0031-3998/95/3803-0336$03.00/0
PEDIATRIC RESEARCH

Vol. 38, No. 3, 1995
*Printed in U.S.A.*

# Soluble L-Selectin (sCD62L) Umbilical Cord Plasma Levels Increase with Gestational Age

CHRISTOPH BÜHRER, DIETGER STIBENZ, JOHANNES GRAULICH, ULRIKE GERNHOLD, EUGENE C. BUTCHER, JOACHIM W. DUDENHAUSEN, AND MICHAEL OBLADEN

*Departments of Neonatology [C.B., D.S., J.G., U.G., M.O.] and Obstetrics [J.W.D.], Rudolf Virchow Medical Center, Berlin Free University, D-14059 Berlin, Germany, and Department of Pathology [E.C.B.], Stanford University Medical Center, Stanford, California 94305*

## ABSTRACT

L-Selectin (CD62L) is a leukocyte surface membrane glycoprotein involved in extravasation and homotypic aggregation which is rapidly cleaved off after cellular activation. From culture supernatants and body fluids, soluble L-selectin (sCD62L) has been recovered with its functional activity retained. We devised a sensitive enzyme-linked immunoassay for quantitation of sCD62L which was used to measure sCD62L in umbilical cord plasma of 255 human newborns with a gestational age (GA) of 23–43 wk (median 38 wk). sCD62L levels ranged from 1.14–13.8 pmol/mL (median 7.2 pmol/mL) and showed strong correlations with GA ($r = 0.71$, $p < 0.001$), birth weight ($r = 0.66$, $p < 0.001$), and absolute neutrophil cell counts (ANC) ($r = 0.62$, $p < 0.001$) obtained from a peripheral vein within the first 6 h of life ($n = 153$), whereas there was a weak inverse correlation with absolute normoblast counts ($r = -0.27$, $p < 0.001$). In multiple regression analysis, only GA and ANC retained a significant association with sCD62L levels ($p < 0.001$). Decreased sCD62L levels were found to be associated with multiple gestation (4.8 ± 2.4 pmol/mL *versus* 7.7 ± 2.3 pmol/mL, $p < 0.05$) also when considering GA and ANC as covariates. In contrast, increased sCD62L levels in infants born from meconium-stained amniotic fluid, and decreased levels in newborns with acute bacterial infection could be fully attributed to differences in GA and ANC. Umbilical cord blood sCD62L levels of healthy, term, vaginally born singletons ($n = 38$) were significantly lower (8.5 ± 2.2 *versus* 11.8 ± 1.9, $p < 0.0001$) than cubital vein levels of healthy adults ($n = 20$). We conclude that sCD62L levels display a strong increase during fetal maturation. (***Pediatr Res* 38: 336–341, 1995**)

**Abbreviations**

**CD62L,** L-selectin
**sCD62L,** soluble L-selectin
**CRP,** C-reactive protein
**TBS,** Tris-buffered saline
**PMSF,** phenylmethylsulfonyl fluoride
**GA,** gestational age
**ANC,** absolute neutrophil count

CD62L is a surface membrane glycoprotein expressed by nucleated blood and bone marrow cells (1). By initiating reversible attachment of leukocytes to endothelial cells lining the wall of postcapillary venules (2, 3), CD62L is pivotal in directing neutrophils into areas of acute inflammation (4), and lymphocytes into secondary lymphoid organs (5) and sites of chronic inflammation (6). CD62L has also a role in homotypic aggregation of neutrophils (7) and lymphocytes (8) and has been implicated in the adhesion of flowing neutrophils to immobilized platelets (9). The function of CD62L present on $CD34^+$ bone marrow hematopoietic progenitor cells (10, 11) is presently unknown.

The extracellular domain of CD62L is rapidly cleaved off proteolytically after leukocyte activation *in vitro* (12–16) and *in vivo* (17, 18). Human serum has been shown to contain sCD62L with its functional activity retained (19). There is little information available on the diagnostic and physiologic significance of serum sCD62L levels. In adults, high levels have been reported in patients with acute leukemia (20), whereas modest elevations were found in patients with sepsis, AIDS (21), and recent-onset insulin-dependent diabetes mellitus (22). In contrast, patients with multiple trauma, perforated bowel, or acute pancreatitis presented with plasma sCD62L levels on admission that were lower than those of healthy controls (23).

We have shown previously that surface-expressed CD62L is decreased in newborns with acute bacterial infection irrespective of GA (17, 18). Hypothesizing that sCD62L levels might be conversely increased in conditions associated with generalized activation of the immune system, we developed an ELISA to quantitate sCD62L in umbilical cord vein plasma of human term and preterm newborns. The ELISA and the data obtained of cord blood sCD62L levels with respect to maturity, hematologic, and obstetrical conditions are the subject of the following report.

Received September 6, 1994; accepted March 14, 1995.
Correspondence: Dr. Christoph Bührer, Kinderklinik KAVH, Virchow-Klinikum, Humboldt-Universität, Heubnerweg 6, D-14059 Berlin, Germany.
Supported by a grant from the German Research Council (DFG, Bu 623/2-3).

## METHODS

### Subjects

sCD62L levels were determined in umbilical cord vein blood of 255 neonates with a GA of 23–43 wk (median 38 wk), and a birth weight ranging from 420 to 5100 g (median 3080 g). Antepartum and intrapartum data were collected prospectively and included gender (145 boys), multiple gestation ($n$ = 40), gestational hypertension ($n$ = 13), gestational diabetes ($n$ = 21), meconium-stained amniotic fluid ($n$ = 69), umbilical artery pH, and mode of delivery. Asphyxia (umbilical artery pH < 7.15) occurred in 15 newborns. One-hundred and fifty newborns were born vaginally (53 assisted by vacuum or forceps), 75 by elective, and 30 by emergency cesarean delivery. Healthy, mature (GA ≥ 37 wk), vaginally born singletons without any of the risk factors gestational hypertension, gestational diabetes, meconium-stained amniotic fluid or asphyxia comprised 38 of the 255 newborns. Although all infants were born at the same tertiary perinatal center, the distribution of risk factors is not necessarily representative for that of the institution.

Laboratory work-up was initiated at the discretion of the attending neonatologist in 189 of the 255 newborns. No venipunctures of the infant were made for investigational purposes. In 153 newborns, a white blood cell count and differential was obtained within the first 6 h of life (24). As further markers of inflammation, CRP serum peak values obtained within the first 36 h of life (25), and platelet count nadir values within the first 72 h of life were recorded (26). A decision of transferal to the children's hospital within the first 24 h of life was made in 112 newborns. For the purpose of this study, a diagnosis of acute bacterial infection was made ($n$ = 15) if clinical symptoms leading to the institution of antibiotics within the first 24 h of life were confirmed by a positive venous blood culture and at least one of the four laboratory parameters outlined below, or at least two parameters in culture-negative newborns placed on i.v. antibiotics after antibiotics had been administered i.v. to the mother before delivery for suspected amnion infection syndrome. Laboratory markers of presumed acute bacterial infection were neutropenia (ANC ≤ 2 000/μL; $n$ = 13), a pathologic neutrophil immature/total ratio (≥0.2; $n$ = 16), peak CRP serum levels of 10 mg/L or more ($n$ = 27), and thrombocytopenia (nadir < 150 000/μL; $n$ = 17).

Umbilical cord vein blood was prevented from clotting by addition of sodium citrate immediately after birth and centrifuged within 6 h after delivery. The plasma was stored at −20°C until analysis. After thawing, plasma samples were diluted 1/25 to obtain a measure in the linear range of the assay developed. Raw data obtained from plasma were corrected for hematocrit and the volume of sodium citrate added.

For comparison, sCD62L levels were quantitated in cubital vein plasma ($n$ = 20) of adults without apparent inflammatory disease. In matched samples of 96 or 127 newborns, respectively, umbilical cord blood sCD62L levels were compared with lymphocyte, or granulocyte and monocyte CD62L surface expression measured by flow cytometry described previously (17, 18).

### Determination of sCD62L Levels

***MAb.*** The anti-CD62L MAb DREG-55 and DREG-200 hybridomas (27) were grown in serum-free medium (Ultroser, GIBCO, Eggenstein, Germany). Supernatants were purified by protein G affinity chromatography using the MAbTrapG-Kit (Pharmacia, Freiburg, Germany). Purity was checked by SDS-PAGE under reducing and nonreducing conditions (PhastSystem, Pharmacia). Purified DREG-200 was biotinylated in 0.1 M borate buffer (pH 8.8) with a freshly prepared 1% (wt/vol) solution of biotinamidocaproate-*N*-hydroxysuccinimide ester (Sigma Chemical Co., Deisenhofen, Germany) in DMSO for 20 h at room temperature, at a molecular ratio of biotin ester:IgG = 30:1, followed by blocking of unreacted succinimide ester groups by addition of 1 M $NH_4Cl$.

***Preparation of an sCD62L-enriched standard.*** A DREG 55-Sepharose 4B column (2.8 mg DREG 55 per mL gel) was prepared according to the manufacturer's instructions from BrCN-activated Sepharose 4B (Pharmacia) and equilibrated with PBS containing 0.05% $NaN_3$, 2 mM EDTA, and 0.2 mM PMSF. Pooled plasma of healthy adult blood donors was cleared by centrifugation (2 h at 0°C and 2500 × $g$), filtered through 0.2 μm filters (Schleicher & Schüll, Dassel, Germany), and diluted 1:1 with PBS (0.05% $NaN_3$, 2 mM EDTA, 0.2 mM PMSF). After passage over the DREG-55 affinity column and elution with 0.1 M glycine buffer (pH 2.7), fractions were immediately neutralized with 1 M Tris buffer (pH 9.0). Peak protein fractions were concentrated and transferred into PBS (0.05% $NaN_3$, 2 mM EDTA, 0.2 mM PMSF) by ultrafiltration (Centricon 10 unit; Amicon, Beverly, MA) yielding 5–11 μg protein/mL plasma (Micro-BCA-method with BSA as the standard; Pierce, Oud-Beijerland, The Netherlands).

The sCD62L preparation was characterized by 12.5% SDS-PAGE followed by blotting on Hybond ECL cellulose membranes (0.45 μm, Amersham Buchler, Braunschweig, Germany) using the PhastSystem including the Semi-dry Transfer Kit (Pharmacia). Protein bands were visualized by silver staining, parallel blots were immunostained with biotinylated MAb DREG-55 (dilution 1:500) followed by ExtrAvidin alkaline phosphatase conjugate (dilution 1:3000) and development with NBT/BCIP reagent (*p*-nitro blue tetrazolium chloride/5-bromo-4-chloro 3-indolyl phosphate toluidine salt; Bio-Rad, Munich, Germany) following the manufacturer's instructions.

***Standardization of the plasma sCD62L preparation by competition titration.*** Polystyrene Falcon tubes (5-mL; Becton Dickinson, Heidelberg, Germany) were blocked with 1% BSA in TBS with 0.02% $NaN_3$ for 3 h at room temperature and washed three times, with careful aspiration after the last washing step but without drying the tubes. Then 100 ng of MAb DREG 55, or DREG 200, and 100 μL of the standard plasma sCD62L preparation containing 150, 100, 50, 12.5, 6.25, 1.25, 0.625, 0.125, 0.0125, 0.00125, and 0 μg (negative control) of protein were pipetted into the tubes, vortexed, and preincubated for 1 h at room temperature in the presence of 0.05% $NaN_3$, 2 mM EDTA, and 0.2 mM PMSF. A leukocyte suspension was prepared by washing a fresh buffy coat three times at 600 × $g$ and twice at 180 × $g$ for 10 min in excess RPMI 1640,

with a final resuspension in PBS containing 0.2% $NaN_3$, 0.15% BSA, and 0.1% human IgG. To each coated tube, 3 $\times$ $10^5$ leukocytes in 50 $\mu$L were added, vortexed, and allowed to react for 10 min at room temperature and 30 min at 4°C. The cells were washed three times with the IgG-containing buffer and incubated with 7 $\mu$L of goat-anti-mouse-IgG(H+L)F(ab')$_2$-FITC for 30 min at 0°C (Dianova, Hamburg, Germany). After addition of 4 mL of FACS, lysing solution (Becton Dickinson), vortexing, and 10-min incubation at 0°C, the samples were centrifuged, the supernatants were aspirated, and the samples were washed again with PBS. Finally, the samples were postfixed by addition of 350 $\mu$L of 2% paraformaldehyde in 1.5 $\times$ PBS (1 h at 5°C). Using a FACScan flow cytometer (Becton Dickinson), median fluorescence intensities ($10^4$ cells/point) were measured and plotted against the logarithm of the protein amount of the standard plasma sCD62L preparation. The content of immunologically active sCD62L of the standard sCD62L preparation was calculated from the protein amount at the inflection point of the sigmoid neutralization curves, assuming a molecular mass of 150 kD for IgG and a binding ratio of 2 CD62L molecules per anti-CD62L MAb.

***ELISA.*** The wells of black microwell plates (F16 black maxisorp immuno modules; Nunc, Roskilde, Denmark) were coated each with 100 $\mu$L of DREG 55 (10 $\mu$g/mL) in 50 mM carbonate buffer (pH 9.85) at 4°C for 18 h. After three washes, the wells were blocked with 1% BSA in TBS for 2 h at 37°C. Quadruplicate serial dilutions of the CD62L standard (0.075–0.746 pmol/mL) and 1:25 dilutions of plasma samples were incubated for 1 h at 37°C. After washing with TBS containing 0.05% Tween 20, the wells were incubated with 100 $\mu$L of DREG 200-biotin conjugate for 1 h at 37°C, washed again, and incubated for 1 h at 37°C with 100 $\mu$L/well of a 1:3000 dilution of ExtrAvidin alkaline phosphatase conjugate (Sigma Chemical Co.). After three further washing steps, the wells were finally incubated with 100 $\mu$L of a 1:50 dilution of the luminescent substrate Lumigen PPD (Boehringer Mannheim, Mannheim, Germany) in 0.1 M Tris buffer (pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) at room temperature. The luminescence (relative light units) was read with an ML 1000 luminometer (Dynatech, Denkendorf, Germany) in the plateau phase after 30 min at 24–28°C.

***Statistical analysis.*** Spearman correlation coefficients were calculated to measure the strength of association between numerical variables. Differences between groups defined by categorical variables were assessed by the $\chi^2$ test, Mann-Whitney $U$ test, or Kruskal-Wallis test for more than one group. Analysis of variance was used for multiple regression analysis of several numerical variables and to assess the influence on numerical variables of categorical variables in the presence of numerical covariates. The Kolmogorov-Smirnov goodness of fit test was used to assess normal (Gaussian) distribution. Means $\pm$ SD are given when the distribution of values did not differ significantly from a normal distribution. All statistical tests were based on a significance level of 0.05 with two-sided $p$ values.

## RESULTS

The ELISA developed to quantitate sCD62L in human plasma has a sensitivity (negative control + 3 SD) of 17 fmol/mL. The intraassay coefficient of variation is <5%, the interassay coefficient of variation is <8%, and the optimal working range is 0.06–0.6 pmol/mL. In adult cubital vein plasma, sCD62L levels were 12.4 $\pm$ 2.1 pmol/mL (mean $\pm$ SD, $n$ = 20).

In cord blood samples of 255 newborns, sCD62L levels were 7.2 $\pm$ 2.5 pmol/mL. Compared with peripheral vein plasma of adults, cord blood plasma of term and postterm newborns (GA $\geq$ 37 wk, $n$ = 170) had significantly ($p <$ 0.0001) lower sCD62L levels (8.4 $\pm$ 1.9 pmol/mL). Samples of premature newborns (GA 31–36 wk, $n$ = 59) and extremely premature newborns (GA 23–30 wk, $n$ = 26) had considerably less sCD62L (5.4 $\pm$ 1.5 pmol/mL, or 3.7 + 2.4 pmol/mL, respectively). Differences between all groups were significant ($p < 0.001$). sCD62L levels of healthy, mature (GA $\geq$ 37 wk), vaginally born singletons without any of the risk factors gestational hypertension, gestational diabetes, meconium-stained amniotic fluid or asphyxia ($n$ = 38) were similar (8.5 $\pm$ 2.2 pmol/mL) to those of the other term newborns but differed from adult cubital vein levels ($p <$ 0.0001). In all groups described, the distribution of sCD62L levels did not differ significantly from that expected for a normal distribution ($p >$ 0.1)

Linear regression analysis confirmed a strong correlation between cord blood sCD62L level and GA ($r$ = 0.71, $p <$ 0.001), as well as birth weight ($r$ = 0.66, $p < 0.001$, Fig. 1). A positive correlation of cord blood sCD62L levels was also found with ANC ($r$ = 0.62, $p <$ 0.001) obtained from a peripheral vein within the first 6 h of life in 153 newborns, whereas there was a weak inverse correlation of sCD62L levels with absolute normoblast counts ($r = -0.27$, $p < 0.001$). Cord blood sCD62L levels, GA, birth weight, ANC, and absolute normoblast counts were found to be highly interrelated (Table 1). By multiple regression analysis, GA and ANC both appeared to be independently associated with sCD62L levels ($p$ < 0.001) but not birth weight and absolute normoblast counts ($p > 0.1$).

No correlation ($-0.25 < r < 0.25$) was seen between sCD62L levels and umbilical artery pH, hematocrit, absolute lymphocyte/monocyte counts, neutrophil immature/total ratio, platelet count nadir within the first 72 h of life, CRP serum level peak during first 36 h of life, or umbilical cord blood CD62L surface expression (17, 18) of lymphocytes ($n$ = 96), granulocytes ($n$ = 127), or monocytes ($n$ = 127), either by single comparison or by multiple regression analysis considering GA and ANC as covariates.

There was no apparent relationship ($p > 0.05$) between cord blood sCD62L levels and the categorical variables gender, gestational hypertension, gestational diabetes, or asphyxia (umbilical artery pH < 7.15) either by direct comparison or when GA and ANC were considered as covariates. Infants born from meconium-stained amniotic fluid appeared to have increased sCD62L levels ($p <$ 0.01). This complication was mostly seen in term and postterm infants ($p <$ 0.0001). When

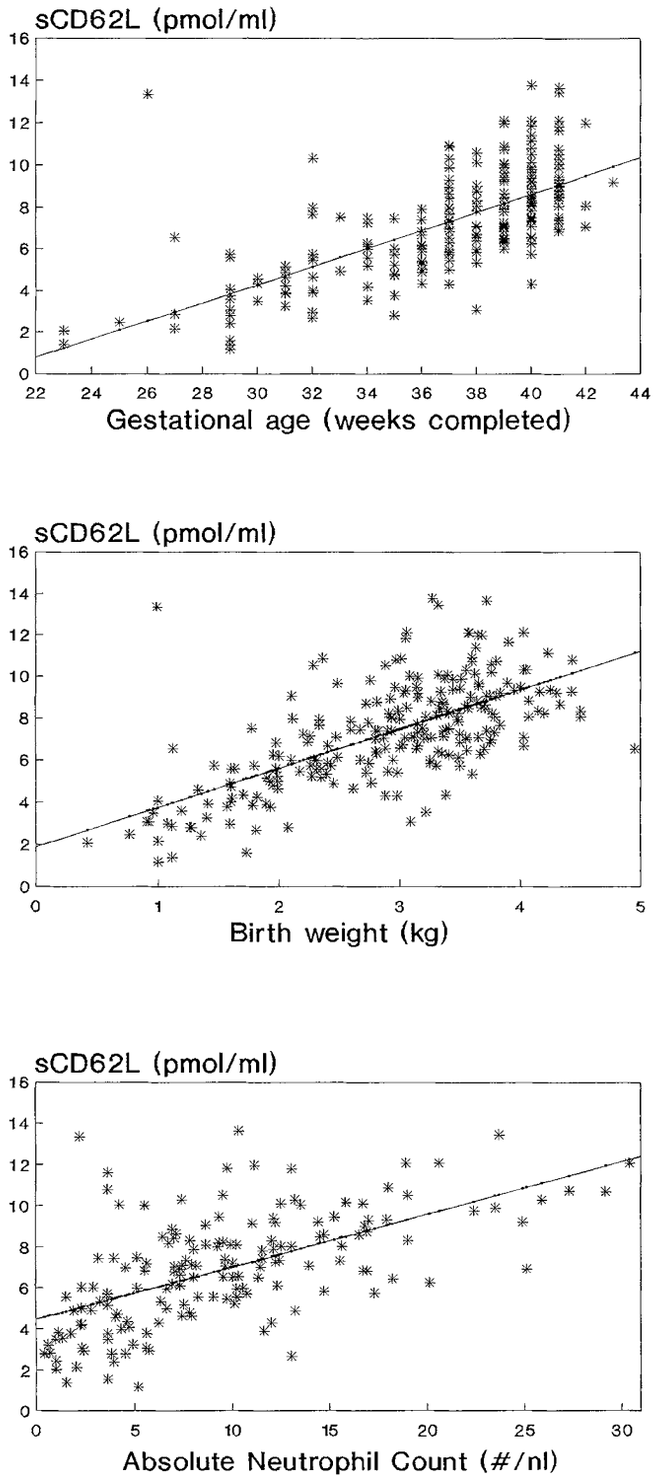


**Figure 1.** Scatter plots with regression lines showing association of umbilical cord plasma sCD62L levels and GA (top), birth weight (middle), and ANC (bottom).

GA and ANC were considered as covariates, the difference was no longer significant ($p > 0.1$).

The most striking feature of newborns with acute bacterial infection was their higher immature/total ratio of the first differential (median 0.26 *versus* 0.03) compared with controls ($p < 0.0001$). In addition, newborns with acute bacterial infection had significantly ($p < 0.05$) lower ANC (median 4500/μL *versus* 9200/μL), a lower birth weight (median 1945 g *versus* 3120 g), a lower GA (median 34 *versus* 38 wk), lower platelet count nadirs (median 196 000/μL *versus* 260 000/μL), higher CRP peak levels (median 21 mg/L *versus* 3 mg/L), and lower sCD62L levels (median 5.56 *versus* 7.35 pmol/mL). The lower sCD62L levels in newborns with acute bacterial infection were found to be insignificant ($p > 0.1$) when GA and ANC were considered as covariates. There was no significant association ($p > 0.1$) of cord plasma sCD62L levels and a pathologic immature/total ratio (≥0.2), with or without correction for GA and ANC. Newborns with platelet counts nadirs below 150 000/μL had slightly lower sCD62L levels but the significance of this association was lost after correction for GA and ANC ($p > 0.1$). Cord blood sCD62L levels were higher in newborns who had peak CRP serum levels of 10 mg/L or more during the first 36 h of life than in newborns whose CRP levels were consistently below 10 mg/L (8.3 ± 2.3 pmol/mL *versus* 7.1 ± 2.5 pmol/mL, $p < 0.05$ without, $p < 0.01$ with correction for GA and ANC).

**Table 1.** *Spearman correlation coefficients of sCD62L levels, GA, birth weight, ANC, and normoblast counts*

| Variable | sCD62L level | GA | Birth weight | ANC | Normoblast count |
|---|---|---|---|---|---|
| sCD62L level | 1 | 0.71 | 0.66 | 0.62 | −0.27 |
| GA | | 1 | 0.78 | 0.66 | −0.27 |
| Birth weight | | | 1 | 0.65 | −0.36 |
| ANC | | | | 1 | −0.29 |

For all coefficients, 2-sided $p < 0.001$.

Samples from twins and triplets had lower cord blood sCD62L levels than singletons (4.8 ± 2.4 pmol/mL *versus* 7.7 ± 2.3 pmol/mL, $p < 0.0001$ by uncorrected comparison, $p < 0.01$ when taking GA and ANC into account). Cord blood sCD62L levels were lower in samples of neonates born by elective cesarean section (5.6 ± 2.6 pmol/mL) than by any other mode of delivery (7.9 ± 2.1 pmol/mL, $p < 0.01$). However, 29 of 40 twins and triplets were born by elective cesarean delivery, compared with 46 of 215 singletons ($p < 0.0001$), and GA and ANC were lower in infants born by elective cesarean delivery ($p < 0.0001$) compared with other modes of delivery. When considering GA, ANC and multiple gestation as confounding variables, no impact of the mode of delivery on sCD62L levels could be demonstrated ($p > 0.1$).

## DISCUSSION

In the absence of purified recombinant sCD62L, efforts to quantitate sCD62L in human plasma or serum have yielded slightly conflicting results. Using three different methods (densitometry of SDS-PAGE bands of partially purified sCD62L, or purified recombinant CD62L-IgG chimeras, or Scatchard plot quantitation of surface CD62L expressed in CD62L cDNA transfectants followed by phorbol 12-myristate 13-acetate-induced shedding) (21), the sCD62L content of a standard plasma has been estimated between 0.95 and 3 μg/mL (11.9–37.5 pmol/mL). Our competition titration experiments favor the lower end of that range. Others have abandoned absolute

quantitation, giving only relative values instead (25, 28). Efforts are under way to express human cDNA-derived CD62L in a prokaryotic system to allow a more precise quantitation of human sCD62L. However, this will not change the validity of conclusions reached by comparing values obtained by the same assay.

The increase of sCD62L plasma levels during the course of fetal maturation could be related to several factors. Nucleated lymphohematopoietic cells are the only known source of sCD62L. Circulating neutrophil numbers and sCD62L levels were indeed found to be closely related in human newborns. A close correlation of serum sCD62L levels to peripheral leukocyte counts has been found in a study of patients with chronic leukemia undergoing bone marrow transplantation, with no detectable sCD62L during periods of severe leukopenia (28). Peripheral white blood cell counts are only a rough estimate of the total number of leukocytes in the body, most of which express CD62L. To a variable extent, CD62L is also found on progenitor cells in the spleen, thymus, and bone marrow which are not amenable to quantitative anaylsis under normal circumstances (10, 11).

sCD62L levels can be expected to be heavily influenced by basal leukocyte CD62L expression and CD62L shedding rates. CD62L expression of neutrophils of term newborns is lower than that of adults (18, 29–31). However, neutrophils in cord blood of immature newborns and in fetal samples obtained percutaneously express CD62L at adult levels (18, 32). CD62L expression of monocytes and lymphocytes is not different between adults and newborns and does not decrease with GA (28, 30). In addition, there is no apparent relationship between sCD62L levels and surface CD62L expression of any major circulating leukocyte population. CD62L shedding in response to stimulation *in vitro* has been shown to occur at similar rates in monocytes of adults and term newborns (30). In neutrophils, stimulation-induced CD62L down-regulation is lower in term newborns than in adults (29–31). However, fetal neutrophils shed CD62L at adult rates in response to stimulation (31). The low levels of sCD62L in premature human newborns thus do not reflect decreased basal leukocyte surface expression nor an impaired shedding response to stimulation.

CD62L shedding appears to occur also without activation at low levels, generating steady state plasma levels already under normal conditions. Nothing is known about baseline CD62L turnover, the mechanism of clearance and the half-life of circulating sCD62L. Measuring circulating sCD62L is likely to miss shed sCD62L bound to CD62L ligand-bearing cells. Enhanced expression of such ligands can be assumed to result in decreased levels of circulating sCD62L but the distribution of such ligands in fetal tissue including the placenta is unknown.

Proteolytic cleavage of CD62L from the cell surface is rapidly accelerated after activation *in vitro* (12–16) and *in vivo* (17, 18). In human newborns, acute bacterial infection is reflected in a reduced CD62L surface membrane expression of lymphocytes, granulocytes, and monocytes, but not in increased sCD62L levels. Neutropenia is common in neonatal early onset sepsis, and increased shedding rates are likely to be counteracted by decreased numbers of cells serving as a source of sCD62L. In addition, activation-induced endothelial expression of CD62L ligands may lead to enhanced sequestration of sCD62L, contributing to a further decline of sCD62L levels.

Depending on the actual plasma level, sCD62L may interfere with leukocyte extravasation. Anti-CD62L MAbs or CD62L-Ig chimeric molecules have be used successfully to protect rats against neutrophil-mediated lung injury (33, 34). In adult patients at risk for the development of acute respiratory distress syndrome, a significant correlation has been found between low initial sCD62L levels and indices of subsequent lung injury, such as requirement for ventilation and degree of respiratory failure, as well as patient mortality (23). It is tempting to speculate that the high susceptibility of premature newborns to disseminated inflammatory lung disease may be related in part to their low levels of circulating sCD62L.

***Acknowledgments.*** We would like to thank Evy Waiß for expert technical assistance, Boris Metze and Reinhard Hartmann for help with data management and statistical advice, and Günter Henze for access to flow cytometry equipment.

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