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Developmental Changes in Circulating IL-8/CXCL8 isoforms in Neonates

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Abstract

Interleukin-8 (IL-8/CXCL8) is widely expressed in fetal tissues although inflammatory changes are not seen. Circulating IL-8 is comprised of an endothelial-derived [ala-IL-8]₇₇ isoform and another, more potent [ser-IL-8]₇₂ secreted by most other cells; [ala-IL-8]₇₇ can be converted into [ser-IL-8]₇₂ by proteolytic removal of an N-terminal pentapeptide from [ala-IL-8]₇₇. In this study, we show [ala-IL-8]₇₇ is the predominant circulating isoform of IL-8 in premature neonates but not in term neonates/adults, who have [ser-IL-8]₇₂ as the major isoform. This isoform switch from the less potent [ala-IL-8]₇₇ to [ser-IL-8]₇₂ correlates with a maturational increase in the neutrophil chemotactic potency of plasma IL-8. The emergence of [ser-IL-8]₇₂ as the major isoform is likely due to increased plasma [ala-IL-8]₇₇-convertase activity and/or changes in the cellular sources of IL-8. Developmental changes in IL-8 isoforms may serve to minimize its inflammatory effects in the fetus and also provide a mechanism to restore its full activity after birth.

Keywords

Interleukin-8; chemokines; isoforms; neonate; neutrophil

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Conflicts of Interest: None.

INTRODUCTION

Interleukin-8 (IL-8/CXCL8) is an important effector of neutrophil functions.¹ In the fetus, IL-8 is widely expressed and has important maturational/angiogenic roles^{2–4} but is not associated with inflammatory changes. IL-8 and its cognate receptors are constitutively expressed in various cell types in the human fetal intestine,^{4,5} hematopoietic system,⁶ and other organ systems.^{7,8} However, despite the expression of IL-8 by fetal cells at biologically relevant levels, there is no increase in the influx of inflammatory cells into fetal tissues. This absence of neutrophils and other inflammatory cells in fetal tissues is intriguing because in rodent models, focal expression of IL-8 is sufficient to recruit neutrophils into intact tissues.⁹

IL-8 is comprised of two major isoforms: a 77-amino acid, endothelial-derived [ala-IL-8]₇₇, and a 72-amino acid [ser-IL-8]₇₂ secreted by monocytes and other cells.^{10,11} [ala-IL-8]₇₇ carries an *N*-terminal pentapeptide extension [alanine-valine-leucine-proline-arginine (AVLPR)], that is cleaved by arginine proteases to convert [ala-IL-8]₇₇ into [ser-IL-8]₇₂.^{12,13} The two isoforms may have different physiological roles; [ala-IL-8]₇₇ has a lower neutrophil chemotactic potency than [ser-IL-8]₇₂ but has specific pro-apoptotic and chemotactic activity for malignant cells.^{11,14–17} In this study, we investigated the hypothesis that IL-8 expression in fetal tissues is not associated with inflammation because the neutrophil chemotactic potency of IL-8 is low in the fetus/premature infant due to the prevalence of the [ala-IL-8]₇₇ isoform. To elucidate the involved mechanisms, we compared [ala-IL-8]₇₇-convertase activity in neonatal and adult plasma and then used an animal (porcine) model to determine developmental changes in IL-8 production by endothelial cells and monocytes, the two most important cellular sources of IL-8 in the body.¹⁸

Materials and methods

Plasma

Cord blood was obtained from 25 preterm (24–32 weeks gestation) and 25 term infants (37–42 wks gestation) delivered by Cesarean section for preeclampsia, abnormal fetal position, and previous sections; pregnancies with possible chorioamnionitis (maternal fever, uterine tenderness, and/or leukocytosis) were excluded. We also obtained plasma samples from 15 healthy adults. The study was approved by the local Institutional Review Board.

Western blots

IL-8 was immunoprecipitated from plasma by using a murine monoclonal antibody (R&D, Minneapolis, MN) and sepharose-immobilized protein A/G (Pierce). To resolve the two isoforms of IL-8, we loaded 50 µg total protein per lane in 15% polyacrylamide gels.¹³ IL-8 was detected using a polyclonal goat anti-IL-8 antibody (R&D) and standard detection systems (Pierce, Rockford, IL).

Enzyme-linked immunosorbent assay (ELISA)

Total IL-8 was measured by a commercially available kit (R&D; detects both [ala-IL-8]₇₇ and [ser-IL-8]₇₂). [ala-IL-8]₇₇ ELISA has been described previously.¹⁹ Both assays have a 10–2000 pg/mL range.

Neutrophil chemotaxis

We immobilized the aforementioned anti-IL-8 antibody in agarose columns (Seize immunoprecipitation kit, Pierce). Immunoprecipitation and elution recovered ≥70% of plasma IL-8.

Neutrophil chemotaxis to IL-8 purified from plasma samples was measured in microchemotaxis chambers (NeuroProbe, Gaithersburg, MD).²⁰ Neutrophils from healthy adults were fluorescence-labeled by using Calcein AM (2 μ M; Invitrogen, Carlsbad, CA).²¹ We used 0.1, 1, 10 and 100 ng/mL of recombinant [ala-IL-8]₇₇ and [ser-IL-8]₇₂ as standards (R&D). In some wells, we placed IL-8 after treatment with α -thrombin (20 nM, 30 min at 37°C; Sigma, St. Louis, MO).¹³ The number of cells that migrated through the filter in 1 hr was calculated by plotting the fluorescence signal in test wells on a standard curve generated from known numbers of labeled cells (485/545 nm).

[ala-IL-8]₇₇ convertase activity of plasma was measured by incubating IL-8 purified from preterm infants in neonatal/adult plasma remaining after immunoprecipitation of IL-8 (37°C, 1 h). In some samples, we added antithrombin III (20 nM, Sigma) to inhibit thrombin and plasmin. Prothrombin and plasminogen concentrations were measured by ELISA (AssayPro, St. Charles, MO).

Porcine aortic endothelial cells (PAECs) and monocytes were harvested from mixed-breed porcine fetuses (80% gestation), neonates, and adult animals (n=3 each). The study was approved by the local Institutional Animal Care and Use Committee.

PAECs were isolated by trypsinization of aortic segments and cultured in M199 media with 10% fetal calf serum (Invitrogen).²² We have shown recently that endothelial IL-8 production is regulated by shear stress, the tangential hemodynamic force exerted on the vascular endothelium by flowing blood.²³ We also showed that low-intensity shear stress (<5 dynes/cm²) resulting from the slower rates of blood flow in the microvasculature is most efficient in inducing IL-8 production in endothelial cells. Therefore, to investigate developmental changes in IL-8 expression, we measured endothelial IL-8 production under static conditions and in the presence of low-intensity shear stress, which is a non-inflammatory, physiologically-relevant stimulus. Endothelial monolayers were exposed to a laminar-flow shear stress of 4 dynes per cm² for 18 h by using a cone-plate apparatus.²⁴ Porcine IL-8 was measured by ELISA (R&D).

Peripheral blood mononuclear cells (PBMCs) were isolated on Percoll gradients after blood sedimentation in dextran.²⁵ Monocytes were separated by adherence to polystyrene (2 h at 37°C). IL-8 production was measured under basal conditions and after stimulation with *E. coli* lipopolysaccharide (LPS; 1 μ g/mL $\times$ 18h; Sigma).

Statistical methods

Non-parametric measures were computed using the software package Sigmapstat 3.1.1 (Systat, Point Richmond, CA).

Results and Discussion

Plasma IL-8 in preterm infants is dominated by the less-potent [ala-IL-8]₇₇ isoform

[ala-IL-8]₇₇ was the major isoform in preterm neonates (gestation mean $\pm$ standard error 26.4 $\pm$ 3.2 weeks), which contrasted with the dominance of [ser-IL-8]₇₂ in adults. Plasma IL-8 from term infants (38.1 $\pm$ 1.2 weeks) was intermediate in composition (Fig. 1A).

We next measured plasma [ala-IL-8]₇₇ and total IL-8 concentrations by ELISA. [ala-IL-8]₇₇ constituted a larger fraction of plasma IL-8 in preterm infants (median 60.5%, range 0–93%) than in term neonates (14.6%, range 0–70.8%) and adults (7.78%, range 0–60.4%; p <0.05; Fig. 1B).

The neutrophil chemotactic potency of IL-8 purified from preterm plasma was lower than that of IL-8 from term infants and adults (Fig. 1C). These differences were abrogated upon treatment with thrombin, a known [ala-IL-8]₇₇ convertase (inset).

[ala-IL-8]₇₇ convertases

To determine the mechanisms involved in the emergence of [ser-IL-8]₇₂ as the dominant IL-8 isoform during maturation, we first investigated developmental changes in plasma [ala-IL-8]₇₇-convertase activity. We incubated IL-8 purified from preterm neonates (rich in [ala-IL-8]₇₇) with neonatal and adult plasma samples and measured total IL-8 and remaining [ala-IL-8]₇₇ after 1 h. The [ala-IL-8]₇₇-convertase activity of adult plasma was higher than that of preterm neonates (Fig. 2A). Consistent with existing literature,^{26,27} the concentrations of prothrombin and plasminogen, the two best-known [ala-IL-8]₇₇ convertases in plasma, were lowest in preterm plasma and increased with maturation (inset). The [ala-IL-8]₇₇ convertase activity of adult plasma was blocked by antithrombin III, which inhibits both thrombin and plasmin (Fig. 2A), thereby emphasizing the importance of these two proteases.

Besides [ala-IL-8]₇₇ convertases, the isoform composition of circulating IL-8 may also be affected by developmental changes IL-8-producing cells. Unlike endothelial cells, monocytes and most other cells efficiently cleave the *N*-terminal AVLPR extension of [ala-IL-8]₇₇ and secrete mainly [ser-IL-8]₇₂ into their microenvironment.^{13,18} Therefore, we next compared developmental changes in IL-8 production by monocytes, which are the major [ser-IL-8]₇₂ secretors in the body,¹³ and the endothelium. We switched to an animal (porcine) model for these studies to obtain primary aortic endothelial cells (AECs) and monocytes at specific developmental stages, which was not possible in human subjects. The porcine model was chosen (over rodents) because of the high degree of similarity between porcine and human IL-8.²⁸

Developmental differences in endothelial and monocyte IL-8 production

We compared AECs and monocytes obtained from the porcine fetus, neonate and adult for IL-8 production *in vitro*. Whereas fetal AECs produced more IL-8 than cells from the neonate and adult, IL-8 production by fetal monocytes was low and increased with maturation (Fig. 2). The observed maturation of IL-8 production by porcine monocytes is similar to human neonates²⁹ and provides an additional mechanism for increased [ser-IL-8]₇₂ in term neonates and adults.

In conclusion, this is the first report of developmental changes in the functional potency of a cytokine. Although developmental differences in isoform composition may minimize the inflammatory effects of IL-8 in the fetus, lower chemotactic potency of IL-8 may contribute to the impaired neutrophil chemotaxis seen in premature neonates.^{20,30} Considering that the *N*-terminally truncated [ser-IL-8]₇₂ isoform may lack some of the biological activities of [ala-IL-8]₇₇,^{11,14–17} differential expression of the two IL-8 isoforms during fetal period may also reflect still unknown developmental role(s) of [ala-IL-8]₇₇.

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Fig. 1. [ala-IL-8]₇₇ is the predominant circulating isoform of IL-8 in preterm infants

A: Western blot of IL-8 immunoprecipitated from neonatal and adult plasma shows that [ala-IL-8]₇₇ is the predominant form of IL-8 in preterm neonates, which contrasts with the dominance of [ser-IL-8]₇₂ in adults. Term infants showed variable amounts of both isoforms. IL-8 isoforms were immunoprecipitated from plasma by using a murine monoclonal anti-IL-8 antibody/immobilized protein A/G, resolved in 15% polyacrylamide gels, and detected using a polyclonal goat anti-IL-8 antibody. The blot shows 3 individual subjects in each group and is representative of at least 3 separate runs with different samples.

B: Dot plots showing that the [ala-IL-8]₇₇ fraction in total plasma IL-8 (expressed as percentage) was significantly higher in preterm neonates (n=25) than in term neonates (n=25) and adults (n=15). The [ala-IL-8]₇₇ fraction in term infants was not significantly different from adults. [ala-IL-8]₇₇ and total IL-8 concentrations were measured by ELISA. Median total

plasma IL-8 concentrations in premature neonates, full-term neonates, and adults were 714 pg/mL (range <10–1503 pg/mL), 440 pg/mL (<10–1364 pg/mL), and 45 pg/mL (<10–321 pg/mL), respectively. Median [ala-IL-8]₇₇ concentrations in these groups were 310 pg/mL (range <10–1299 pg/mL), 41 pg/mL (<10–1054 pg/mL), and 12 pg/mL (<10–69 pg/mL), respectively. The [ala-IL-8]₇₇: total plasma IL-8 ratio was computed as percentage for each sample. Median values are depicted as horizontal bars. Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant.

C. IL-8 purified from preterm plasma has lower neutrophil chemotactic activity than IL-8 from full-term infants and adults. These differences were partially corrected upon treatment with thrombin. Neutrophil chemotaxis was measured in a fluorescence-based microchemotaxis assay against a range of concentrations of IL-8 (0.1–100 ng/mL). Depicted data include the mean number ($\pm$ standard error of the mean) of neutrophils migrating to 1 ng/mL starting total IL-8 concentration. Standards included media alone (not shown), recombinant human [ala-IL-8]₇₇ (rh[ala-IL-8]₇₇), rh[ser-IL-8]₇₂, and rh[ala-IL-8]₇₇ pre-treated with thrombin. Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant. Data represent 5 subjects in each group and two separate experiments.

Inset: Total IL-8 and [ala-IL-8]₇₇ concentrations after thrombin treatment in the rh[ala-IL-8]₇₇ standard and the test IL-8 samples purified from preterm plasma that were used in the chemotaxis assay. Consistent with its activity to convert [ala-IL-8]₇₇ to the more potent [ser-IL-8]₇₂, thrombin reduced the [ala-IL-8]₇₇ fraction in both sets of samples. These data indicate that the lower chemotactic potency of preterm IL-8 is due to the relative abundance of [ala-IL-8]₇₇. Data are representative of at least 3 separate runs with different samples. Groups were compared by the Mann-Whitney *U* test; * denotes $p < 0.05$.

Fig. 2.

2A: Plasma [ala-IL-8]₇₇ convertase activity increases with maturation. We incubated rh [ala-IL-8]₇₇ standard and test samples of IL-8 purified from preterm plasma (that have a large [ala-IL-8]₇₇ fraction) with plasma from preterm neonates, term neonates, and adults for 1 h and compared the remaining [ala-IL-8]₇₇ fraction. IL-8 was immunoprecipitated from preterm plasma by using a murine monoclonal anti-IL-8 antibody immobilized on agarose columns. Bar diagram shows mean ($\pm$ standard error of the mean) total IL-8 and [ala-IL-8]₇₇ concentrations. The [ala-IL-8]₇₇-converting activity was the lowest in preterm plasma, intermediate in term infants, and was the highest in plasma from adult subjects. The [ala-IL-8]₇₇-converting activity of adult plasma was blocked by antithrombin III, which inhibits

thrombin and plasmin, the two best-known [ala-IL-8]₇₇ convertases in plasma. Data represent >5 different samples in each group and at least 3 separate runs with different samples. Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant.

Inset: Prothrombin and plasminogen concentrations were low in the study samples from preterm infants and increased with maturation. Data include samples from preterm neonates ($n=25$), term neonates ($n=25$), and adults ($n=15$). Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant. Prothrombin and plasminogen concentrations correlated negatively with the [ala-IL-8]₇₇: total IL-8 ratios in these samples (Spearman's $r = -0.612$ and -0.542 , respectively; $p < 0.05$; not depicted).

2B, 2C: Developmental changes in the site of IL-8 production may contribute to the emergence of [ser-IL-8]₇₂ as the dominant isoform of IL-8 during maturation. 2B:

Endothelial IL-8 production is high during fetal period and decreases with maturation. Bar diagrams (means $\pm$ standard errors) show basal and shear-activated IL-8 production by porcine aortic endothelial cells isolated from fetal, neonatal, adult pigs ($n=3$ each) *in vitro*. Shear stress, a physiological activator of IL-8 production in endothelial cells, was applied by using a cone-plate apparatus. Porcine IL-8 was measured in supernatants by ELISA and was normalized against total protein concentrations. Endothelial cell viability was monitored by trypan blue exclusion. Data are representative of 3 separate runs with different samples. Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant.

2C. Monocyte IL-8 production increases with maturation. Bar diagrams (means $\pm$ standard errors) show basal and LPS-stimulated IL-8 production by blood monocytes from fetal, neonatal, adult pigs ($n=3$ each) *in vitro*. Porcine IL-8 was measured in supernatants by ELISA and was normalized against total protein concentrations. Cell viability was monitored by trypan blue exclusion. Data are representative of 3 separate runs with different samples. Groups were compared by the Kruskal-Wallis *H* test/Dunn's multiple-comparison post-test; * denotes $p < 0.05$, *N.S.* indicates data not significant.