Membrane Skeleton Involvement in Cell Fusion Kinetics: A Parameter That Correlates with Erythrocyte Osmotic Fragility

Martin Baumann and Arthur E. Sowers
Department of Pathology, School of Medicine, University of Maryland at Baltimore, Baltimore, Maryland 21201 USA

ABSTRACT Spectrin levels in erythrocytes have been related to several biomechanical and biophysical membrane properties essential to the survival and function of the cell. Populations of erythrocytes display a natural and finite range of sensitivities to osmotic shock that has been directly correlated, in studies from other laboratories, to the presence of spectrin. We used a procedure to isolate subpopulations of 1) the osmotically most sensitive and 2) the osmotically most resistant erythrocyte membranes in an attempt to select for membranes enriched and depleted in spectrin (and/or a related component). The mechanical function of the spectrin-based membrane skeleton was further explored in these two subpopulations by searching for any effect on the time-dependent increase in fusion zone diameter in pairs of electrofused erythrocyte ghosts as a model for cell fusion. The results clearly show that the diameter expansions in fusions of membranes from osmotically resistant erythrocytes are faster in the early stage (up to 9 to 10 s after fusion) but do not thereafter expand as far as in fusions of membranes from osmotically sensitive membranes.

INTRODUCTION

The fusion of two cells, or two pure lipid vesicles, is usually illustrated by a sequence showing two circles that come into contact and then develop a "fusion pore" at the point of contact (membrane fusion). Afterward, the diameter of that fusion pore increases as the two component circles change shape into a single larger circle (this is often described as cell fusion) (Knutton and Pasternak, 1979). Whereas earlier papers devoted attention to the overall process of cell fusion, research became more focused, starting in the early 1980s, on the membrane fusion step (White, 1992), and the subsequent cell fusion, or rounding-up process, was essentially taken for granted. Indeed, one fundamental question in membrane fusion, the concept of hemifusion (the merging of the outer leaflets of two bilayers in contact as an event separate from the complete formation of a fusion pore) is still of major fundamental interest (Kemble et al., 1994; Song et al., 1991). Although the emphasis on membrane fusion ignored the cell fusion process, cell fusion is recognized in many in vitro studies of, for example, interactions between cells and enveloped viruses in terms of syncytium formation, a major and common observable end point.

A series of recent papers from our laboratory showed evidence of a substantial if not dominant and specific role for the spectrin-based membrane skeleton in controlling and possibly regulating the cell (i.e., post-membrane) fusion process (Wu et al., 1994a,b; Sowers, 1995). We also presented data that implicate spectrin, alone or in connection with a spectrin-related component, in controlling the cell fusion phenomenology and lead to an important clarification of the kinetics of the cell fusion process. We recognized at least two new quantitative variables: 1) fusion zone expansion signature and 2) flat diaphragm lifetime. Fig. 1 illustrates this clarification (see Wu et al., 1994b, for a complete description). We have previously shown that such fusion zone expansion "signatures" are highly sensitive to both chemical and biologically relevant experimental conditions. Our work also showed that these two variables can be linked or unlinked, depending on experimental conditions. It is important to note that in much prior work (Knutton and Pasternak, 1979) as well as our own (Chernomordik and Sowers, 1991), there appear both micrographs and line drawings that show that multiple fusion pores can be arranged in a double membrane fusion zone.

The involvement of the spectrin-based membrane skeleton is important because it is present in all eukaryotic cells (Bennett, 1990). It is generally accepted that the function of this array is to provide the membrane with strength, flexibility, and cell shape (Elgsaeter et al., 1986). Additional, but more specific and functional roles include, for example, the basis for much if not most of the membrane's elastic modulus (Waugh and Agre, 1988) and correlation with osmotic fragility (Agre et al., 1986). Moreover, resistance to malarial parasite invasion in the erythrocyte has been shown to have a strong genetic control factor originating in the erythrocyte precursor genome (Hadley et al., 1983) and another control factor originating in the malarial parasite genome (Cranston et al., 1984). Some mutations in spectrin and other membrane skeleton protein components are known to be correlated with hemolytic diseases (Palek and Lux, 1983).

The present paper reports that two subpopulations of rabbit erythrocyte ghost membranes, one derived from intact erythrocytes that are sensitive to osmotic shock and one

Received for publication 26 February 1996 and in final form 11 April 1996.

Address reprint requests to Dr. Arthur E. Sowers, P.O. Box 489, Georgetown, DE 19947; E-mail: arthures@access.digex.net.

Dr. Baumann's permanent address is Dozentur fur Biomedizinische Systemanalytik, Institute fur Physiologie, Klinikum der RWTH Aachen, Pauwelsstrasse 30, 52057 Aachen, Germany. E-mail: martin@physiology.rwth-aachen.de.

0006-3495/96/07/336/05 $2.00

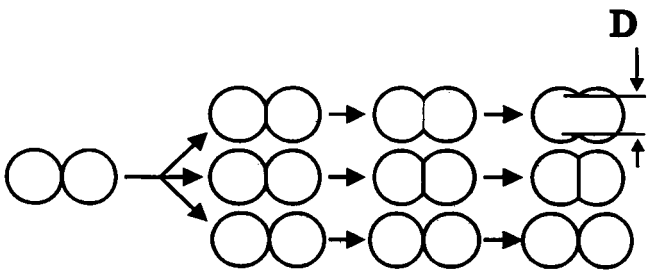

FIGURE 1 Three cell fusion scenarios, as they have been shown to appear in phase-contrast optics for a doublet of erythrocyte ghosts in close contact (see text). Membrane fusion occurs at the point of close contact (starting at far left), according to one of three pathways: (*top*) producing a flat diaphragm fusion zone that expands in diameter, *D*, without diminution in intensity; (*middle*) producing a flat diaphragm fusion zone that expands in diameter but with diminution in intensity and ending in an open lumen; (*bottom*) producing irreversible attachment of two membranes but no appearance of an expanding flat diaphragm or open lumen. Fusion zone diameter can be measured regardless of whether it is a flat diaphragm type or an open lumen type.

derived from intact erythrocytes that are resistant to osmotic shock, can be easily obtained and display quantitatively different time-dependent fusion zone expansion signatures. Because prior published work has linked osmotic fragility with spectrin concentration (Agre et al., 1986), the present paper further implicates spectrin concentration as a major determinant in controlling the fusion zone expansion signature.

MATERIALS AND METHODS

Materials

All operations were carried out at a temperature of 0–4°C unless otherwise specified. Rabbit whole blood was obtained from Truslow Farms (Chestertown, MD) as a mixture (1:1, v/v) in Alsevers (trisodium citrate dihydrate, 8 g/liter; citric acid, 0.55 g/liter; sodium chloride, 4.2 g/liter; D-glucose, 20.5 g/liter).

Osmotic fragility determination

Osmotic fragility curves were obtained for the purpose of determining the osmolarity that hemolyzed only about 10% and about 90% of the entire erythrocyte population. The sensitivity of these curves to erythrocyte heat treatments at 45 and 48°C (20 mins) before exposure to the osmotic shock was determined to provide evidence that the native versus any denatured state of spectrin could be linked to sensitivity to osmotic shock. Osmotic fragility was determined as follows. The rabbit whole blood/Alsevers mixture was spun into a pellet at 300 × *g* for 20 min, the buffy coat was removed, and the mixture was resuspended in isotonic sodium phosphate buffer at pH 7.4 and then spun again into a pellet. This pellet was then resuspended with a buffer containing a volume ratio of 1) phosphate-buffered saline (PBS) (mix of 20 mM Na mono- and dibasic phosphates to get pH 7.4 in 0.9% NaCl) and 2) distilled water. About 20–50 μl of pellet was resuspended in 5 ml of the PBS/water mixture, transferred to a microscope, and observed with phase optics. In single fields of view, the numbers of both ghost membranes (clear or light-gray circles) and intact (black) circles were counted within 1 h after placement on the slides. Plots of the ratio of numbers of clear/black circles against the ratio of PBS/water were used to determine the ratios of PBS/water for the fractionation protocol (see below).

Erythrocyte heat treatment

Heat treatments of washed erythrocytes involved exposure to 45°C or 48°C for 20 min before being resuspended in the subosmolar solutions. The heat treatments were carried out in a temperature-controlled water bath.

Fractionation by osmotic sensitivity

Erythrocyte ghost fractions were prepared from the most osmotically fragile ("weak") as well as the most osmotically resistant ("strong") erythrocytes. This procedure was similar to that previously described (Abidor et al., 1994). The details of this procedure were as follows. A quantity (18 ml) of the rabbit whole blood/Alsevers mixture was centrifuged (Sorvall RC5B; DuPont Instruments) at 300 × *g* for 10 min (the slow spin) using a swinging bucket rotor (HB-4), resuspended with a buffer composed of 0.9% NaCl and 20 mM monobasic and dibasic sodium phosphate (NaP_i) mixed in a ratio to obtain pH 7.4 (PBS), then centrifuged again into a pellet. This step was repeated for a second wash. After each washing step, the supernatant and the remaining buffy coat were discarded. This yielded a volume of about 4 ml of packed red blood cells (RBCs). The packed cell pellet was resuspended with another buffer composed of 54.4% PBS and 45.6% distilled and deionized water (v/v) vortexed and incubated for 20 min. Subsequently, a slow spin was used to spin all remaining hemoglobin-containing RBCs into a pellet while leaving all ghost membranes in the supernatant. These were from the osmotically most fragile (i.e., "weak") erythrocytes. The supernatant was designated W and was saved for further processing as described below. The collected pellet was resuspended with a third buffer composed of 41.1% PBS and 58.9% distilled and deionized water (v/v), vortexed, and then incubated for 20 min. Another slow spin was used to spin down the remaining hemoglobin-containing cells. The supernatant from this step, containing all ghosts with an osmotic fragility intermediate between weak and strong, was removed. The pellet containing the most osmotically resistant erythrocytes was diluted with 5 mM NaP_i buffer, vortexed, and incubated for 20 min, which caused all remaining osmotically resistant (i.e., "strong") cells to hemolyze. This membrane suspension was designated as S. Both the W and the S suspensions were centrifuged into pellets with a fast spin (10,000 × *g* for 20 min) using a fixed-angle rotor (SS-34 head). The pellets from both of these spins were pink in color, and each had a volume of 200–400 μl. Supernatants were discarded, both pellets were kept separate, and each was resuspended in 5 mM NaP_i buffer for 20 min. Both were centrifuged in a fast spin into pellets that in both cases were white. Supernatants were removed and the pellets were resuspended in 20 mM NaP_i (pH 7.4) for storage. On the day of the experiment, the ghosts were spun with a fast spin and the pellets were used to produce an observation solution with a hematocrit of 0.5% in 20 mM NaP_i buffer. Fusions were induced and observed at room temperature.

Fusion apparatus

The fusion chamber used for the experiment was designed for reuse with recyclable parts (Fig. 2). Square microslides (25 mm × 25 mm) were cut from normal 25 mm × 75 mm glass microscope slides and cleaned. A square sheet of parafilm (American Can Company) sized 13 mm × 13 mm with a rectangular slotted hole of 1.5 mm × 5 mm in the center is sandwiched between the glass piece and a clear plastic layer 15 mm × 15 mm with two drilled holes of diameter 1.5 mm and centers 3.5 mm apart. The three-layer sandwich was bonded together by heating and moderately melting the parafilm while pressing all layers against each other. Chambers were stored dust free until used. The chamber was filled by dispensing solution from a pipette (the tip contained about 10 μl of solution) into one hole until the meniscus propagated to and filled up the second hole. The electrodes used to establish the necessary electric field strengths were made of platinum wire of 0.5 mm diameter with rounded tips. These were lowered by a micropositioner through both holes until they touched the lower glass surface. This ensured that they were in physical contact with the solution. The distance between both electrodes was always kept at 3 mm. The chamber was used in horizontal orientation. After usage, the

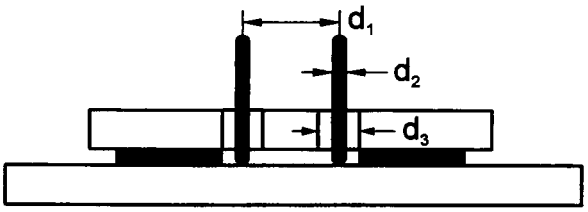

FIGURE 2 Observation chamber cross section. The chamber is composed of a sheet of 15 mm × 15 mm × 1.3 mm thick plexiglas with two holes with diameter d_3 = 1.5 mm each, spaced with centers at d_1 = 3.5 mm, and platinum electrodes with diameter d_2 = 0.5 mm. The plexiglas wall was fastened to a 25 mm × 25 mm × 1 mm glass microslide with a sheet of parafilm heated with a hot plate and then allowed to cool (Sowers, 1989; 1992; 1993).

chamber was disassembled and the parafilm removed. The plastic and glass components were cleaned and reused.

Fusion protocol

The fusion chamber was filled with the membrane suspension, and the platinum electrodes were lowered into the solution. The chamber containing the ghost suspension was placed on the stage of a phase optics microscope (Olympus IMT-2). Ghost membranes were brought into contact by dielectrophoresis (Sowers, 1989, 1992, 1993), using an AC sine wave field with an effective field strength of E = 5 V/mm at 60 Hz for 30–60 s. Prior work (Sowers, 1989, 1992, 1993) showed that the field inhomogeneity near electrodes had an insignificant effect on bulk homogeneity in the center of the observation chamber, although microscopic inhomogeneity in close proximity to membranes is expected. A pair of ghosts was chosen and fused by application of a single exponentially decaying high-voltage pulse that produced a maximum field strength of E = 400 V/mm (calculated from a 1200-V pulse divided by 3-mm electrode spacing) and a decay half-time of 0.5 ms. The AC field was not turned off during the observation time. The experiment was recorded on VHS tape using a standard VCR with the tape started 10 s before the application of the pulse and stopped no less than 95 s afterward. A continuously running video timer alphanumeric signal was inserted in the top left corner of the video signal, providing date and time information readout with 17-ms resolution. A device was connected that made the timer alphanumeric vertical position shift within 17 ms of the time of the application of the pulse (60 256-line scans were recorded per second).

Fusion zone expansion measurement

After the experiment the tape was replayed, and selected single frames were examined and the fusion zone diameters measured with Java Software on images frozen with a frame grabber adapter card. Frames measured included the frame just before the pulse and frames at 0.5, 1, 2, 5, 10, 30, 60, and 90 s after the pulse. A contrast-enhanced picture (provided by Java) was used to measure the fusion zone diameters of the fused pairs.

RESULTS AND DISCUSSION

The osmotic fragility curve (Fig. 3) is similar in shape to those found in prior studies (de Bruijne and van Steveninck, 1979; Shand and Noble, 1981; Ho and Lin, 1991; Gjedrem et al., 1991), but erythrocyte heat treatment causes the curve to shift toward a higher sensitivity with progressively higher temperature. This is consistent with at least one prior study (de Bruijne and van Steveninck, 1979) with a slightly different heat treatment protocol. Specifically, half of the control erythrocytes hemolyze at 48% PBS/52% water, but after 48°C for 20 min, half of the erythrocytes hemolyze at even less water (52% PBS/48% water). Studies have shown that spectrin is the most heat-sensitive protein in the erythrocyte and that temperatures need to be at 48–49°C or higher to have broader and more extensive effects on intact erythrocytes (such as hemolysis and shape change) (Ho and Lin, 1991; Crum et al., 1979). Heat treatment effects can begin to be observed in isolated erythrocyte ghost membranes at temperatures as low as 42°C, but require a low ionic strength medium (Wu et al., 1994a,b). Because heat treatments can cause other effects on membranes, inducing, for example, blebbing and shape distortions (Ho and Lin, 1991; Crum et al., 1979), the participation of other heat-induced changes in altering the fragility curve cannot be completely ruled out at this time.

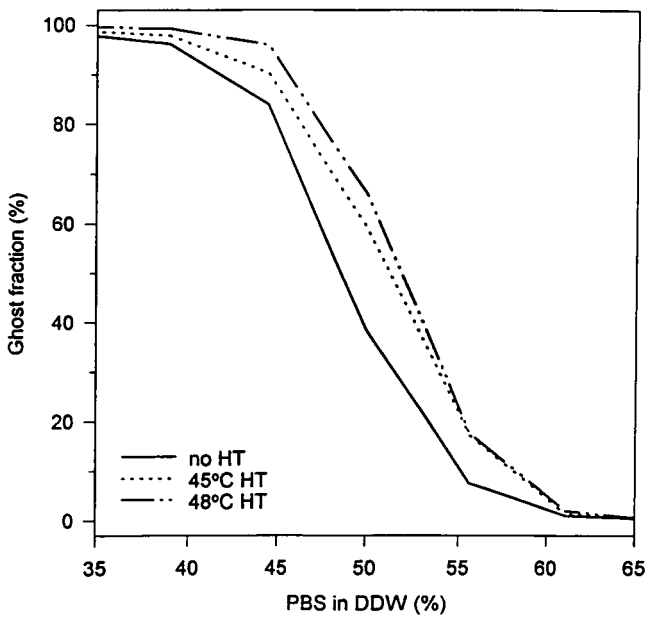

FIGURE 3 Osmotic fragility of erythrocytes. Fraction, in percent, of erythrocytes transforming into the ghost morphology within 1 h after being resuspended from a pellet with a mixture (v/v) of phosphate-buffered saline (PBS) and distilled and deionized water (DDW). Curves for control and 45°C and 48°C heat treatments (20 mins) are as shown.

The osmotic fragility curve was obtained, in the present study, for the purpose of selecting a PBS/water ratio to obtain the fragility-based fractionation of the erythrocyte membranes as described in Materials and Methods. We aimed for the isolation of ghost membranes from approximately the 1/10 pellet volume of most osmotically fragile intact erythrocytes and the 1/10 pellet volume of most osmotically resistant intact erythrocytes in a population from rabbit whole blood. We discarded the 8/10 pellet volume of ghosts from the erythrocytes with intermediate osmotic sensitivity.

The fusion zone diameter versus time curves for erythrocyte ghost electrofusion in the W and S fractions were

small but consistently different (Fig. 4). From measurements of 31 fusions of "weak" ghosts and 30 fusions of "strong" ghosts, calculated standard deviations ranged from 0.06 to 0.19 μm and averaged 0.07 μm for both populations. Although average differences between the populations were small compared to light microscope resolution, the fact that the respective curves that plotted the averages came out so smooth and consistent suggests that we were, indeed, recovering a "signal" from the "noise" and further work would be justified.

The fusion zone expansion curve shows that in the first 9–10 s after fusion, the expansion rate was higher for the "strong" ghosts, but after 9–10 s the expansion rate became progressively higher for the "weak" ghosts. This changeover in the kinetic signature was in the same time range after membrane fusion is induced, as is seen when heat-treated fusion zone expansion signatures begin to differ from expansion signature from non-heat-treated (control) ghost membranes (Wu et al., 1994a). This implies a kinetic process or a force generation mechanism with at least two components, and each is dominant in its respective period. Because the two fractions are based on a method (osmotic fractionation) that has been linked in prior work to spectrin concentration (Agre et al., 1986), this finding provides an additional new line of evidence that allows us to hypothesize the involvement of spectrin and/or spectrin-related proteins in the fusion zone expansion signature. It is clear that actual measurements of spectrin and/or spectrin-related proteins would be needed to confirm this hypothesis. It should also be acknowledged that manipulation of other physicochemical variables (e.g., time of exposure to a low-osmolarity medium) during the hemolytic fractionation might shed additional light on the relationship between the mechanical properties of the membrane skeleton and the fusion zone expansion signature during cell fusion.

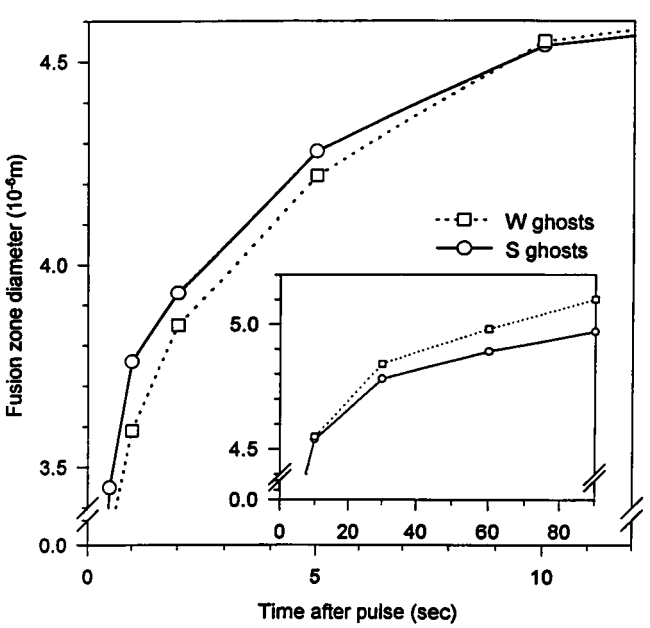

FIGURE 4 Expansion of diameter of flat diaphragm fusion zone in pairs of fused erythrocyte ghosts, as a function of time, in populations of "weak" (W) and "strong" (S) ghost membranes obtained from osmotically sensitive and resistant erythrocytes (see text). Number of measurements on fusions: $N = 31$ for fusions of "weak" ghosts and $N = 30$ for fusions of "strong" ghosts (see text). Neither the erythrocytes nor the ghosts received a heat treatment.

In addition to the above-mentioned role of the membrane skeleton in cell fusion, the above observation relates to the effect of heat treatment on plasma membrane rigidity. It has been known from early experiments that heat treatments cause an increase in the rigidity of erythrocytes, as measured by the micropipette (de Bruijne and van Steveninck, 1979; Rakow and Hochmuth, 1975; Mohandas et al., 1978). Our studies on the expansion of fusion zones in fusing erythrocyte ghosts showed that a heat treatment known to partially denature spectrin would lead to a small but reproducible acceleration in the fusion zone expansion (in the diameter expansion in the period starting at least 10–15 s after membrane fusion was induced) and a consistently higher acceleration if the heat treatment were such as to completely denature spectrin (Wu et al., 1994a). The higher acceleration is in apparent contradiction to the heat-induced increase in rigidity. However, this apparent paradox may be due to the possibility that heat may induce more changes (other than an increase in rigidity) in one or more mechanical properties. For example, the bending moment may also be changed. Although it remains for future work to further clarify and quantify the mechanistic origins and identify the molecular entities for the above phenomenon, the present study indicates that spectrin and/or a spectrin-related component can play a role in the fusion zone expansion signature.

This research was supported by ONR grant N00014-92-J-1053 and National Institutes of Health grant R03RR07764.

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