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Developmental Patterns of Antioxidant Defense Mechanisms in Human Erythrocytes

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ABSTRACT. To obtain a profile of erythrocyte antioxidant defense potential during late fetal development, we studied selected antioxidant parameters in blood samples from 65 neonates with birth wt between 520 and 4210 g and from 12 healthy adults. Erythrocyte superoxide dismutase activity did not change significantly with maturation and no significant differences were observed among preterm infants grouped in increasing birth wt categories, term neonates, and adults. Erythrocyte catalase and glutathione peroxidase, as well as plasma vitamin E levels, showed highly significant positive correlations ($p < 0.001$) with increasing fetal wt and gestational age; by term, CAT activity reached a level similar to the adult control group, but glutathione peroxidase activity, as well as plasma vitamin E levels, were markedly lower in all the preterm and in the term groups than in adults ($p < 0.01$). Erythrocyte glutathione S-transferase activity showed a negative correlation with increasing gestational age ($p < 0.01$) and the adult values were considerably lower than any of the neonatal levels ($p < 0.001$). The role of glutathione S-transferase in erythrocyte metabolism remains obscure. Maturational changes in the activity of the red cell enzymes that were studied and in the plasma vitamin E level were apparent from about 31–36 wk of gestation, suggesting that the stimulation for these changes may have commenced from about 28–31 wk. (*Pediatr Res* 26: 366–369, 1989)

Abbreviations

CAT, catalase
SOD, superoxide dismutase
GSH-Px glutathione peroxidase
GSH-Px[H_2O_2], glutathione peroxidase with hydrogen peroxide as substrate
GSH-Px[t-bh], glutathione peroxidase with t-butyl hydroperoxide as substrate
GST, glutathione S-transferase

The evolution of oxidative metabolic processes within mammalian cells has necessitated the concomitant development of mechanisms to protect vital cell components against oxygen-induced damage that could result from oxygen free-radical species produced during metabolism. These antioxidant systems include both enzymatic and nonenzymatic mechanisms. Vitamin E appears to be the most significant non-enzymatic antioxidant, and CAT, SOD, and selenium-dependent GSH-Px are believed to be the primary enzymatic systems; however, a role has also been identified recently for nonselenium-dependent glutathione peroxidase activity (1), and this appears to be a function of the GST (2).

During fetal development, antioxidant defense mechanisms have generally been considered to be less active than those of the mature newborn infant or of the adult (3–5). It has been suggested that this might reflect a lower requirement for cellular antioxidant activity because of the relatively protective intrauterine environment and the lower perfusing oxygen tension in the fetus (3, 6). Inadequate antioxidant protection has been postulated as a prime factor in pathologic states such as bronchopulmonary dysplasia (6) and retinopathy (7) in premature infants, and an enhanced susceptibility to hemolysis in both preterm and term infants (8). Many studies have evaluated levels of one or more of the antioxidant enzymes in erythrocytes of term infants as compared with adults (3, 8–15), and several reports have described levels of specific enzymes in premature infants or fetuses (16–19). Developmental patterns of antioxidant enzymes have been well documented in fetal rat and rabbit lung (4, 5, 20, 21) and in rat small intestine (22); however, few human developmental data are available on these enzyme systems.

To obtain a profile on antioxidant defense potential in human erythrocytes during late fetal development, we studied blood samples from prematurely born infants with birth wt between 500 and 2500 g, and compared them with results of assays on blood from term infants and adults. We had also previously reported preliminary results on developmental patterns of erythrocyte GST (23), and we sought to expand these data and to evaluate whether a possible relationship could be established between GST and nonselenium-dependent GSH-Px activity.

The study was approved by the Institutional Review Board and appropriate consent for blood sampling was obtained.

MATERIALS AND METHODS

The subjects consisted of 65 neonates with birth wt between 520 and 4210 g and 12 healthy adult volunteers. In the neonates, most of the blood samples were drawn from the cord at the time of delivery, and the remainder via a cubital vein or an indwelling umbilical catheter within approximately 12 h of birth. None of the neonates was known to have hemolytic disease or any other symptomatic hematologic disorders, nor did they manifest any major congenital anomalies. Dry heparin was used as the anticoagulant.

The erythrocytes were separated by centrifugation in a Sorvall RC5 refrigerated centrifuge (Du Pont Co., Biotechnology Systems, Wilmington, DE), and plasma was removed and frozen for later determination of vitamin E levels (24). Erythrocytes were washed three times in cold isotonic saline and resuspended to an hematocrit of approximately 40%. The actual hematocrit was verified for calculation of enzyme activity after assay. Enzyme activities were measured either in freshly prepared hemolysates or from portions frozen for later determination. Stability at −15°C was established in each assay procedure. In all cases

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enzyme activity was determined at 25°C in a Zeiss PM6 spectrophotometer (Carl Zeiss Inc., Thornwood, NY) and was expressed both as U/mL erythrocytes and as U/g Hb.

GSH-Px activity was assayed with H_2O_2 as substrate to determine only selenium-dependent activity (9), and also with t-bh to determine total GSH-Px activity, *i.e.* both selenium-dependent and nonselenium-dependent peroxidase activity (25). The latter assay was carried out according to the method of Beutler (26) modified to give a final substrate concentration of 2.5×10^{-4} M, which resulted in higher activity and also improved assay proportionality considerably. Glutathione S-transferase was measured with 1-chloro 2,4-dinitrobenzene (CDNB) as substrate (27). Superoxide dismutase activity was determined on Hb-free extracts (28) using the xanthine oxidase reaction at a pH of 10.2 as described by Kirby and Fridovich (29). Catalase was assayed according to the procedure of Beutler (30), but readings were taken at 30-s intervals and only the initial 2.5 to 3 min of the reaction was used to calculate enzyme activity. Hb was measured by the cyanmethemoglobin technique (31).

For evaluation of developmental changes, the neonates were grouped in increasing birth wt categories, 500–1000 g ($n = 23$) (P1), 1001–1500 g ($n = 14$) (P2), 1501–2500 g ($n = 14$) (P3), and >2500 g ($n = 14$) (T). The wt groups were then compared by a one-way analysis of variance with respect to each of the parameters that were studied; when a significant intergroup difference was found, Duncan's multiple range test ($p < 0.05$) was performed to determine where the differences occurred. The adult values were compared separately with each neonatal wt group by Student's t tests with Bonferoni's correction for multiple comparisons.

RESULTS

With the exception of SOD, clear developmental patterns emerged for the other antioxidant enzymes (Fig 1). We observed no significant differences in mean activity of erythrocyte SOD among the different premature infant groups studied, nor were there significant differences among the low birth wt groups, term infants, and adults. However, a considerably broader range of enzyme activity was noted within the groups of low birth wt infants.

Erythrocyte catalase activity, GSH-Px activity (with either H_2O_2 or t-butyl hydroperoxide as substrate) and plasma vitamin E levels all showed highly significant positive correlations with increasing birth wt and gestational age ($p < 0.001$). However, GST showed a significant negative correlation with increasing birth wt ($p < 0.01$). By term, fetal erythrocyte catalase activity had reached a level similar to that of the control adult population. However, erythrocyte GSH-Px activity (with either substrate), as well as plasma vitamin E levels, were markedly higher in the adult controls than in the term or preterm populations ($p < 0.01$). Erythrocyte GST activity was considerably lower in the adult group than in term or preterm infants ($p < 0.001$), with no overlap between adult and neonatal values.

Analysis of specific preterm wt groups showed no statistically significant differences between the 500–1000 g (P1) and 1001–1500 g (P2) groups in any of the parameters studied; differences emerged only with advancing gestational age and wt more than about 1500 g, as shown in Figure 1, where enzyme activity is expressed as U/g Hb. When enzyme activity was expressed as U/mL erythrocytes, the findings were very similar. Mean values for the respective groups P1, P2, P3, T and Adults were as follows: Catalase, 33.8×10^3, 36.2×10^3, 38.1×10^3, 44.7×10^3, 44.3×10^3; SOD, 4.94×10^3, 4.61×10^3, 4.99×10^3, 4.74×10^3, 5.32×10^3; GSH-Px[t-bh], 7.18, 6.84, 7.63, 10.73, 18.57; GSH-Px[H_2O_2], 5.69, 5.65, 7.19, 8.14, 10.08; and GST, 2.02, 2.22, 1.88, 1.67, 0.51. Statistical analysis of these results was very similar to that in which enzyme activity was expressed as U/g Hb (Fig. 1); the only significant differences were the observations that for catalase activity A > P2 ($p < 0.05$) in addition to A > P1 ($p < 0.01$), and that for GST activity, significant variability ($p < 0.05$) among the neonatal groups could be demonstrated only between T and P2 (T < P2).

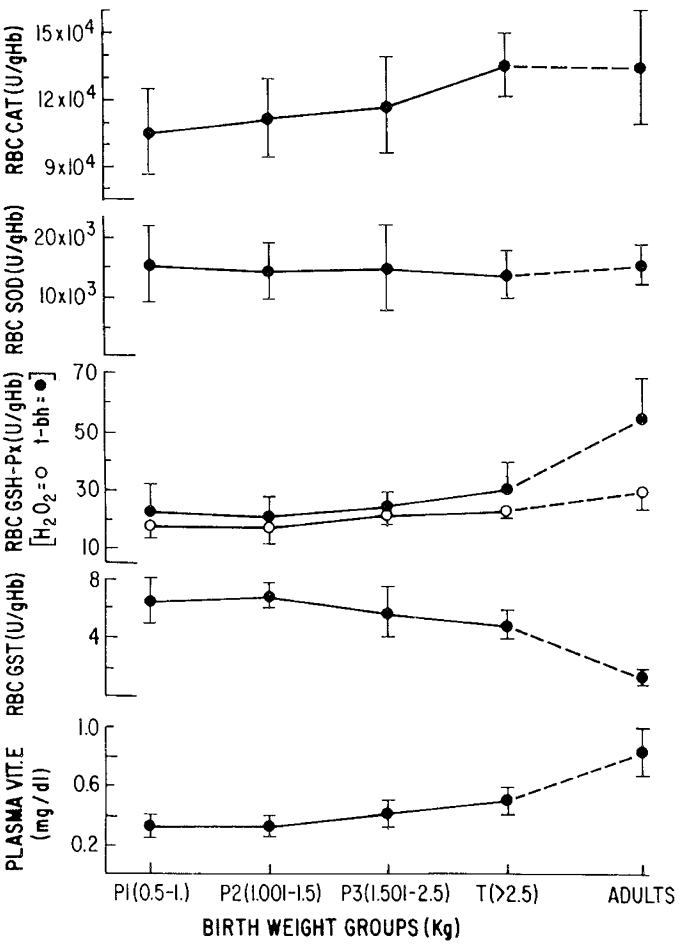

Fig. 1. Developmental patterns of human erythrocyte (RBC) antioxidant enzymes and plasma vitamin E. Points represent group means ± SD. Significant differences among groups: 1) neonatal groups—analysis of variance (ANOVA) and Duncan's multiple range test; and 2) adult *versus* neonatal groups—Student's t test with Bonferoni's correction (See "Materials and Methods.") CAT, *1)* ANOVA: $F = 7.57$, $p < 0.001$; T > P1, T > P2, T > P3; *2)* A > P1($p < 0.01$). SOD, *1)* NS; *2)* NS; GSH-Px[t-bh], *1)* ANOVA: $F = 6.28$, $p < 0.001$; T > P1, T > P2, T > P3; *2)* A > P1, A > P2, A > P3, A > T ($p < 0.01$). GSH-Px[H_2O_2], *1)* ANOVA: $F = 10.13$, $p < 0.001$; T > P1, T > P2, P3 > P1, P3 > P2; *2)* A > P1, A > P2, A > P3, A > T ($p < 0.01$). GST, *1)* ANOVA: $F = 4.05$, $p = 0.01$; T < P1, T < P2; *2)* A < P1, A < P2, A < P3, A < T ($p < 0.001$). Vitamin E, *1)* ANOVA: $F = 18.49$, $p < 0.001$; T > P1, T > P2, T > P3, P3 > P1, P3> P2; *2)* A > P1, A > P2, A > P3, A > T ($p < 0.001$).

On comparing GST and GSH-Px activities, a highly significant overall negative correlation was observed between the two enzymes ($p < 0.001$). When this was examined in each of the wt groups separately to minimize the influence of gestational age, this negative correlation persisted only in the term infants ($p < 0.05$) with GSH-Px[H_2O_2] and with GSH-Px-[t-bh]), and marginally in the 500–1000 g (P1) group ($p < 0.05$ with GH S-Px[t-bh]). No correlation was found in adults between GST and GSH-Px activities. The ratio of enzyme activities of GSH-Px[t-bh]:GSH-Px[H_2O_2] revealed marked differences ($p < 0.01$) between adults and each of the infant groups. (Adult mean ratio = 1.84 ± 0.22 SD; P1 = 1.29 ± 0.53; P2 = 1.28 ± 0.37; P3 = 1.08 ± 0.26; $T = 1.31 \pm 0.22$). No significant differences were detected among the neonatal groups.

DISCUSSION

The results of our investigation demonstrate that antioxidant defense mechanisms in human fetal erythrocytes manifest specific developmental patterns that are somewhat different from those that have been described in other tissues studied in animals. In the fetal rat lung, a marked increase in activity of GHS-Px has been demonstrated during the last 3 d of gestation, with a smaller increase of catalase and SOD activities commencing 2 d before birth and continuing postnatally (4). Similar late gestational changes in lung antioxidant enzymes have been reported in fetal rabbits (5). In rat small intestine, a maturational increase in catalase and SOD activity has also been observed, with most of the increase occurring postnatally (22).

The mature human erythrocyte, in contrast to cells in other tissues, lacks a nucleus and is therefore incapable of protein synthesis and hence of new enzyme formation. This biologic distinction might dictate ontogenic differences between erythrocytes and other cells during fetal development. The mean life span of red cells produced during the last 2 mo of gestation has been estimated to be between 45 and 70 d (32), so that adaptation to the extrauterine environment would require developmental changes in erythrocytes to commence well ahead of term. The measurement of enzyme activity in a sample of fetal erythrocytes at any gestational age reflects the mean value of a constantly changing population of cells, therefore it can be assumed that a variation in enzyme activity of the sample as a whole implies changes produced by an increasing proportion of younger erythrocytes in the sample. Thus, it is likely that the stimulus for an augmentation or reduction in enzyme synthesis must commence several weeks before a significant overall increase or decrease in enzyme activity would be detectable.

Although we did not study fetuses less than 500 g, our observations suggest that most of the changes in antioxidant activity that we investigated were detectable from about 31 to 34 wk gestation, equivalent to a fetal wt of approximately 1500–2000 g (Fig. 1). This would appear to indicate that the stimulus for such changes probably occurs by at least 28 to 30 wk.

In contrast to the other antioxidant indices, we were unable to demonstrate any significant developmental changes in erythrocyte SOD. Our findings were similar to those of Haga and Kran (16), who included a relatively small sample of preterm infants; however, these investigators did report a small but significantly higher value in red cells of adults compared with full-term neonates when their results were expressed as U/g Hb, but not when expressed as U/RBC. Varga *et al.* (18) found higher activity in erythrocytes of term infants than prematures, although relatively few preterm infants were studied. Autor *et al.* (3) reported no significant differences between whole blood SOD activity in healthy preterm infants, sick premature infants, and healthy term infants, but found higher values in adults. However, studies on washed erythrocytes by several other investigators were unable to document significant differences in activity between healthy term neonates and adults (11–13). It appears likely that variations in methodology might account for some of the conflicting results reported on erythrocyte SOD activity.

Erythrocyte catalase activity increased progressively with increasing birth wt, but showed a highly significant rise only at the end of gestation. The levels at term were similar to those in adults. These findings in fullterm neonates are in accordance with those reported by Yoshioka *et al.* (15) and contradict much earlier observations that suggested significantly lower red cell catalase activity in term infants than in adult controls (8, 10). The development of more precise methodology has probably improved assay techniques for catalase.

The developmental patterns of GSH-Px and of GST in human erythrocytes are of particular interest. Previous studies had demonstrated that erythrocyte levels of selenium-dependent GSH-Px, with H_2O_2 as substrate, were higher in adults than in neonates (8, 9), and were almost invariably higher in the mother than in her own fetus (9). Our study shows a developmental increase in GSH-Px detectable only after about 32–35 wk gestation, and appears to extend the observations of Lestas and Rodeck (19), who found no significant change in the level of activity of this enzyme in fetal erythrocytes obtained by fetoscopy between gestational ages ranging from 17–24 wk. At that gestational age they noted mean fetal GSH-Px activity to be about half that of their adult subjects, which included pregnant mothers.

The metabolic function of erythrocyte GST is not clear. The glutathione S-transferases are a group of isoenzymes present in various tissues, including liver and erythrocytes. They have been shown to be capable of detoxifying various endogenous and exogenous substances by conjugation with glutathione, and might act as ligands for nonsubstrate compounds such as heme and bilirubin (33). They also exhibit variable levels of nonselenium-dependent GSH-Px activity against organic hydroperoxides, but not against H_2O_2 (2, 33). Human red cells have been said to contain only GSTπ, a form of transferase that has a relatively low sp act with cumene hydroperoxide and therefore poor glutathione peroxidase activity (33). However, in our study the adults' erythrocytes exhibited a proportionately greater increase in reactivity of GSH-Px with t-bh than with H_2O_2, as compared with the fetal red cells (Fig. 1). This suggested the possibility of a significant level of nonselenium-dependent peroxidase activity, and appeared to conflict with our own observations of lower erythrocyte GST activity in adults and with Strange's studies that were unable to detect differences between fetal and adult erythrocyte GST (34). An alternative and more likely explanation for the discrepancy between fetal and adult erythrocytes might be found in the possible occurrence of different forms of selenium-dependent GSH-Px during development. Indeed, Ganther and Kraus (35) have described the existence of at least three forms of the selenium-dependent enzyme according to the degree of oxidation or reduction of the enzyme. We are currently investigating this possibility to account for the difference observed between fetal and adult cells.

Overall, the maturational changes in the antioxidant factors that we investigated, manifesting as they do from about 31–34 wk gestation, raised the possibility that they may represent a commencing adaptation to the higher extrauterine oxygen environment. However, the presence of generally adequate erythrocyte SOD activity at 25–28 wk gestation is at variance with the relatively low levels of CAT, GSH-Px, and plasma vitamin E at that gestational period, as well as with the low levels of SOD reported in human fetal lung tissue at midgestation (3). This suggests that fetal levels of antioxidant enzyme activity might not be determined by the intrauterine oxygen tension alone, and that additional factors might play a role.

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