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Endogenous erythropoietin varies significantly with inflammation-related proteins in extremely premature newborns

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Abstract

Introduction—Erythropoietin, a pluripotent glycoprotein essential for erythropoiesis, fetal growth, and development, has recently been implicated in innate immune regulation. Data from the ELGAN Study allowed us to evaluate relationships between endogenous erythropoietin and 25 inflammation-related proteins in extremely premature newborns.

Methods—We measured the concentrations of 25 inflammation-related proteins and of erythropoietin in blood spots collected on postnatal days 1, 7, and 14 from 936 infants born before 28 weeks gestation. We calculated the odds that infants with an inflammation-related protein in the highest quartile for gestational age and collection day had an erythropoietin concentration in the highest or lowest quartile.

Results—The proportion of children with inflammation-associated protein concentrations in the top quartile tended to increase monotonically with increasing quartile of EPO concentrations on 2 of the 3 days assessed. To a large extent, on each of the 3 days assessed, the odds ratios for an erythropoietin concentration in the top quartile were significantly elevated among those with an inflammation-related protein concentration in the top quartile.

Conclusions—Our findings suggest that in very preterm newborns, circulating levels of endogenous erythropoietin vary significantly with circulating levels of inflammation-related proteins. Elevation of endogenous erythropoietin might not be an epiphenomenon, but instead might contribute to subsequent events, by either promoting or reducing inflammation, or by promoting an anti-injury or repair capability.

Keywords

Infant; Premature; Protein; Acute-phase; Cytokine

1. Introduction

Erythropoietin (EPO), a pluripotent glycoprotein essential for erythropoiesis as well as fetal growth and development, [1–3] has unique biochemical and epigenetic properties that are beneficial in critical illness [4,5]. Animal research provides evidence that EPO opposes the actions and/or sequelae of inflammation, [6] apoptosis, [7] oxidative stress, [8] and hypoxia-ischemia [9]. Moreover, EPO appears to have neurotropic [10,11] and neuroprotective [12] properties that could be useful in the prevention and repair of brain injury [6,13].

Maternal EPO does not cross the placenta in pregnancy. Elevations in cord blood levels, then, are related to fetal production, and may be a marker for intra-uterine exposures and

processes associated with preterm birth [14,15]. In one study, infants with elevated serum concentrations of EPO were admitted to the intensive care unit six times more frequently than those with normal EPO concentrations; these infants had lower umbilical artery pH, oxygen tension, and APGAR scores [16,17].

Exogenous EPO is currently used to reduce or prevent the need for red blood cell transfusions in preterm infants [14]. When first administered after postnatal day 8, EPO reduces the number of transfusions required in infants [15]. However, preterm and/or low birth weight infants given exogenous EPO appear to be at heightened risk of retinopathy of prematurity (ROP) [16], a disorder that may be triggered by inflammation [17].

Endogenous EPO might also be associated with inflammation. In adult humans with sepsis, septic shock, or severe acute inflammation, circulating concentrations of EPO appeared to function as an acute phase reactant [18,19]. Following lipopolysaccharide exposure, mice display a dramatic rise in serum concentrations of EPO [20].

Is endogenous EPO also related to inflammation in very preterm newborns? Data from the ELGAN Study of infants born before the 28th week of gestation [21] allowed us to evaluate, for the first time, relationships between EPO and 25 inflammation-related proteins in blood spots obtained during the first two postnatal weeks.

2. Methods

2.1. The ELGAN study

The ELGAN study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in extremely low gestational age newborns [21]. During the years 2002–2004, women who delivered before 28 weeks gestation at one of 14 participating institutions were asked to enroll in the study. The enrollment and consent processes were approved by the individual institutional review boards. A full description of the methods is provided elsewhere [21].

A total of 939 infants had blood proteins measured on at least one day. On day 1 there were 861 measures; on day 7 there were 867 measures; on day 14 there were 786 measures. These measures comprise the samples for the assessments presented here of the relationships between EPO concentrations, presented as quartiles, and the top quartile concentration of blood protein indicators of systemic inflammation.

2.2. Newborn variables

The gestational age estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14th week (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks (29%), last menstrual period (LMP) without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

The birth weight Z-score is the number of standard deviations the infant's birth weight was above or below the median weight of infants at the same gestational age in referent samples not delivered for preeclampsia or fetal indications [22,23].

2.3. Blood spot collection

Drops of blood were collected on filter paper on the first postnatal day (range: 1–3 days), the 7th postnatal day (range: 5–8 days), and the 14th postnatal day (range: 12–15 days). All blood was from the remainder of specimens of blood collected for clinical indications. Dried blood spots were stored at -70°C in sealed bags with desiccant until processed, usually 6–8 years later.

2.4. Protein measurement

Details about elution of EPO and the 25 inflammation-related proteins from blood spots and their measurement with the Meso Scale Discovery (MSD) electrochemiluminescence system are provided elsewhere [24]. Validated by comparisons with traditional ELISA, [25,26] this system has high content validity, and inter-assay variations that are invariably less than 20% [24,27–30].

The Laboratory of Genital Tract Biology of the Department of Obstetrics, Gynecology and Reproductive Biology at Brigham and Women's Hospital, Boston measured the following 26 proteins using commercially available platforms: EPO, IL-1 β (interleukin-1 beta), IL-6 (interleukin-6), IL-6R (interleukin-6 receptor), TNF-alpha (tumor necrosis factor-alpha), TNF-R1 (tumor necrosis factor-alpha-receptor1), TNF-R2 (tumor necrosis factor-alpha-receptor2), IL-8 (CXCL8) (interleukin-8), MCP-1 (CCL2) (monocyte chemotactic protein-1), MCP-4 (CCL13) (monocyte chemoattractant protein-4) (CCL13), MIP-1 β (CCL4) (Macrophage Inflammatory Protein-1beta) (CCL4), RANTES (CCL5) (regulated upon activation, normal T-cell expressed, and [presumably] secreted), I-TAC (CXCL11) (Interferon-inducible T cell Alpha-Chemoattractant), ICAM-1 (CD54) (intercellular adhesion molecule-1), ICAM-3 (CD50) (intercellular adhesion molecule-3), VCAM-1 (CD106) (vascular cell adhesion molecule-1), E-SEL (CD62E) (E-selectin) (CD62E), MMP-1 (matrix metalloproteinase-1), MMP-9 (matrix metalloproteinase-9), CRP (C-reactive protein), SAA (serum amyloid A), MPO (myeloperoxidase), VEGF (vascular endothelial growth factor), VEGF-R1 (vascular endothelial growth factor-receptor1), VEGF-R2 (vascular endothelial growth factor-receptor2), and IGFBP-1 (insulin-like growth factor binding protein-1). Concentrations were measured in duplicate and the mean served as the basis for all tables and analyses. Because the volume of blood spots can vary in ways that we cannot easily measure, measurements of each protein were normalized not to volume, but to mg total protein. The measurements are picograms/mg protein or international units/mg protein. Total protein was measured using the BCA assay (Thermo Scientific, Rockford, IL) as previously described [30].

2.5. Data analysis

EPO concentration was not normally distributed and varied with gestational age and with the day of blood collection. This lead us make the decision to calculate quartiles of the concentration within gestational-age (23–24, 25–26, and 27 weeks) and collection-day

(postnatal day 1, 7, and 14) strata. We created Table 1 (for postnatal day 1), 2 (for postnatal day 7), and 3 (for postnatal day 14), to see if the tendency to have a measure of each inflammation-related protein in the top quartile varied with the quartile of EPO concentration. Relationships between EPO and each inflammation-related protein were considered monotonic if the proportion of children with a protein concentration in the top quartile varied consistently in the same direction as the quartile of EPO concentration increased.

We evaluated the null hypothesis that EPO measurements in the blood do not vary with blood measurements of inflammation-associated proteins. Using multinomial logistic regression models that adjusted for gestational age category (i.e. 23–24, 25–27, and 27 weeks) (Fig. 1), we calculated the odds (and their 99% confidence intervals) that children who had an EPO concentration in the bottom or top quartile had a concentration of each inflammation-related protein in the top quartile compared to children who had an EPO concentration the middle two quartiles. We used logistic regression models, adjusted for gestational age, to calculate the odds that children who had inflammation-related proteins in the top quartile on one day or on two or more days a week apart had an EPO concentration in the top quartile on day 14 (Fig. 2).

3. Results

Overall, EPO concentrations tended to be elevated among children with elevated concentrations of inflammatory proteins (Tables 1–3). By and large, the relationships between EPO and inflammation-related proteins were statistically significant on each of the 3 days assessed (Fig. 1). Additionally, children who had elevated concentrations of inflammation-related proteins on two separate days tended to have elevated odds of a day-14 EPO measurement in the top quartile (Fig. 2) as did, to a lesser extent, those with elevations on any single day.

3.1. Day 1 (Table 1)

With only one protein exception (MMP-1), children who had a day-1 highest quartile EPO measurement tended to have, at the same time, inflammation-related protein measurements in the top quartile as well. For 20 of the 25 proteins, the proportion of children with measurements in the top quartile increased mono-tonically with increasing quartile of EPO measurement (CRP, SAA, MPO, IL-1 β , IL-6, IL-6R, TNF- α , TNF-R1, TNF-R2, MCP-1, MIP-1 β , RANTES, I-TAC, ICAM-1, ICAM-3, VCAM-1, E-SEL, MMP-1, VEGF-R2, IGFBP-1).

3.2. Day 7 (Table 2)

IL-6R, RANTES, and VCAM-1 are the only three proteins that did not have the highest proportion of day-7 measurements in the top quartile among children with the highest day-7 EPO quartile. Seventeen inflammation-related proteins showed the day-1 pattern of monotonic increase in the proportion of children with increasing EPO measurements on day 7 (CRP, SAA, IL-1 β , IL-6, TNF- α , TNF-R2, IL-8, MCP-1, MCP-4, I-TAC, ICAM-1, ICAM-3, E-SEL, MMP-1, MMP-9, VEGF, VEGF-R2).

3.3. Day 14 (Table 3)

All but four of the inflammation-related proteins showed a monotonic pattern of increasing proportion of children with day-14 concentrations in the top quartile with increasing quartile of day-14 EPO concentrations (RANTES, I-TAC, MMP-1, VEGF-R1).

3.4. Odds ratios of low and high EPO measurements on each day (Figs. 1 and 2)

The middle two quartiles serve as the referent group for all comparisons in Figs. 1 and 2, which depict the odds ratios (and 99% confidence intervals) for having a protein concentration (left) in the top quartile on postnatal days 1, 7, and 14, associated with an EPO concentration in the bottom (Fig. 1) or top (Fig. 2) quartile for that same day. On all three of the days assessed, newborns with top quartile concentrations of CRP, IL-1 β , IL-6, TNF- α , IL-8, and E-SEL each had the pattern of significantly reduced risks of bottom quartile EPO, and significantly increased risks of top quartile EPO. MMP-1 did not show any statistically significant relationship on any day, while IGFBP-1 top quartile concentrations were associated with significantly increased EPO only on day 1.

Top quartile concentrations of 18 other proteins were associated with significantly increased risks on at least one of the three days assessed (SAA, MPO, IL-6R, TNF-R1, TNF-R2, MCP-1, MCP-4, MIP-1 β , ITAC, ICAM-1, ICAM-3, VCAM-1, MMP-9, VEGF, VEGF-R1, and VEGF-R2). Top quartile concentrations of MCP-4 and I-TAC are noted on at least 2 of the three days assessed. However, IL-6R and VCAM-1 were increased on only one of the three days (day 1), and there were no significant elevations of three proteins, RANTES, MMP-1, and IBFBP-3, on any of the three days assessed (Fig. 2).

3.5. Odds ratios of elevated EPO concentrations in newborns who had inflammation proteins elevated on one versus two or more days a week apart (Fig. 3)

The odds of an EPO concentration in the top quartile on day 14 were elevated for 10 inflammation-related proteins that had a concentration in the top quartile on at least one day. The odds of a day-14 EPO elevation were even higher if the concentrations of these inflammation proteins were elevated on two or more days a week apart, and were significant for a total of 17 proteins.

4. Discussion

Our main finding is that, in extremely preterm newborns, the concentration of endogenous EPO is high when the concentrations of inflammation-related proteins are also high, and the odds of elevated EPO concentrations are even higher when systemic inflammation is evident on two separate days, a week apart. These findings are in keeping with several lines of evidence that elevated concentrations of endogenous EPO frequently accompany or precede inflammation [18–20].

We suspect that the relationships we found in extremely low gestational age newborns might also be true for term newborns, given similar birth conditions. However, we are unable to offer evidence to support this notion, as our sample is restricted to infants born less than 28 completed weeks gestation.

In our sample, the concentrations of 18 of the 25 inflammation-related proteins were in the top quartile when EPO measures were in the top quartile, on each of the three days assessed. Further, 6 of the inflammation-associated protein measurements (CRP, IL-1 β , IL-6, TNF- α , IL-8, and E-SEL) were in the top quartile when EPO measurements were in the top quartile, and vice versa, a measurement of these same proteins in the top quartile was least likely when EPO measures were in the bottom quartile.

We speculate that EPO is probably best classified as an acute phase protein with modulatory effects on inflammation. Since pregnancy disorders and fetal distress, which frequently accompany preterm delivery, are associated with elevated EPO concentrations in this and prior studies, we speculate that endogenous EPO may also be a marker for perinatal stress. The following discussion, however, includes three mutually-compatible explanations for our findings.

4.1. Endogenous EPO as an anti-inflammatory agent

A crucial response to injury is the attenuation of inflammation, [31] and the literature provides ample evidence that EPO possesses anti-inflammatory properties [5,31]. One finding that supports an anti-inflammatory role for EPO is that the biologic effects of EPO and TNF- α are antagonistic, each capable of suppressing the synthesis and activity of the other [31]. In addition, several animal studies have documented that the concentrations of pro-inflammatory proteins are reduced following EPO administration, [31–37] as is the expression of the genes for these proteins [5,38]. The pattern we see, then, could represent an anti-inflammatory response to the presence of pro-inflammatory cytokines, waxing and waning with the degree of inflammation.

4.2. Endogenous EPO may be an acute phase protein

Synthesized in the liver in ELGANS, [39] EPO concentrations in our sample varied closely with those of C-reactive protein (CRP), the most classic example of an acute phase protein, also synthesized in the liver [40]. EPO concentrations also paralleled those of the acute phase protein SAA, but did not achieve statistical significance on days 7 and 14.

Some acute phase proteins participate in initiating and sustaining inflammation, while others appear to inhibit it. CRP, for example, activates the complement system and induces inflammatory cytokines and tissue factor in monocytes [41,42]. Similarly, EPO stimulates the NF- κ B pathway (a pro-inflammatory pathway) in the retina of mice, causing apoptosis of neuronal cells [43]. On the other hand, some acute phase proteins have inhibitory (anti-inflammatory) properties, including the anti-oxidants haptoglobin, hemopexin, and α -1-anti-chymotrypsin, which protect the host from reactive oxygen species [40,44]. Therefore, the pattern we see, i.e. EPO varying directly with the acute phase proteins CRP and SAA, suggests the possibility that EPO is acting as an acute phase protein, with both stimulatory and inhibitory effects on inflammation.

4.3. Endogenous EPO as a cytoprotective molecule

A third explanation is that EPO promotes the healing and repair of injured tissues independent of its pro-inflammatory and anti-inflammatory properties. While the

cytoprotective effects of EPO have sometimes been attributed to EPO's *indirect effects* on inflammation (anti-inflammatory), human and animal studies suggest that EPO has direct cytoprotective properties via signal-transduction pathways that inhibit apoptosis and stimulate neurogenesis [45,46]. In one study, EPO had no direct effect on IL-6 or TNF-alpha production, but did reduce other manifestations of inflammation, suggesting that the association between exogenous EPO and reduced inflammation was likely indirect and related to EPO's innate cytoprotective properties, not to its effects on inflammation [47]. Further support for EPO's cytoprotective effects can be seen in two studies of hypoxia-ischemia in rats. In one study, pre-treatment with EPO inhibited apoptosis in the brain, consistent with induction of gene expression [7]. In the second, exogenous EPO reduced both histologic and functional injury to the retina, providing evidence that cytoprotection was mediated by anti-apoptotic mechanisms [48]. The relationship we see, then, between endogenous EPO and inflammation-related proteins which frequently accompany biologic injury, is compatible with the possibility that endogenous EPO plays a role in the prevention and/or repair of tissue injury.

5. Conclusions

Our findings convey information only about preterm infants. In our sample of extremely low gestational age newborns, endogenous EPO concentrations varied with the concentrations of inflammation-related proteins (i.e., cytokine, chemokines, adhesion molecules, matrix metalloproteinases, etc.). We offer three mutually compatible explanations for this. The first two possibilities suggest that alterations in EPO concentrations are not merely epiphenomena, but contribute to subsequent events by either promoting or reducing inflammation. The third possibility is that EPO is an anti-injury/healing-promoting response to tissue injury, which is frequently preceded or accompanied by inflammation. To what extent each of these three possibilities applies to extremely low gestational age newborns remains to be seen.

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Fig. 1.

Odds ratios (and 99% confidence intervals) for having a protein concentration (left) in the bottom quartile on days 1, 7, and 14, associated with an EPO concentration in the bottom quartile for that same day. The referent group for all comparisons consists of infants whose EPO concentration was in the middle two quartiles.

Fig. 2.

Odds ratios (and 99% confidence intervals) for having a protein concentration (left) in the top quartile on days 1, 7, and 14, associated with an EPO concentration in the top quartile for that same day. The referent group for all comparisons consists of infants whose EPO concentration was in the middle two quartiles.

Fig. 3.

Odds ratios (and 99% confidence intervals) for having a day-14 concentration of erythropoietin in the top quartile associated with a blood concentration of the protein listed on the left in the top quartile on one day only, or on two or more days a week apart. The models are adjusted for gestational age.

Table 1

Percent of all children whose day-1 concentration of erythropoietin was in the quartile listed at the top of the column, who also had a concentration of the protein listed on the left in the highest quartile on the same day. These are column percentages.

Protein in top quartile	Erythropoietin, day 1			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
CRP	9	18	30	44
SAA	9	16	29	46
MPO	16	20	25	38
IL-1 β	2	8	24	66
IL-6	8	18	25	49
IL-6R	15	21	29	36
TNF- α	1	4	22	73
TNF-R1	12	13	26	50
TNF-R2	12	12	25	51
IL-8 (CXCL8)	4	3	21	72
MCP-1 (CCL2)	19	22	25	34
MCP-4 (CCL13)	21	20	26	33
MIP-1 β (CCL4)	16	22	24	38
RANTES (CCL5)	20	22	27	31
I-TAC (CXCL11)	13	20	28	39
ICAM-1 (CD54)	11	13	25	52
ICAM-3 (CD50)	14	20	25	41
VCAM-1 (CD106)	14	18	30	39
E-SEL (CD62E)	10	13	23	54
MMP-1	20	24	28	28
MMP-9	21	23	21	36
VEGF	13	21	20	46
VEGF-R1	24	15	24	37
VEGF-R2	14	15	27	44
IGFBP-1	19	22	25	34
Total N	214	216	215	216

Table 2

Percent of all children whose day-7 concentration of erythropoietin was in the quartile listed at the top of the column, who also had a concentration of the protein listed on the left in the highest quartile on the same day. These are column percentages.

Protein in top quartile	Erythropoietin, day 7			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
CRP	13	22	24	41
SAA	18	18	22	42
MPO	16	14	27	43
IL-1β	8	12	21	59
IL-6	8	13	23	56
IL-6R	18	22	31	30
TNF-α	3	8	27	62
TNF-R1	15	14	27	44
TNF-R2	16	18	27	39
IL-8 (CXCL8)	5	6	29	61
MCP-1 (CCL2)	16	24	25	35
MCP-4 (CCL13)	19	23	27	31
MIP-1β (CCL4)	18	13	27	42
RANTES (CCL5)	15	23	34	29
I-TAC (CXCL11)	15	22	27	37
ICAM-1 (CD54)	19	21	22	39
ICAM-3 (CD50)	15	15	31	40
VCAM-1 (CD106)	17	20	34	30
E-SEL (CD62E)	10	21	28	41
MMP-1	20	21	29	30
MMP-9	15	19	28	38
VEGF	13	16	33	39
VEGF-R1	22	14	27	38
VEGF-R2	13	21	26	39
IGFBP-1	22	25	22	34
Total N	215	217	217	218

Table 3

Percent of all children whose day-14 concentration of erythropoietin was in the quartile listed at the top of the column, who also had a concentration of the protein listed on the left in the highest quartile on the same day. These are column percents

Protein in top quartile	Erythropoietin, day 14			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
CRP	13	21	27	39
SAA	19	19	27	35
MPO	13	16	28	43
IL-1β	6	12	22	61
IL-6	7	16	21	57
IL-6R	16	25	27	33
TNF-α	3	8	21	68
TNF-R1	7	16	27	51
TNF-R2	8	18	30	45
IL-8 (CXCL8)	2	6	22	70
MCP-1 (CCL2)	14	19	27	40
MCP-4 (CCL13)	16	22	26	36
MIP-1β (CCL4)	15	19	29	37
RANTES (CCL5)	21	26	32	22
I-TAC (CXCL11)	16	20	35	30
ICAM-1 (CD54)	16	20	24	40
ICAM-3 (CD50)	12	18	30	41
VCAM-1 (CD106)	11	25	27	37
E-SEL (CD62E)	9	19	28	44
MMP-1	25	26	24	25
MMP-9	11	19	29	41
VEGF	13	23	27	38
VEGF-R1	19	14	25	42
VEGF-R2	15	22	26	37
IGFBP-1	17	26	26	32
Total N	195	197	196	198