Membrane proteins in senescent erythrocytes

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The examination of erythrocyte senescence has been facilitated by recent advances in techniques for the isolation of aged red cells. One of these methods, which uses biotinylated rabbit erythrocytes, has been used to examine the state of membrane proteins in effete cells. These aged red cells were found to have normal ratios of α -spectrin and β -spectrin as well as normal levels of ankyrin. The observation concerning ankyrin is particularly important due to the sensitivity of this protein to proteolysis and the postulated action of proteinases in the aging process. The senescent erythrocytes were also found to have an altered ratio of bands 4.1a and 4.1b without any apparent change in the total level of 4.1. In addition, the analysis of the aged cell membranes did not show any large-molecular-mass aggregated protein at the origin of the SDS/polyacrylamide gels, indicating a lack of transglutaminase activity in the senescence process for rabbit erythrocytes. These results indicate that aging of the rabbit erythrocyte is not accompanied by gross proteolytic degradation or transglutaminase-catalysed cross-linking of membrane components.

INTRODUCTION

The mammalian erythrocyte has a well-defined life-span, which varies between species but is exceptionally constant within a species (Vacha, 1983). The nature of the biological clock which determines this life-span is unknown, although a variety of hypotheses have been proposed (Clark, 1988). Some of the more prominent theories include modification of surface glycoproteins (Galili *et al.*, 1985; Low *et al.*, 1985; Lutz *et al.*, 1987), loss of calcium homeostasis (Clark, 1988), activation of cellular proteinases (Kay *et al.*, 1983; Morrison *et al.*, 1987), and loss of deformability through the action of transglutaminases (Lorand *et al.*, 1979; Swislocki & Tierney, 1985). Until recently the examination of erythrocyte senescence was hindered by the inability to isolate definitively aged cells for biochemical examination (Beutler, 1985; Clark, 1988). However, the introduction of two experimental techniques (Ganzoni *et al.*, 1971; Suzuki & Dale, 1987) and one experiment of nature (Beutler & Hartman, 1985) have now made it easier to examine these cells and ask significant questions concerning the nature of red-cell senescence.

The current study was undertaken to examine the integrity of several membrane proteins in senescent erythrocytes. The primary protein studied was ankyrin, also known as band 2.1, which is a 215 kDa molecular mass protein and a key component of the erythrocyte's skeleton (Bennett & Stenbuck, 1980). This molecule serves as an anchor for the attachment of the skeletal matrix to the membrane, and modification of ankyrin has been shown to affect dramatically the deformability of the erythrocyte (Jinbu *et al.*, 1984). The critical aspect of ankyrin, which makes it the focus of this study, is its extreme sensitivity to proteolysis. Various workers have demonstrated this susceptibility to proteolysis with the common observation that isolation of stroma in the absence of proteinase inhibitors results in very poor recoveries of ankyrin (Bennett & Stenbuck, 1980; Weaver & Marchesi, 1984). Perhaps the most graphic

report involved incubation of isolated stroma at 0 °C for 30 s with low levels of trypsin and which resulted in total cleavage of ankyrin (Jinbu *et al.*, 1984).

With this background the level of native ankyrin in senescent cells was measured to determine if proteolytic cleavage had occurred *in vivo*. Specifically, rabbit erythrocytes were biotinylated by a previously reported method (Suzuki & Dale, 1987) and the biotinylated erythrocytes recovered after prolonged circulation *in vivo* (Suzuki & Dale, 1988). These aged cells were then examined for the level of ankyrin and other proteins in their skeletons.

MATERIALS AND METHODS

Isolation of senescent erythrocytes

Rabbit erythrocytes were biotinylated, reinfused and isolated as previously described (Suzuki & Dale, 1987, 1988). Briefly, rabbits were injected with 25 mg of phenylhydrazine hydrochloride on days –13, –11 and –10 to produce young cohorts of red cells. Erythrocytes were then collected and biotinylated on day 0 (Suzuki & Dale, 1987). The biotinylated erythrocytes were infused into the original donor animal and subsequently recovered on avidin supports on the days specified (Suzuki & Dale, 1988). The cells that were originally biotinylated represent a population ranging from 0 to 10 days old, since phenylhydrazine eliminated all previously existing erythrocytes; therefore, the approximate mean age of the recovered biotinylated cells is the day that they were recovered plus 5 days. The results below are reported for the actual day of recovery of the cells.

The recovered red cells were washed twice in phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.2) and then lysed in 20 vol. of cold 0.5 mM-EGTA/10 mM-Tris, pH 7.4, containing 0.5 mM-phenylmethanesulphonyl fluoride (PMSF). The stroma were pelleted by centrifugation at 23000 *g* for 10 min and washed again with the same lysing buffer until the pellet was white.

Abbreviations used: SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride.

Quantification of ankyrin

For the separation of ankyrin from the two spectrin proteins, SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) was performed in 4% (w/v) acrylamide using a continuous buffer system (Weber & Osborn, 1969; Ballas, 1987). Briefly, this system uses 100 mM-sodium phosphate, pH 7.0, in all compartments. The gel itself was 1.5 mm wide with 8 mm sample wells. The stroma was added to an equal volume of 20 mM-sodium phosphate, pH 7.0, containing 2% SDS, 1% 2-mercaptoethanol, 0.5% Bromphenol Blue and 20% (v/v) glycerol. After boiling the sample for 3 min, 10–20 μ g of protein was applied to the gel. The sample was allowed to enter the gel at 30 mA for approx. 1 h after which the amperage was increased to 60 mA for a total of 7 h.

The gel was stained in 0.05% Coomassie Blue in methanol/acetic acid/water (50:7:43, by vol.). Destaining was with methanol/acetic acid/water (30:7:63, by vol.). The stained gel was photographed and the photographic negative used for scanning by a laser densitometer (Biomed Instruments, Fullerton, CA, U.S.A.). Individual peaks from the densitometry scan were cut and weighed to determine the relative amounts of a given peak within each electrophoretic lane.

Quantification of bands 4.1a and 4.1b

The estimation of bands 4.1a and 4.1b was found to be more precise when a Laemmli buffer system was used, similar to that described previously (Mueller *et al.*, 1987). Briefly, a 9% (w/v) acrylamide gel was run in the same apparatus as above with specified buffers (Laemmli, 1970). The stroma sample was added to an equal volume of 65 mM-Tris, pH 6.8, with 4% SDS, 1% 2-mercaptoethanol, 0.5% Bromphenol Blue and 20% (v/v) glycerol. After boiling the sample for 3 min, approx. 10–20 μ g of protein was added to each well. The electrophoretic system was maintained at 70 V for the first hour and then raised to 200 V until the Bromphenol Blue dye reached the bottom of the gel. The staining and quantification of this gel was identical to that detailed above, except that to increase the sensitivity of the system further, the gels were also treated with a commercial silver-stain kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) according to the manufacturer's recommendations.

RESULTS

A typical SDS/PAGE separation of rabbit erythrocyte ankyrin and the two spectrin molecules is shown in Fig. 1. As found by other workers (Bennett & Stenbuck, 1980; Weaver & Marchesi, 1984), the quantitative recovery of ankyrin is dependent on the inclusion of PMSF to inhibit proteinases during isolation and analysis of the skeletal proteins (results not shown). Using the biotinylation method, which we have previously described for the recovery of senescent erythrocytes (Suzuki & Dale, 1987, 1988), cells of various ages were isolated from a total of five rabbits. Fig. 1 lane 2 shows an SDS/PAGE analysis of a representative senescent-erythrocyte sample recovered 54 days after biotinylation and reinfusion and, therefore, within 6 days of its scheduled death *in vivo*.

To quantify the actual levels of intact ankyrin and spectrin in these various erythrocyte preparations, laser

Fig. 1. SDS/PAGE analysis of spectrin and ankyrin in senescent erythrocyte membranes

Erythrocyte stroma were isolated from control (Con) and aged (Day 54) erythrocytes as detailed in the Materials and methods section. SDS/PAGE was performed in 4% (w/v) acrylamide gels in the continuous buffer system described; staining was with Coomassie Blue. The middle lane represents stroma from erythrocytes 54 days after infusion of the biotinylated cells back into the rabbit.

densitometry of the individual bands was performed. The data in Fig. 2 show the ratio of α - and β -spectrin in the aging red cells. There is no change in the ratio of these two proteins, which indicates that there is no preferential loss of either of these molecules during the aging process. It is possible that both α - and β -spectrin are lost in unison as the cell ages and, as a result, the ratio of these two proteins would remain constant; however, this possibility seems remote.

Fig. 3 presents the level of ankyrin in the senescent cells relative to the total spectrin content for each stroma sample. These data demonstrate that the ratio of ankyrin to total spectrin does not significantly change during the life-span of the rabbit erythrocyte. There is a trend towards a lower ankyrin:spectrin ratio during the aging process, but this change is not statistically significant.

Fig. 4 shows an SDS/PAGE analysis of bands 4.1a and 4.1b in aged erythrocytes isolated from rabbits. Densitometry was used again to quantify the ratio of bands 4.1a to 4.1b and these data are shown in Fig. 5. There is a dramatic shift in the ratio of these two proteins as the erythrocyte ages with band 4.1a becoming more prominent in the senescent cell. These data are in agreement with those obtained in mice (Mueller *et al.*, 1987) and humans (Ravindranath *et al.*, 1987). Addi-

Fig. 2. Ratio of α -spectrin to β -spectrin in control and aged erythrocytes

The SDS/PAGE analyses were similar to that shown in Fig. 1 and were quantified by laser densitometry. The ratio of band 1 (α -spectrin) to band 2 (β -spectrin) was determined and plotted as a function of time after infusion of the biotinylated cells into the rabbit (●). The ratio for control cells (random population) was 1.14 ± 0.16 (mean $\pm$ 1 S.D.) and is shown as the stippled area.

Fig. 3. Ratio of ankyrin to total spectrin in control and aged erythrocytes

The analysis shown here is similar to that in Fig. 2. The ratio of ankyrin to total spectrin in the control cells was 0.215 ± 0.059 (mean $\pm$ 1 S.D.) and is shown as the stippled area.

tionally, the total level of band 4.1 was compared with that of band 4.2 within each membrane protein analysis. The ratio of $(4.1a + 4.1b)/4.2$ was 1.19 ± 0.47 (mean $\pm$ 1 S.D., $n = 15$) for control cells and 1.00 ± 0.26 ($n = 15$) for all aged cells (cells more than 39 days after biotinylation and infusion). This same type of analysis was done to compare the levels of total 4.1 to total spectrin; again there was no difference between the

Fig. 4. SDS/PAGE analysis of band 4.1 in control and senescent erythrocytes

Erythrocyte stroma from control (Con) and aged stroma (Day 39) were isolated and SDS/PAGE was performed in 9% (v/v) acrylamide gels as described in the Materials and methods section. Gels were first stained with Coomassie Blue and then with a silver stain to increase sensitivity. Note the shift in relative intensity of bands 4.1a and 4.1b in the aged versus control cells.

control and aged cells (results not shown). Therefore, the altered ratio of 4.1a to 4.1b in aged erythrocytes does not appear to result from a preferential loss of 4.1b during the senescence process but rather from a conversion of 4.1b to 4.1a.

DISCUSSION

The mammalian erythrocyte has an exquisite internal clock which allows the cell to circulate for extended periods of time with apparently full functional capabilities and then to be quickly and reliably removed from the circulation. As detailed in the Introduction, the molecular basis for this erythrocyte clock is not known and even the examination of the question has been hindered by the difficulty of isolating aged erythrocytes. Recently, we have introduced a new procedure for the isolation of senescent erythrocytes from the rabbit (Suzuki & Dale, 1987, 1988). This technique involves biotinylation of erythrocytes, infusion of the derivatized cells back into

Fig. 5. Ratio of band 4.1a to total band 4.1 in control and aged erythrocytes

Gels similar to that in Fig. 4 were scanned as detailed in Fig. 2, and ratio of band 4.1a to total 4.1 was calculated. The control value was 0.290 ± 0.055 (mean $\pm$ 1 S.D.) and is shown as the stippled area.

the donor animal and the eventual isolation of the biotinylated cells by their affinity for an avidin support. The ability of these biotinylated erythrocytes to survive normally allows the recovery of cells after extended periods of time and, therefore, the isolation of cells within specific time intervals of their expected death *in vivo*.

This biotinylation method has been used to examine the integrity of membrane proteins in the senescent erythrocyte. Ankyrin was a primary focus of the study due to its extreme sensitivity to proteolytic cleavage (Bennett & Stenbuck, 1980; Weaver & Machesi, 1984; Jinbu *et al.*, 1984) and the resulting negative impact this proteolysis has on the deformability of the erythrocyte membrane (Jinbu *et al.*, 1984). Any loss of ankyrin in the aging cell would have implications for the longevity of the cell and would address the proposal that proteolytic enzymes play an important role in erythrocyte senescence (Kay *et al.*, 1983; Morrison *et al.*, 1987).

The data presented above demonstrate that ankyrin is present in the senescent erythrocyte in apparently normal quantities (Fig. 3). The oldest erythrocyte sample examined was 57 days after biotinylation and infusion and, therefore, within 3 days of expected death *in vivo*. The possibility cannot be excluded that a dramatic activation of proteinases in the last 3 days of the erythrocyte's life might occur; however, this seems unlikely at this time.

Also shown in this report is a shift in the ratio of bands 4.1a and 4.1b in the senescent erythrocyte. This change has been previously found in mice (Mueller *et al.*, 1987) and humans (Ravindranath *et al.*, 1987) and is now the

sole documented example of an alteration that occurs during red cell aging in three different species. The biochemical reason for this shift is not clear. Band 4.1a is approx. 2000 Da heavier than 4.1b as measured by SDS/PAGE and the mechanisms which can add this apparent mass to a protein in the erythrocyte are not known. Three possible explanations for the different migrations of 4.1a and 4.1b have been proposed, including proteolysis, phosphorylation, or an unknown post-translational modification (Goodman *et al.*, 1982). Clearly, proteolysis cannot explain the increase in molecular mass seen with a temporal switch of 4.1b to 4.1a; however, the other two possibilities cannot be differentiated at this time. Recently, investigators have demonstrated that band 4.1 is *O*-glycosylated with *N*-acetylglucosamine (Holt *et al.*, 1987); however, both 4.1a and 4.1b were found to contain this modification, so that it is unlikely that this glycosylation can explain the molecular mass difference. These findings, together with the knowledge that 4.1 is encoded by a single gene (Tchernia *et al.*, 1981) and that no protein synthesis occurs in the mature erythrocyte, make this modification of the 4.1 protein with erythrocyte age particularly difficult to understand. The physiological significance of this change also is unknown since few data are available on the relative functional capabilities of 4.1a versus 4.1b (Goodman *et al.*, 1982; Conboy *et al.*, 1986). It is unlikely, however, that this switch from 4.1b to 4.1a is significant for the longevity of the erythrocyte since the change occurs relatively early in the cell's life [Fig. 5; Mueller *et al.* (1987)].

A final point concerning the electrophoretic patterns shown in Fig. 1 is the lack of aggregated or large-molecular-mass material at the origin of the gel for the aged erythrocytes. None of the 11 samples analysed from aged cells showed any stainable protein with a molecular mass higher than that of α -spectrin. Several reports have suggested that calcium-activated transglutaminases can cross-link membrane or skeletal proteins in the aging red cell (Lorand *et al.*, 1976; Anderson *et al.*, 1977; King & Morrison, 1977; Swislocki & Tierney, 1985). This cross-linking would result in decreased deformability and longevity of the cell (Lorand *et al.*, 1979; Swislocki & Tierney, 1985). One additional point concerning the action of transglutaminases is the apparently normal levels of band 4.1 in the senescent cells. Studies *in vitro* have shown that band 4.1 is an excellent substrate for the calcium-activated transglutaminases and one of the first proteins to disappear during transglutaminase action (King & Morrison, 1977; Anderson *et al.*, 1977). Therefore, the normal levels of band 4.1 in aged cells seen in this report would also suggest that the transglutaminase activation is not a significant factor in erythrocyte senescence in the rabbit.

The data presented in this report demonstrate that senescent erythrocytes from the rabbit do not have gross abnormalities in their membrane-skeleton complex. Additional functional studies will be required to determine if more subtle alterations occur in the erythrocyte membrane during cellular aging.

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