisons, Mann-Whitney tests were performed. Correlation coefficients assessed relationships between plasma and serum sCD40L.

Results—Plasma sCD40L levels were higher in neonates than in all other age groups, ($p<0.001$). All grouped pediatric plasma levels were significantly higher than in adults ($p<0.0001$). There were no significant differences in plasma sCD40L between pediatric age groups. Serum levels were significantly higher in neonates than in any other age group ($p <0.0001$). Pediatric and adult serum sCD40L levels were not significantly different.

Conclusions—Plasma sCD40L levels are highest at birth and remain higher than those in adults throughout childhood. Reasons for such developmental changes remain to be investigated. Age appropriate reference ranges should be used when sCD40L is being evaluated in pediatric disorders.

Keywords

platelets; platelet activation; pediatric; reference values

Originally identified on B lymphocytes, CD40 is a membrane receptor protein belonging to the tumor necrosis factor (TNF) receptor family (1,2,3). Its ligand, CD40L (formally CD154), is a TNF family trimeric transmembrane protein that was initially discovered on cells of the immune system (3,4). Both CD40 and CD40L have been identified on macrophages, endothelial cells, vascular smooth muscle, fibroblasts, and most notably, platelets (3,4,5,6,7).

When platelets are activated, CD40L moves from intracellular sites to the platelet surface (1,3,4). Membrane CD40L may then be cleaved, producing a soluble trimeric fragment known as sCD40L. Over 95% of circulating CD40L derives from platelets, suggesting that these cells contribute substantially to the biological functions initiated by CD40L (1,3).

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CD40L has been shown to initiate inflammatory responses including the expression of adhesion receptors and tissue factor (TF), as well as the release of chemokines (3,8,9). CD40L also plays a role in thrombosis by inducing TF expression on endothelial cells and monocytes, operating as a platelet agonist, and stabilizing arterial thrombi (1,3,8,10). Notably, these functions are not mediated exclusively via CD40L-CD40 interactions, because the integrin receptors αIIb βIII and α5β1 have also been found to bind CD40L (10,11).

Given the involvement in inflammation and thrombosis, it is not surprising that CD40L has been implicated in the pathophysiology of many disorders. Increased sCD40L levels have been found in adults with acute coronary syndromes and peripheral arterial occlusive disease, and are strongly associated with an increased risk of cardiovascular events in healthy women and in adults with cardiovascular disease (8,12,13,14). Soluble CD40L has also been found to be elevated in adults with sickle cell anemia (15), systemic lupus erythematosus (16), rheumatoid arthritis (17) and some types of pulmonary arterial hypertension (18). Plasma levels of sCD40L are elevated following cardiopulmonary bypass (19) and are predictive of restenosis following percutaneous coronary angioplasty (20).

To date, the CD40L system is virtually unexplored in children and there are no published pediatric sCD40L reference ranges. It is unknown whether, like coagulation proteins and platelet function, there are developmental changes in sCD40L levels. As observed in adults, CD40L may also play a role in childhood inflammatory diseases and hypercoagulable states and levels may correlate with disease severity or risk. We therefore pursued a study of age dependent levels of sCD40L in healthy pediatric and adult populations in order to: (1) determine whether sCD40L levels vary with age and (2) identify reference ranges to be employed in future studies of childhood diseases.

METHODS

Subjects

Cord blood samples were obtained from 25 full-term newborns. The mean (± standard deviation) time between birth and specimen processing was 132 ± 43 minutes. Blood was also obtained from 74 healthy pediatric patients (3 months –14.99 years) presenting for elective day-surgery. Twenty healthy adult volunteers (age 22–58 years) also provided specimens.

Procedures

Study approval was obtained from the Research Subjects Review Board at the University of Rochester Medical Center (URMC). Parents provided witnessed written informed consent for their child's participation at the outpatient visit that preceded the pediatric surgical procedure. For children over 13 years, witnessed written assent was acquired. An approved informed consent procedure was also pursued for the procurement of cord blood samples. The adult volunteers were recruited by the URMC blood bank for the purpose of sCD40L assay quality control.

For all subjects, exclusion criteria included: (1) chronic medical conditions; (2) medication use; (3) recent (preceding week) or current fevers; (4) acute illness; (5) recent (preceding week) or current use of non-steroidal anti-inflammatory drugs; and (6) aspirin use in the past month. Participating children were re-evaluated at the time of surgery to ensure that they were still eligible. Cord bloods were excluded from analysis if they were greater than 3 hours old or of indeterminate age.

Within approximately 1–2 minutes following delivery, cord blood (from both the umbilical veins and arteries) was dripped into an untreated (red top) and ethylenediaminetetraacetic acid (EDTA) treated (lavender top) tube using a plastic funnel. Samples were then sent to the blood

bank where they were immediately centrifuged at a relative centrifugal force of 1300g for 5 minutes. Platelet poor supernatants were separated and stored at −80°C for later analysis.

For pediatric collections, a tourniquet was applied to the upper arm and a 20- or 22-gauge angiocatheter was inserted into a peripheral vein. Blood was allowed to flow directly into an untreated (red-top) and citrated (blue-top) tube (1–2 ml in each). Care was taken to ensure that only blood from the initial angiocatheter insertion was utilized so as to avoid any confounding effects that might be produced by prolonged tourniquet use. In some instances, for children under 8 years, inhalation anesthetics (but no other medications) were given prior to the blood draw. Samples were transported within minutes to the blood bank for processing in the manner described above. In all pediatric cases, care was taken not to collect or transfer blood through a needle as such a maneuver could potentially activate platelets, causing enhanced sCD40L or cytokine release. Samples were processed quickly to minimize platelet activation.

In adults, a 21G vacutainer blood collection needle (Becton Dickinson and Company, Franklin Lakes, NJ) was inserted into an antecubital vein and blood was drawn into untreated (red-top), citrated (blue-top), and EDTA treated (lavender top) tubes. A tourniquet was employed and samples were drawn exclusively from the first attempt. Specimens were then handled as previously outlined.

Soluble CD40L was assayed using an enzyme-linked immunosorbent assay (ELISA) that captures monomeric and multimeric forms of the protein. (21).

Statistical Methods

Sample data were grouped according to subject age (Newborn, 0–5 years, 6–10 years, 11–14 years, 20 years and above) and the median, quartiles, and range (5^{th} to 95^{th} percentiles) of sCD40L values were determined. Individual group comparisons were made using the Mann-Whitney rank order tests. In order to assess the linear relationship between serum and plasma sCD40L, as well as the relationship between platelet count and serum or plasma sCD40L, correlation coefficients were determined. A correlation coefficient was also calculated to assess the linear relationship between citrate and EDTA prepared sCD40L plasma levels in adults. For pair-wise comparisons among the five age groups, a Bonferroni-adjusted significance level of $0.05/10 = 0.005$ was used. For all other analyses, a significance level of ≤ 0.05 was employed. Analyses were performed using the statistical programs MedCalc version 9.2 for Windows (Mariakerke, Belgium), and SPSS 14.0 for Windows, Chicago, Illinois.

RESULTS

De-identified cord blood was used for the newborn sCD40L assays. Although samples likely reflect the demographics of our local population, actual male/female and racial/ethnic percentages are unknown. Of the 74 pediatric subjects, 29 were female and 45 were male. Of the 20 adults, 11 were female and 9 were male.

The median, quartiles, and range (5^{th} to 95^{th} percentiles) of sCD40L for each group are presented in the Table. Comparisons of median values between groups are illustrated in Figure 1. Median plasma sCD40L was significantly higher in newborns than in all other age groups ($p<0.001$). Median plasma sCD40L levels were significantly higher in each of the pediatric groups than in the adult cohort ($p<0.0001$). Median levels did not differ significantly between the pediatric age groups. Median serum levels were significantly higher in cord blood than in any other age group ($p <0.0001$). Pediatric and adult serum sCD40L levels were not significantly different.

There was a statistically significant correlation between plasma and serum sCD40L levels in cord blood specimens and in those from each pediatric age group: cord blood group, n = 25, r = 0.98 (95% CI 0.95 to 0.99), p < 0.0001; 0–5 year group, n = 23, r = 0.99 (95% CI 0.97 to 0.99), p < 0.0001; 6–10 year group, n = 27, r = 0.95 (95% CI 0.89 to 0.98), p < 0.001; 11–14 year group, n = 23, r = 0.97 (95% CI 0.93 to 0.99), p <0.0001. Median serum values were always higher than median plasma values. No correlation was apparent between plasma and serum sCD40L in adults [n = 20, r = 0.045 (95% CI −0.44 to 0.45), p = 0.98]. Notably, adult plasma obtained from EDTA and citrate anti-coagulated samples were highly correlated [n = 20, r = 0.90 (95% CI 0.75–0.95)] (Figure 2; available at www.jpeds.com).

Platelet counts were only available in the adult group. There were no statistically significant correlations between platelet count and serum or plasma sCD40L, although the existence of these relationships cannot be ruled out given the small sample size and correspondingly wide confidence intervals (data not shown).

DISCUSSION

The present study demonstrates that plasma sCD40L levels are high at birth and remain significantly higher than those of healthy adults throughout childhood. The median cord plasma sCD40L level is also significantly higher than the median level of any pediatric group. Neonatal serum sCD40L is significantly higher than in all of the older age groups.

When compared with those in older subjects, decreased neonatal platelet responses have repeatedly been identified. Larger, more active von Willebrand multimers in newborn plasma, as well as a higher hematocrit at birth, are believed to offset this deficit, resulting in shorter cord blood Platelet Function Analyser –100 closure times (22,23). It is conceivable that, given its ability to bind platelet associated CD40L, αIIb βIII, and α5β1, the increased neonatal sCD40L found in this study may further contribute to the newborn's ability to form a primary hemostatic plug (3,10,11).

Although lower than in cord blood, pediatric plasma sCD40L levels are still significantly higher than in normal adults. During childhood and adolescence many platelet and coagulation functions become more like those of adults so that higher sCD40L during this period is not as easily ascribed to compensating hemostatic abilities. When considering developmental changes in coagulant and inhibitor levels, Monagle and colleagues hypothesized that these proteins might have additional functions that dictate age-related differences in concentration (24). It could be that there is a greater necessity at birth and during the early years of life for certain non-hemostatic CD40L activities, mandating higher levels during these periods.

Children and neonates may have fewer CD40, αIIb βIII, and α5β1expressing cells (although platelet counts are higher than in adults) or fewer receptors per cell, resulting in higher levels of circulating ligand. Alternatively, differences in ligand levels might be simply attributed to differences in platelet number because historic studies of platelet ranges reveal that platelet counts in all pediatric age groups are statistically higher than in adults (25). Although there appeared to be no correlation between an individual adult's platelet count and the plasma sCD40L level in this study, the small sample size and wide confidence intervals undermine the confidence with which such a statement can be made. Correlations have been noted in the setting of thrombocytosis (26), but other investigations have failed to show a direct relationship between normal platelet counts and sCD40L levels (27).

Mean serum sCD40L was higher than mean plasma sCD40L in all specimens, although the degree of difference was not consistent. In addition, several of the statistically significant differences in pediatric versus adult plasma sCD40L levels were not recapitulated in the serum specimen results. Soluble CD40L is released into the supernatant during in vitro clotting, which

likely accounts for more elevated serum levels. The process of clotting, and associated platelet activation, is no doubt prone to some variability, making differences between serum and plasma, as well as between age groups, less easily identified. In all, serum measurements may not provide an accurate representation of in vivo circumstances. Furthermore, although serum sCD40L is frequently reported, its validity has previously been called into question. Conde et al found that most of the CD40L in serum was associated with microparticles instead of actually representing the soluble form (28). Conversely, practically all of the CD40L in plasma was soluble, and plasma sCD40L is likely the more reliable measure.

The study has some limitations. Newborn samples were obtained from cord blood and may not represent levels present in the first few days of life. Further studies will be needed to clarify whether such high sCD40L levels are sustained during this time period. Our oldest pediatric subjects ranged from 11–14.99 years; therefore many were likely pre-pubertal. As it is possible that pubertal changes may result in lower sCD40L levels more similar to those of adults, the inclusion of an older adolescent age group would have been informative.

Pediatric and adult plasma specimens were collected in citrate, whereas cord blood samples were anticoagulated with EDTA (cord blood was collected for a number of other studies that required EDTA). Although some investigators have questioned the suitability of EDTA prepared plasma (29) for the sCD40L assay used in this study, Blake et al compared anti-coagulants and found that though citrate provided the highest reproducibility, the variation coefficient for EDTA treated samples was < 10% (30). We compared plasma sCD40L from the same adult subject's blood separately treated with citrate or EDTA, and the results were well correlated ($r = 0.90$). In addition, both adult plasma EDTA and citrate samples produced significantly lower levels than cord plasma EDTA samples ($p = 0.0001$).

When compared to pediatric and adult levels, plasma sCD40L is high at birth. Although lower than in the neonate, plasma sCD40L levels in children remain higher than those of adults. Future research may help to answer questions regarding the underlying reasons for developmental changes in the concentration of this plasma protein. Age appropriate reference ranges should be used when sCD40L is being evaluated in pediatric disorders.

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List of Abbreviations

sCD40L
: soluble CD40 ligand

ELISA
: enzyme-linked immunosorbent assay

EDTA
: ethylenediaminetetraacetic acid

URMC
: University of Rochester Medical Center

TNF
: tumor necrosis factor

TF

tissue factor

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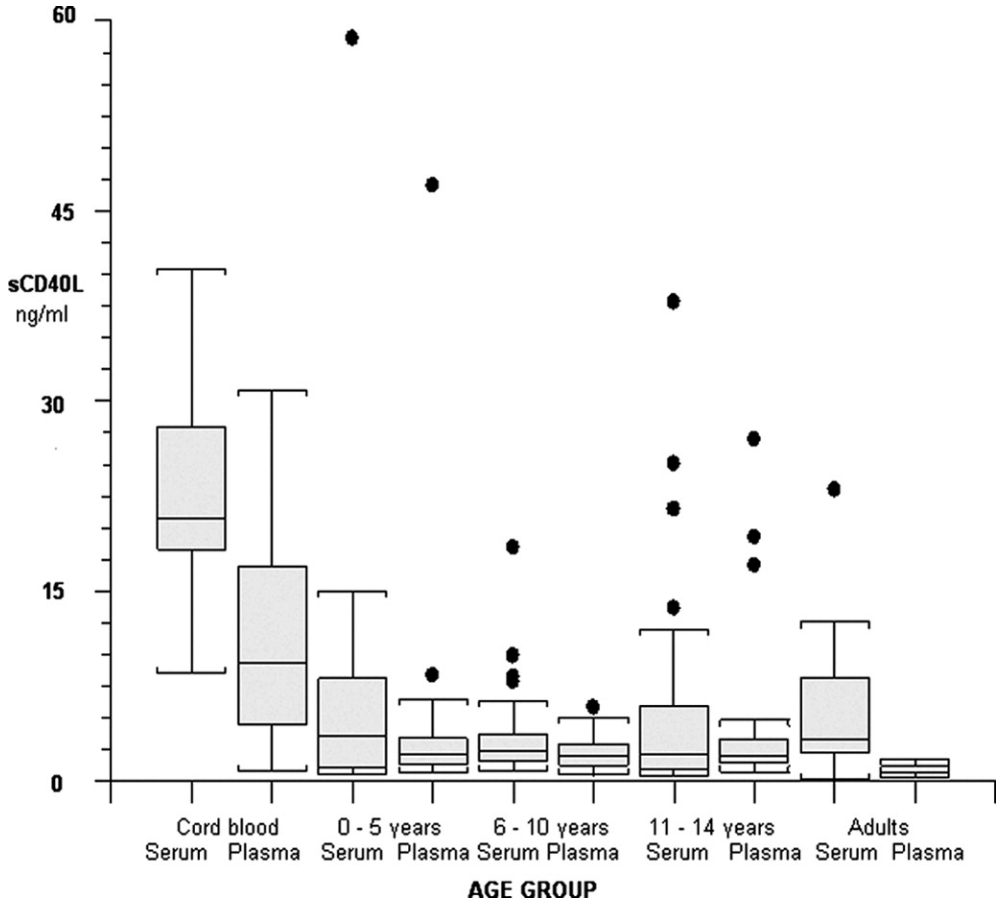

Figure 1.
sCD40L in serum and plasma by age group. The line in each box represents the median value. The 25th and 75th percentile are at each perimeter and range/outliers are displayed.

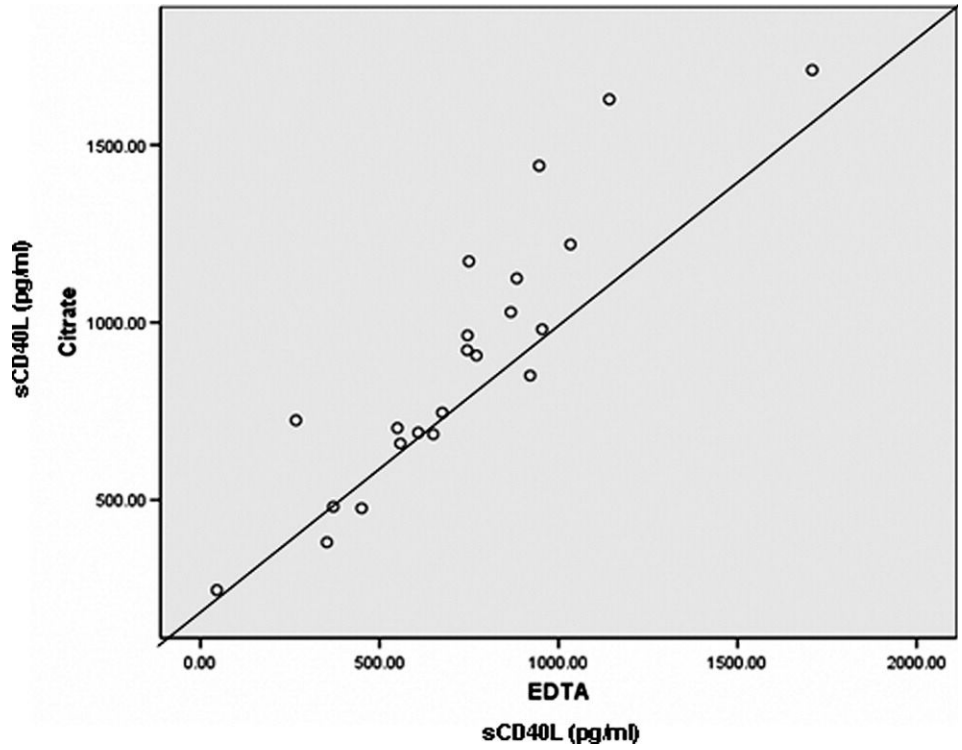

Figure 2.
sCD40L in EDTA vs. Citrate treated adult samples.

Table 1

Plasma and serum sCD40L (ng/ml) values.

Age group	Sample	1st Quartile 25th percentile	Median 50th percentile	3rd Quartile 75th percentile	5th–95th percentiles
Cord blood N = 25	Plasma	5.0	9.4	15.9	1.5–25.8
	Serum	18.3	20.9	27.0	12.3–33.8
0–5 years N = 23	Plasma	1.5	2.5	3.4	0.6–8.4
	Serum	1.3	3.7	8.0	0.5–15.0
6–10 years N = 28	Plasma	1.3	2.2	2.9	0.6–5.0
	Serum	1.7	2.6	3.6	0.8–9.9
11–14 years N = 23	Plasma	1.4	2.1	3.3	0.7–19.3
	Serum	1.3	2.3	5.7	0.8–25.1
Adults[α] N = 20	Plasma	0.7	0.9	1.1	0.5–1.6
	Serum	2.4	3.4	7.8	0.8–12.6

[α]Represents citrate-treated samples. For EDTA treated samples: median = 0.7, range =0.3–1.1 ng/ml.