Deterioration of Membrane Morphology, Phospholipids, and Cytoskeletal Protein in Rat Erythrocytes Exposed to tert-Butyl Hydroperoxide: Protection by Exogenous Glutathione Fails in Selenium Deficiency

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Summary Here we report scanning electron microscopy, phospholipid and fatty acid composition and cytoskeleton proteins of erythrocytes from Se-adequate and Se-deficient rats, showing protection by glutathione (GSH) against tert-butyl hydroperoxide (t-BuOOH)-induced hemolysis of erythrocytes from Se-adequate, but not from Se-deficient rats. Without exogenous GSH, erythrocytes incubated with t-BuOOH exhibited remarkable deterioration of cell membranes with diminished membrane phosphatidyl-ethanolamine, -serine, and -inositol and selective loss of a cytoskeletal protein, ankyrin 2-1. Without exogenous GSH these changes occurred in erythrocytes from both Se-deficient and Se-adequate rats. Dietary Se, through provision of glutathione peroxidase (GSH-Px) in erythrocytes as a probable scavenger of t-BuOOH, protects against hemolysis when GSH is available.

Key Words selenium, selenium deficiency, glutathione, tert-butyl hydroperoxide-induced hemolysis, membrane phospholipid, cytoskeleton, glutathione peroxidase, ankyrin

The pliability and elasticity of erythrocytes' membranes are essential to accommodate their continual passage through the circulation for a long time. To keep erythrocytes flexible and durable, membrane phospholipids and cytoskeletal proteins must be protected from oxidative damage by antioxidants such as tocopherol and by enzymatic detoxification system such as glutathione peroxidase (GSH-Px), catalase, superoxide dismutase (1). Once the erythrocyte cell incurs damage to its membrane phospholipids and (or) cytoskeleton protein, by in-
teraction with denatured hemoglobin or active oxygen radicals, the flexibility of the cell is lost and it shows abnormal cell shape.

GSH-Px catalyzes decomposition of hydrogen peroxide and organic peroxides, thereby affording protection against oxidative damage to cells and tissues (2, 3). In selenium (Se)-deficient animals, GSH-Px activity is low (4), because the active center of GSH-Px is selenocysteine (5). In a long-term study of Se deficiency, uncomplicated by other dietary factors, we compared susceptibility of erythrocytes from Se-deficient and Se-adequate rats to hemolysis induced by tert-butyl hydroperoxide (t-BuOOH) under varied conditions (6, 7). We also showed that dietary Se afforded protection against t-BuOOH-induced hemolysis in vitro when exogenous GSH was provided, and that α-tocopherol gave no protection (7, 8). We have further examined membrane morphology and phospholipids, and cytoskeletal proteins of erythrocytes from Se-deficient and Se-adequate rats to determine whether dietary Se protected against membrane damage as well as against hemolysis.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats were fed semipurified Se-deficient and Se-adequate diets for 3 months, as described previously (6).

t-BuOOH-induced hemolysis test. t-BuOOH-induced hemolysis of Se-deficient and of Se-adequate rats’ erythrocytes was followed by the procedure as described previously (6).

Morphological observation of hemolyzed and of nonhemolyzed erythrocytes. Morphological changes of erythrocytes during t-BuOOH-induced hemolysis test were examined by a scanning electron microscope (Hitachi S-450). Erythrocytes were fixed with 1% glutaraldehyde in isotonic 0.45% NaCl–1/30 M potassium-phosphate buffer (pH 7.4) at 0–5°C for 4 h and further fixed with 1% osmium tetroxide overnight at room temperature. In place of 3% solution which is conventionally used, an isotonic 1% glutaraldehyde solution was used to avoid formation of echinocytes due to too high osmotic pressure. The cells were dehydrated stepwise by ethanol with increasing concentration from 30 to 100%, followed by isoamyl acetate. After dehydration, erythrocytes were dried over liquid carbon dioxide by critical point dryer [Hitachi critical point dryer HCP-2], then prepared for coating with gold by an ion coater (Eiko Ion Coater IB-3).

Analysis of phospholipid composition of erythrocytes. Erythrocytes were subjected to total lipid extraction by modified Bligh and Dyer’s method (9) in which 0.001% butylated hydroxytoluene was added to avoid peroxidation of the membrane lipids during lipid extraction. Total phospholipid contents were determined by Bartlett’s method (10). The extracted total lipid was subjected to thin-layer chromatography on Silica Gel G thin-layer plate (250 μm thick, E. Merck) for analysis of membrane phospholipid composition. Samples spotted on the plate were developed by two-step single dimensional development (11); i.e., chloroform : methanol : acetone : acetic acid : water = 100 : 50 : 100 : 4 : 10 (v/v) for

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the first step development, and chloroform : methanol : acetic acid : water = 180 : 150 : 30 : 10 (v/v) for the second step development. The individual phospholipid component was determined by using a densitometer according to the method of Rouser et al. (12).

Analysis of fatty acid composition of phospholipids. After isolation of total phospholipids from the total lipid on thin-layer plates developed with hexane : diethyl ether : acetic acid (70 : 30 : 1, v/v), fatty acids moieties were trans-esterified to fatty acid methyl esters with 0.5 N sodium methoxide in methanol at 60°C for 20 min (13). Fatty acid methyl esters thus prepared were extracted with n-hexane, then subjected to gas-liquid chromatography using a separation column of 2 m x 3 mm i.d. packed with 10% Silar 10C on Chromosorb W (100–120 mesh, acid washed, base washed, and silanized) in a Shimadzu gas chromatograph GC-9A equipped with a flame ionization detector. Column temperature was raised from 160 to 240°C at a rate of 4°C/min. Fatty acid methyl esters were chromatographed and identified on an LKB-9000S gas chromatograph-mass spectrometer equipped with computer system DGB-300. The samples were separated on a column (3 mm i.d. x 2 m) of 10% Silar 10C coated on Chromosorb W (100/120 mesh, acid washed, base washed, silanized). Column temperature was raised from 160 to 240°C at the rate of 4°C/min, and carrier gas (He) flow rate was 30 ml/min. The temperatures of molecular separator and ion source were maintained at 270 and 290°C, respectively. Ionization energy was 22 eV, and acceleration voltage was 3.5 kV.

SDS-polyacrylamide gel electrophoresis (PAGE) of erythrocyte proteins. To examine if any compositional changes of erythrocyte proteins are found in the non-hemolyzed erythrocytes, erythrocytes were subjected to gel electrophoresis on the 10 x 10 cm SDS-polyacrylamide gel (PAG) with linear gradient gel concentration from 4 to 20% (Daiichi Kagaku Chemicals Co., Ltd., Tokyo). After the incubation, erythrocytes were collected by centrifugation, and erythrocyte ghosts were subjected to the electrophoresis. The unhemolyzed cells were subjected to electrophoresis after disruption with water. Procedures of electrophoresis were essentially the same as employed by Laemmli (14). The amount of the protein applied was 50 μg for each sample. Protein concentration was determined by Lowry’s method (15). Electrophoresis was run at a constant current of 60 mA at room temperature. Protein bands were visualized by the silver staining method (16) using silver-stain kit purchased from Daiichi Kagaku Chemical Co., Ltd. (Tokyo).

Miscellaneous analysis. Total hemoglobin was estimated as azide-methemoglobin (17). GSH-Px activity and glutathione reductase activity were measured by the methods of Little et al. (18), and Racker (19), respectively.

Statistic analysis of data. Statistic analyses of data obtained were made by Student’s t-test and Duncan’s multiple range test (20).
RESULTS

Hemolysis of Se-adequate and Se-deficient erythrocytes with or without GSH

Hemolysis of erythrocytes from Se-adequate and Se-deficient rats after 2- and 4-h incubation with t-BuOOH is shown in Table 1. Remarkable suppression of hemolysis was observed only in erythrocytes from Se-adequate rats incubated with externally added 2 mM GSH. Hemolysis rate was only 6 ± 2% after 2-h incubation, and still as low as 11 ± 2% even after 4-h incubation. Without addition of GSH, however, even Se-adequate erythrocytes hemolyzed severely. No effect of external GSH addition was observed for Se-deficient erythrocytes.

Morphological changes of erythrocytes of Se-adequate and of Se-deficient rats during the incubation with t-BuOOH

Scanning electron microscopy was conducted to examine if these erythrocytes show any morphological changes by the incubation with 0.6 mM t-BuOOH (Fig. 1). Erythrocytes from Se-adequate rats hemolyzed, and aggregated severely after 2-h incubation when GSH was not added externally (Fig. 1C), whereas those incubated with externally added GSH maintained their intact structure after 4-h incubation with 0.6 mM t-BuOOH (Fig. 1B).

On the other hand, erythrocytes from Se-deficient rats severely hemolyzed and aggregated after 2-h incubation in spite of addition of GSH (Fig. 1E). Prior to the hemolysis and aggregation, considerable numbers of deformed erythrocytes with spurs (echinocytes) were observed both in the Se-adequate erythrocytes incubated without GSH (Fig. 1G) and in Se-deficient erythrocytes incubated with GSH. It is

Table 1. tert-BuOOH-induced hemolysis\(^a\) of erythrocytes from Se-adequate and Se-deficient rats with and without added GSH.

| Incubation system                  | Hemolysis (%) |
|-----------------------------------|---------------|
|                                   | Se-adequate   | Se-deficient |
| Basal medium\(^b\)                |               |              |
| 2 h                               | 88 ± 4        | 90 ± 5       |
| 4 h                               | 98 ± 2        | 99 ± 1       |
| Basal medium + 2 mM GSH           |               |              |
| 2 h                               | 6 ± 2         | 88 ± 3       |
| 4 h                               | 11 ± 2        | 92 ± 3       |

\(^a\)Values are means ± SE, \(n=4\). \(^b\)Basal medium comprises isolated 10% erythrocytes as hematocrit value, 1 mM KCN, 1 mM NaN\(_3\) in isotonic saline-potassium phosphate buffer (pH 7.4) in 1 ml plastic centrifugation tube. Hemolysis experiment was started by the addition of freshly prepared 0.6 mM t-BuOOH to the medium and incubated at 37°C with continual gentle shaking. GSH was added just before addition of t-BuOOH. Other experimental details are described elsewhere (6).

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unlikely that the high osmotic pressure during the fixation process caused artificially echinocyte-like deformations. Almost no deformation was observed in the Se-adequate erythrocytes incubated with externally added GSH (Fig. 1B).

Compositional changes of membrane phospholipids of erythrocytes incubated with t-BuOOH

Phospholipid composition of erythrocytes from Se-adequate and from Se-deficient rats which were incubated with or without t-BuOOH were compared (Fig. 2). There were no significant differences in phospholipid composition between Se-adequate erythrocytes and Se-deficient erythrocytes just collected after 3-month feeding. On the other hand, hemolyzed erythrocytes, namely, Se-adequate erythrocytes incubated without addition of GSH and those from Se-deficient erythrocytes irrespective of GSH addition in the incubation medium, showed a remarkable decrease of phospholipids, especially of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), after incubation for 4 h. The content of phosphatidylcholine (PC) did not decrease so much except for erythrocytes from Se-deficient rats incubated with GSH. Sphingomyelin (SM) did not decrease in any cases.

Alteration of fatty acid composition of erythrocyte membrane phospholipid by the incubation with t-BuOOH

No differences were recognized between Se-adequate and Se-deficient erythrocytes, in the fatty acid composition of total phospholipid (Fig. 3). However, after they were incubated with t-BuOOH with or without GSH, remarkable differences developed. The Se-adequate erythrocytes without GSH addition and Se-deficient erythrocytes with and without GSH addition, in which severe hemolysis took place by the incubation with t-BuOOH for 4 h, showed remarkable decreases in polyunsaturated fatty acids, arachidonic acid (20:4), docosatetraenoic acid (22:4), docosapentaenoic acid (22:5), and docosahexaenoic acid (22:6) in particular took place. The fatty acid that decreased most was docosahexaenoic acid of Se-deficient erythrocytes incubated with GSH and t-BuOOH. Arachidonic acid, the major polyunsaturated fatty acid, decreased by 50% during the first 2 h of incubation.

Erythrocytes from Se-adequate rats which were incubated with GSH and t-BuOOH did not show any changes in fatty acid composition of total phospholipid.

Fatty acid compositions of PE from Se-adequate erythrocytes without incubation and those after 2-h incubation with t-BuOOH and 2 mM GSH were 15.0 and 11.2% for 16:0, 14.7 and 12.7% for 18:0, 12.2 and 12.1% for 18:1, 7.5 and 7.1% for 18:2, 39.4 and 36.0% for 20:4, 6.1 and 6.6% for 22:5, 4.2 and 2.7% for 22:6, while those for without external GSH addition were 8.7% (16:0), 8.7% (18:0), 8.8% (18:1), 6.7% (18:2), 18.7% (20:4), 2.1% (22:5), and 1.0% (22:6). Because these are data obtained from one measurement, the inferences are merely tentative.

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Fig. 1. Scanning electron micrographs of erythrocytes from Se-adequate and Se-deficient rats, as affected by \( t \)-BuOOH in the presence or absence of GSH.

Figure 1A shows scanning electron micrograph (SEM) of Se-adequate rat's erythrocytes before incubation. Figure 1B shows Se-adequate erythrocytes incubated with 0.6 mm \( t \)-BuOOH and 2 mm GSH for 4 h at 37°C. Figure 1C shows erythrocytes incubated with 0.6 mm \( t \)-BuOOH in the basal medium at 37°C for 2 h. Note severe hemolysis and cells with abraded and disrupted membranes. Figure 1D shows SEM of Se-deficient erythrocytes without incubation. Figure 1E shows Se-deficient cells incubated for 2 h with GSH addition, and Fig. 1F is SEM of Se-deficient cells without GSH addition. Figure 1G shows Se-adequate erythrocytes incubated with \( t \)-BuOOH but without GSH addition (60 min, 37°C). Note erythrocytes with echinocyte-like structures. Bar lengths represent 5 or 0.5 \( \mu \)m, respectively.
Compositions of erythrocyte membrane phospholipids from Se-adequate and Se-deficient rats, and their alterations after incubations under different conditions. Bar graphs show contents of total phospholipid and major phospholipids (nmol per mg protein, as mean ± SEM for 4 measurements). Control, phospholipid content without incubation; BM, incubated with 0.6 mM t-BuOOH for 2 h at 37°C in the basal medium; BM+GSH, incubated with 0.6 mM t-BuOOH and 2 mM GSH for 2 h at 37°C in the basal medium. TPL, total phospholipid.

**Altering of composition of erythrocyte protein by incubation with t-BuOOH**

Along with the decrease of phospholipid species as described above, a specific loss of cytoskeleton protein, ankyrin, was observed on the SDS-PAGE pattern of the hemolyzed erythrocytes (Fig. 4). Comparing with the protein patterns of erythrocytes on the SDS-PAG, no significant difference was observed between that of Se-adequate (lane 1) and Se-deficient (lane 4). For the Se-adequate erythrocyte incubated with 0.6 mM t-BuOOH in the basal incubation medium, selective loss of a band corresponding to ankyrin (215 kDa, lane 2) was recognized as early as after 2 h of incubation, then loss of spectrin 2 band (225 kDa) followed. Similar results were obtained from Se-deficient rat erythrocytes incubated with the basal medium (lanes 4–6). On the other hand, Se-adequate erythrocyte incubated with t-BuOOH and externally added GSH sustained their components even after 4-h incubation (lane 8), i.e., ankyrin, spectrin 1 and 2 were clearly detected at 215, 225, and 240 kDa, respectively. Such a protective effect of GSH, however, was not observed with Se-
Fig. 3. Fatty acid composition of total phospholipid fraction in erythrocyte membranes of Se-adequate and of Se-deficient rats under different conditions. The erythrocytes were incubated in the individual medium for 2 h at 37°C as described in MATERIALS AND METHODS. Data are presented as percentages of total fatty acids in total phospholipid fraction of unincubated control erythrocytes (mean ± SEM, n=4). The percentages were corrected for the recovery of margaric acid (17:0) used as internal standard.

deficient erythrocytes (lane 9) at all. No loss other than ankyrin and subsequent spectrin loss during the hemolysis was observed on the SDS-PAGE.

DISCUSSION

In this report we showed that the Se-adequate erythrocytes incubated with 0.6 mM t-BuOOH and 2 mM GSH for 4 h still maintained intact cell shape, whereas those without GSH were severely aggregated and some cells had many holes on the surface (Fig. 1). Morphological changes, especially during the early stage of incubation showed remarkable difference between Se-adequate erythrocytes with GSH and other erythrocytes (Se-adequate erythrocytes without GSH addition and Se-deficient erythrocytes with and without GSH). Sustaining intact morphology of Se-adequate erythrocytes by the external GSH addition strongly suggests that GSH-
Fig. 4. SDS-PAGE pattern of erythrocytes from Se-adequate and Se-deficient rats as effected by incubation with 0.6 mM t-BuOOH under different conditions. Erythrocyte ghosts were applied on the 4 to 20% linear gradient SDS-PAG, and processed for 90 min at 60 mA with constant current. Protein bands from erythrocyte ghosts were visualized by silver staining (16): Lane 1, Se-adequate without incubation; lane 2, Se-adequate, incubated for 2 h in the basal medium (BM); lane 3, Se-adequate, incubated for 4 h in the BM; lane 4, Se-deficient, without incubation; lane 5, Se-deficient, incubated for 2 h in the BM; lane 6, Se-deficient, incubated for 4 h; lane 7, Se-adequate, incubated for 2 h with 2 mM GSH; lane 8, Se-adequate, incubated for 4 h with 2 mM GSH; lane 9, Se-deficient, incubated for 2 h with 2 mM GSH; lane 10, Se-deficient, incubated for 4 h with 2 mM GSH. Arrows A and B identify ankyrin (band 2-1) and spectrin-2 (band 2), respectively.

Px, which decreased in activity to a barely detectable level in Se-deficient erythrocytes (6, 7), plays homeostatic roles in cooperation with GSH.

Erythrocytes that did not hemolyze during incubation with t-BuOOH and GSH sustained their membrane phospholipids and their fatty acid composition. The major phospholipids of erythrocytes from Wistar rats that we used were PE, PC, SM, PS, and PI. The phospholipid composition was similar to that reported by Kameda et al. (21); however, it was considerably different from that reported by Nelson (22). The phospholipid composition of the unhemolyzed erythrocyte did not change at all throughout the incubation (2 and 4 h). The fact that no loss of the phospholipids and their component fatty acids occurred in the Se-adequate erythrocytes when incubated with t-BuOOH and externally added GSH indicates the intactness of erythrocyte membranes even after 4 h. In contrast, PE, PS, and PI, which have been known to localize inside the erythrocyte membrane (23, 24), decreased specifically in the hemolyzed erythrocytes suggesting that damage was initiated intracellularly. Furthermore, in the hemolyzed cells polyunsaturated fatty

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acids, especially arachidonic acid (20:4), remarkably decreased (Fig. 3). Arachidonic acid is the major component of polyunsaturated fatty acids of PE. The preferential disappearance of PE and arachidonic acid are seen in many reports, regardless of what is used to induce hemolysis (25–27). Remarkable decrease of arachidonic acid (20:4) in PE fraction of hemolyzed erythrocytes is consistent with other reports on the hemolyzed erythrocytes (21, 26).

The gross morphological changes into the echinocyte conformation (consisting of spherical cells with spicules over the cell surface) must be caused by oxidative damage to cytoskeleton protein, and probably to membrane phospholipids, especially to PE. Since ankyrin hinges between spectrin and membrane integral protein “band 3” that participates in anion exchange (exchange of bicarbonate and chloride between cytosol and cell membrane), the loss of ankyrin could cause morphological change by failure of support, and may also lead to failure of osmotic balance in the erythrocyte. Morphological changes similar to those we observed were reported by Rice-Evans et al. (28) for t-BuOOH-treated human erythrocytes.

The data obtained from SDS-PAGE are quite informative. The most interesting modification of the protein profile during hemolysis was selective disappearance of the band 2-1, or ankyrin (215 kDa). Ankyrin is an important cytoskeleton protein which binds to β-chain of spectrin to membrane protein, band 3, which is responsible for anion exchanging (29–32). Thus, ankyrin supports a cytoskeleton protein, spectrin, to give durability and flexibility on the one hand, and may also control osmolality (by band 3). Rice-Evans et al. (28) reported a concomitant increase of ankyrin 2-3 band on the SDS-PAGE of human erythrocytes; however, we did not find such a change. Some investigators reported that spectrin first disappeared during t-BuOOH treatment of erythrocytes (33, 34), whereas Sullivan and Stern (35) reported that all the proteins disappeared randomly.

There was no loss of cytoskeleton bands of erythrocytes of Se-adequate rat incubated with externally added GSH and t-BuOOH on the SDS-PAG, in our study. This supports and explains the intact feature of those erythrocytes under the microscopic observation. Our findings showed that ankyrin (not spectrin) is the first cytoskeleton protein that disappeared during hemolysis, and this basically supports the data by Rice-Evans et al. (28).

It has been generally regarded that t-BuOOH represents an ideal model system for elucidating the sequence of intracellular events underlying the oxidative hemolysis that are still largely unknown. And t-BuOOH-treated erythrocytes have been used as a model for studying the effects of endogenously generated lipid hydroperoxides (25, 36). On the contrary, our understanding is that t-BuOOH-induced hemolysis does not elucidate the effect of endogenously generated lipid peroxidation, but it is worthwhile to elucidate the oxidative damage caused by low-molecular-weight hydroperoxide that may not be generated inside the cell; however, hydrogen peroxide is generated inside the cell. Our findings address the importance of GSH-Px as the main protector against oxidative damage inside the cells.

It is clear that the suppression of the hemolysis (in vitro) is closely related to
previous supply of dietary Se \textit{(in vitro)} and availability of GSH during incubation of erythrocytes. However, the disappearance of ankyrin band in \textit{t}-BuOOH-induced hemolysis was not prevented by the \textit{in vitro} addition of Se in the form of Na$_2$SeO$_3$ (data not shown).

Regarding the effect of externally added GSH on the suppression of the hemolysis, its role could be explained by some mechanism(s) other than as substrate for GSH-Px, because GSH cannot be transported through erythrocyte membranes (37). It does not directly act as butoxyl radical scavenger either. Its function could be considered as a stabilizer of erythrocyte in cooperation with GSH-Px. The protective role of GSH to the sulfhydryl (SH) group of membrane protein has been reported (38). Since we could suppress the \textit{t}-BuOOH-induced hemolysis by externally added cysteine (data not shown) but not by glutathione disulfide (6), the role of externally added GSH should be delineated by linkage to the function of GSH-Px as a stabilizer of membrane system. Another hypothesis that externally added GSH acts to transport reducing equivalent of electron into cytosol \textit{via} SH group of membrane proteins may be possible.

On the other hand, criticism of the hypothesis of GSH-Px as lipid peroxide scavenger was raised by Trotta \textit{et al.} (36) in the following terms: that “Se-dependent GSH-Px (the only GSH-Px in red cells) plays a role in reduction of membrane lipid peroxides (39, 40) has come under question since the demonstration that Se-dependent GSH-Px does not utilize phospholipid-bound fatty acid hydroperoxides as substrates (41–44) whether they are in free solution or incorporated into lipid bilayers.” Although we do not have enough data to argue against the scheme presented by Trotta \textit{et al.} (36), their hypothesis seems to have overestimated the protective role of hemoglobin and GSH by themselves, and underestimated the role of GSH-Px. If the methemoglobin molecule does scavenge the butoxyl radical, as they insist, it should be scavenged by methemoglobin which is formed from oxyhemoglobin as shown in their figure. Furthermore, if free GSH scavenges \textit{t}-butoxyl radical, as they insist, the hemolysis of Se-deficient erythrocytes should have been suppressed also in our experiment, because in the Se-deficient erythrocyte the GSH content is two times as much as Se-adequate erythrocyte, and methemoglobin should more easily be formed in the Se-deficient rat’s erythrocytes than in the Se-adequate.

We present here our own concept showing how \textit{t}-BuOOH-induced hemolysis is caused in the Se-deficient erythrocyte and suppressed in the Se-adequate erythrocyte (Fig. 5). The amphiphatic molecule, \textit{t}-BuOOH, penetrates easily inside the erythrocyte, and probably other types of cells. Some may stay inside the membrane. In the Se-deficient erythrocyte, \textit{t}-butoxyl radical (\textit{t}-BuO$^\cdot$), generated from \textit{t}-BuOOH by the interaction with ferrous ion or oxyhemoglobin (27) in the cytosol, attacks ankyrin and polyunsaturated fatty acyl residue of inner membrane phospholipid such as PE. The attacked erythrocyte deforms into an echinocyte-like structure prior to hemolysis. Membrane portions where vitamin E exists are protected from oxidative damage because \textit{t}-butoxyl radical is immediately scav-

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Fig. 5. Representation of erythrocyte membrane treated with t-BuOOH, as affected by dietary selenium deficiency (left side) or selenium adequacy (right side), showing postulated mechanism of t-BuOOH-induced hemolysis and protection afforded by GSH-Px. Left half represents simplified model for erythrocyte membrane and cytoskeleton protein of Se-deficient erythrocyte, and right half is the model for Se-adequate erythrocyte. In these models, cholesterol and major membrane protein except for band 3 are omitted.
enged by vitamin E; however, vitamin E does not have the ability to move rapidly enough to scavenge radicals existing far away on the inner surface of biomembrane. Free GSH, by itself, does not have enough scavenging ability to protect against t-butoxyl radical. The ankyrin attacked by t-butoxyl radical, on the other hand, becomes oxidized and degenerated. Erythrocytes from Se-adequate rats contain enough GSH-Px to maintain their functionality under such oxidative stress when GSH is abundant. However, the mechanism of beneficial effects of extracellular GSH remains unknown, although it has been discussed in terms of increased GSH availability for protection of cells from exogenous and endogenous toxicants through GSH-S-transferase activities (45).

Rats that provided erythrocytes for the present study have been observed throughout early, progressive, and long-term, uncomplicated Se deficiency (Yasumoto, K.; unpublished data). Damage to cells and tissues in Se-deficient rats progressed to overt pathologies, involving far more than erythrocyte susceptibilities, such as cartilage derangement, lens cataracts, and evident tumors, for which protection was afforded by dietary selenite. These findings (8) indicate that the mechanisms discussed in our concept of erythrocyte protection by dietary selenium extend to other cells of the body as well.

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