ERYTHROCYTE MORPHOGENESIS IN THE AXOLOTL, AMBYSTOMA MEXICANUM

Mary F. Ginsburg, Laura H.Twersky, and William D. Cohen
Department of Biological Sciences,
Hunter College, NY, NY 10021

Axolotl erythrocytes were a favored material of early cytologists (Ranvier, 1875; von Smirnow, 1906), and the axolotl is particularly well-suited for the study of erythropoiesis. As pointed out by Duprat and Flavin (1976), red blood cells develop in the early larval amphibian spleen in an apparently homogenous population. The spleen during this time is a closed, red sac attached to the stomach (Fig 1) and easily visible in the living larva. The spleen is normally isolated from the general circulation until about the tenth to twelfth day post-hatching, enabling the isolation of a pure population of developing erythropoietic cells for study at various larval ages.

This laboratory is particularly interested in the role of the marginal band in erythrocyte morphogenesis. The marginal band, a part of the cytoskeleton, consists of microtubules and associated proteins arranged circumferentially around the elliptical cell in the plane of flattening. This structure has been found in all nonmammalian vertebrate erythrocytes (Fawcett, 1959; Goniakowska -Witalinska and Witalinksa, 1977), which are usually nucleated, and in a variety of invertebrate blood cells (Cohen and Nemhauser, 1985). Since Fawcett's description of the marginal band of the toadfish erythrocyte, and his suggestion of its involvement in cell shape maintenance (Fawcett, 1959), several laboratories have investigated such a relationship in various red blood cells (reviewed by Behnke, 1970). More recently, the ability of mature erythrocytes to resist shape changes during mechanical stress has been shown to depend upon the presence of the marginal band (Joseph-Silverstein and Cohen, 1984, 1985).

Cell biologists have begun to focus attention on the dynamic aspects of the cytoskeleton, which assumes varied geometry and composition at different stages of cell division and morphogenesis. A role for the marginal band in the development of erythrocyte morphology has been shown using avian erythrocytes (Barrett and Scheinberg, 1972; Barrett and Dawson, 1974), but the exact morphological sequence is unknown. Murphy et al. (1986) and Kim et al. (1978) recently have examined the formation of the marginal band in chicken erythrocytes and have found microtubules emanating from a microtubule-organizing center at the early stages of band development. However, little information is available on the correlation between changes in microtubule arrangement and evolving cell shape. Such a study of the development of the marginal band and the concomitant morphogenesis from spherical erythroblast to elliptical erythrocyte is of interest in terms of both cytoskeletal function and red blood cell differentiation.

As part of this laboratory's efforts to elucidate the role of the marginal band in erythrocyte morphogenesis, we have developed procedures for removal of the intact spleen from an anesthetized Ambystoma larva (using Finquel, 1:5000 concentration) after

measuring spleen size in the living animal to determine whether it is at the desired stage. Cells may then be cultured or analyzed by any of several means. Phase contrast light microscopic studies on these cells have shown a progression from spherical hemoglobinated cells to flattened ellipses (Ginsburg et al., 1986). As seen below in data obtained from 18-day larvae whose spleens remained isolated from the general circulation (unusual at this age), there is a significant difference in the composition of circulating blood versus spleen blood in terms of cell shape:

TABLE I:
Cell Shapes in Circulating and Splenic Blood of the Same Animal

No.	So.	Cnt.	El	D	Sh	SP	DP
1	C	107	68.6%	26.7%	2.9%	1.9%	0.0%
	S	334	57.2	19.8	7.5	12.0	3.0
2	C	249	79.5	19.3	0.8	0.4	0.0
	S	179	31.3	48.6	12.3	5.6	2.2
3	C	296	67.9	30.4	0.7	0.7	0.3
	S	295	37.3	45.8	5.1	10.5	1.0
4	C	249	79.5	19.3	0.8	0.4	0.0
	S	179	31.3	48.6	12.3	5.6	2.2

(So: Cell Source; C: Circulating Blood; S: Spleen;
Cnt: Number cells counted; Ellipses; D: Discs; Sh: Spheres;
SP: Singly-pointed; DP: Doubly-pointed)

As shown in the table, pointed cells are relatively rare in the circulation, but are common in the developing cell population in the spleen. For comparison, the averages of the percentages in five larvae at least eighteen days post-hatching in which the spleen was connected to the general circulation were: 75.5% ellipses, 21.3% discs, 1.5% spheres, 1.5% singly-pointed, 0.3% doubly-pointed. Though less dramatic, the increased number of pointed cells in the splenic population is again evident.

Cell shapes may be viewed using both phase contrast light microscopy and scanning electron microscopy. A phase contrast micrograph showing the variety of erythrocyte shapes may be seen in Figure 2.

Scanning electron microscopy was used to study the surface morphology of the splenic erythroblasts. Intact larval spleens, after fixation, dehydration, and critical point drying, were cut with a razor blade to reveal the developing cells (Fig. 1 and 3).

The occurrence of pointed cells, of particular interest as possible stages of erythrocyte differentiation, does not seem to be related to spleen size or larval age, as shown in the table below:

TABLE II: Spleens Which Contained Very High Percentages of Singly- and/or Doubly-Pointed Erythroblasts

Larva No.	Total% Pointed	%Singly-Pointed	%Doubly-Pointed	Larval Age*	Spleen Length
1	41.8	27.9	13.9	9	1.0mm
2	19.1	16.7	2.4	5	0.4
3	16.5	15.8	0.7	6	0.7
4	15.7	6.3	9.4	4	0.3
5	13.5	11.8	1.7	5	0.3
6	12.5	12.5	0.0	4	0.3

*Days post-hatching

In contrast, in the thirty-three spleens studied to date, the pointed cells constituted, on average, 7.5% of the total cell population, with a median of 6.4%. Our working hypothesis is that the pointed cells represent intermediate morphological stages between spherical post-mitotic cells and final flattened ellipsoids. We interpret the occurrence of some spleens with very high percentages of pointed cells and others with very low percentages as suggestive of successive waves of mitosis and differentiation with some degree of synchrony, resulting in peaks and valleys in percentages of pointed cells (Cohen and Ginsburg, 1986).

Both whole and lysed cells have been examined. Lysis, when done, is carried out in a microtubule-preserving medium (Cohen, 1978); this permits the viewing of the intact marginal band in addition to the cell nucleus (Fig. 4). In some of those lysed cells which are singly- or doubly-pointed, a pair of dots may be observed when using the 100x oil immersion objective of the phase microscope. Based upon previous work in this laboratory (Nemhauser et al., 1983, and Cohen, 1986), these are of a size and density consistent with their being centrioles (see Figures 5 and 6). We are currently using transmission electron microscopy to determine whether these are in fact centrioles which might be part of a precisely positioned organizing center for marginal band formation.

REFERENCES

Barrett, L.A. and R.B.Dawson (1974). Avian Erythocyte Development: Microtubules and the Formation of the Disk Shape. Dev. Biol. 36:72-81.

Barrett, L.A. and S.L. Scheinberg (1972). The Development of Avian Red Cell Shape. J. Exptl.Zool. 182:1-14.

Behnke, O. (1970). Microtubules in Disk-shaped Cells. Int. Rev. Exp. Path. 9:1-92.

Cohen, W.D. (1978). Observations on the Marginal Band System of Nucleated Erythrocytes. J. Cell. Biol. 78:260-273.

Cohen, W.D. (1986). Associations of Centrioles with the Marginal Band in Skate Erythrocytes. Biol. Bull. 171:338

Cohen, W.D. and M. Ginsberg (1986). A Model for Marginal Band

Formation in Amphibian Erythrocytes. Annual Meeting, American Society for Cell Biology. J. Cell. Biol. 103:547a.

Cohen, W.D. and I. Nemhauser (1985). Marginal Bands and the Cytoskeleton in Blood Cells of Marine Invertebrates. Chap. One, pp. 3-49, in Cohen, W.D., ed., Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology, Alan R. Liss, Inc., New York.

Duprat, A.M. and M. Flavin (1976). In vitro Culture of Larval Amphibian Erythroblasts. Experientia 32:1587-1589.

Fawcett, D.W. (1959). Electron Microscopic Observations on the Marginal Band of Nucleated Erythrocytes. Anat. Rec. 133:379.

Ginsburg, M.F., L.H. Twersky, W.D. Cohen (1986). Studies in the Differentiation of Splenic Erythroblasts in Ambystoma mexicanum Larvae. Annual Meeting, American Society for Cell Biology. J. Cell. Biol. 103:546a.

Goniakowska-Witalinska, L. and W. Witalinksi (1977). Occurrence of Microtubules during Erythropoiesis in Llama, Llama glama. J. Zool. (London) 181:309:313.

Joseph-Silverstein, J. and W.D. Cohen (1984). The Cytoskeletal System of Nucleated Erythrocytes. III. Marginal Band Function in Mature Cells. J. Cell. Biol. 98:2118-2125.

Joseph-Silverstein, J. and W.D. Cohen (1985). Role of the Marginal Band in an Invertebrate Erythrocyte: Evidence for a Universal Mechanical Function. Can. J. Biochem. Cell Biol. 63:621-630.

Kim, S., M. Magendantz, W. Katz, F. Solomon (1987). Development of a Differentiated Microtubule Structure: Formation of the Chicken Erythrocyte Marginal Band in Vivo. J. Cell Biol. 104:51-59.

Murphy, D.B., W.A. Grasser, K.T. Wallis (1986). Immunofluorescence Examination of Beta Tubulin Expression and Marginal Band Formation in Developing Chicken Erythroblasts. J. Cell Biol. 102:628-635.

Nemhauser, I.J., Joseph-Silverstein, W.D. Cohen (1983). Centrioles as Microtubule-Organizing Centers for Marginal Bands of Molluscan Erythrocytes. J. Cell Biol. 96:979-989.

Ranvier, L. (1875). Recherches sur les Elements du Sang. Arch. Physiol. 2:1-15.

von Smirnow, A.E. (1906). Die Prolongierte Osmiummethode nach Fr. Kopsch als ein Mittel zur Darstellung einiger Strukturen in den Erythrocyten des Siredon pisciformis. Anat. Anz. 29:236-241.

FIGURE LEGENDS

Figure 1. The developing axolotl spleen (arrow) attached to the stomach, as seen by scanning electron microscopy. Bar = 100μm.

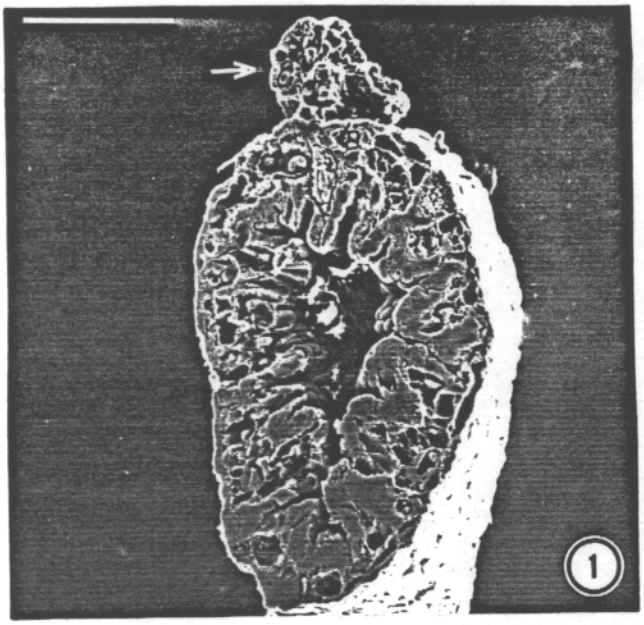
Figure 2. Morphology of cells released from larval spleen of *A. mexicanum*. In addition to flattened, elliptical erythrocytes (E), the population includes significant numbers of spherical (S), singly-pointed (SP), doubly-pointed (DP), and discoidal (D) erythroblasts. Phase contrast; bar = 20μm.

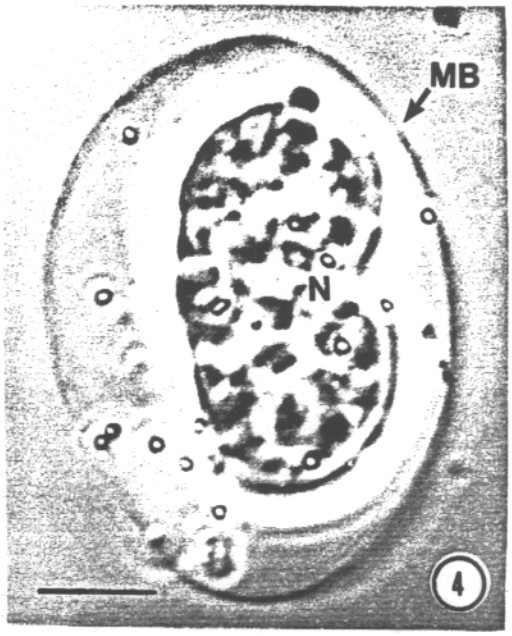
Figure 3. Erythroblasts as seen at higher magnification in the scanning electron microscope. There is a tear-drop shaped singly-pointed cell present (SP), as well as two flattened discs.

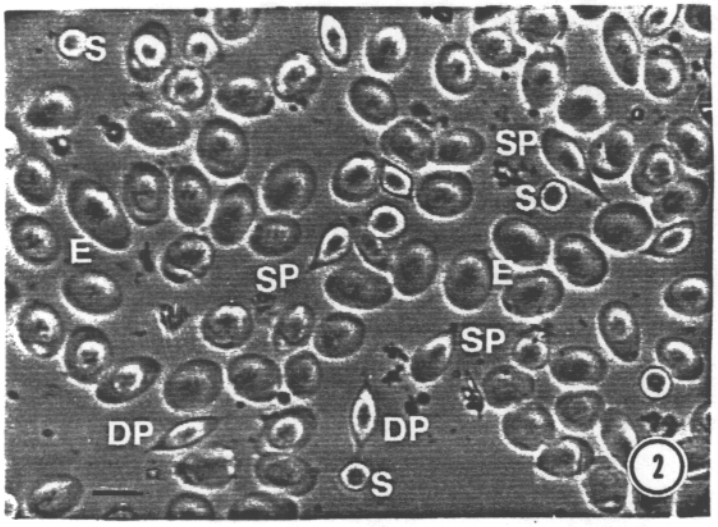
Figure 4. A detergent-lysed splenic erythrocyte (a "cytoskeleton"), showing nucleus (N) and marginal band of microtubules (MB). Phase contrast; bar = 5μm.

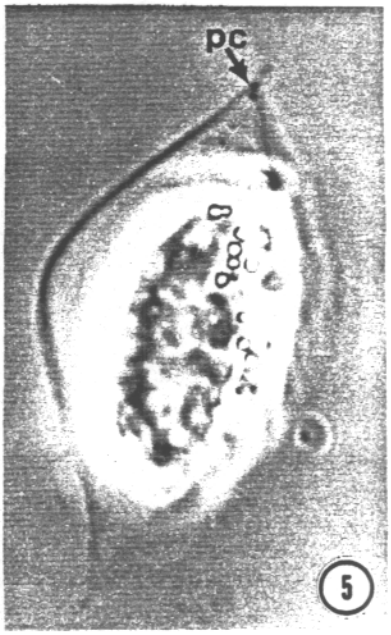
Figure 5. A doubly-pointed erythroblast "cytoskeleton". One end contains a putative centriole pair near tip (PC). Phase contrast; magnification as in Fig. 4.

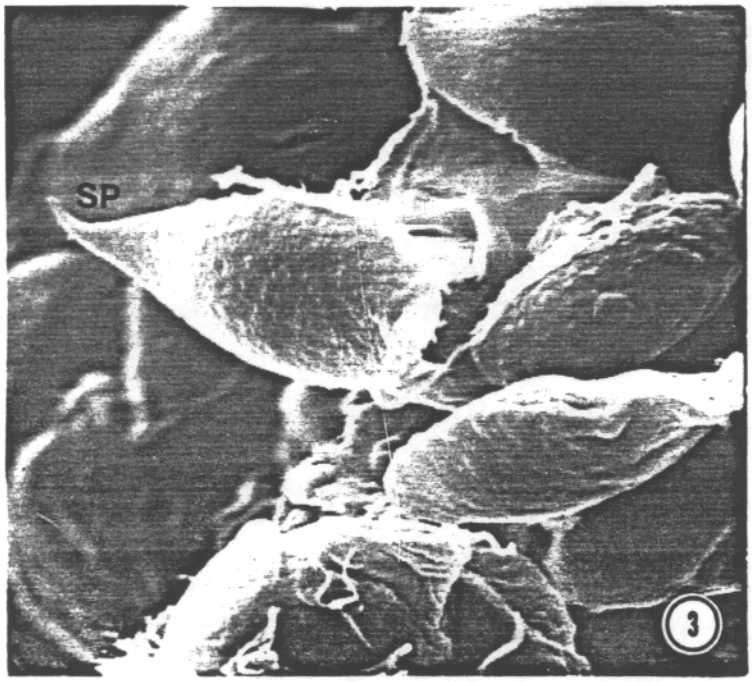
Figure 6. A singly-pointed erythroblast "cytoskeleton", with putative centriole pair (PC) near tip of point. Phase contrast; magnification as in Fig. 4.

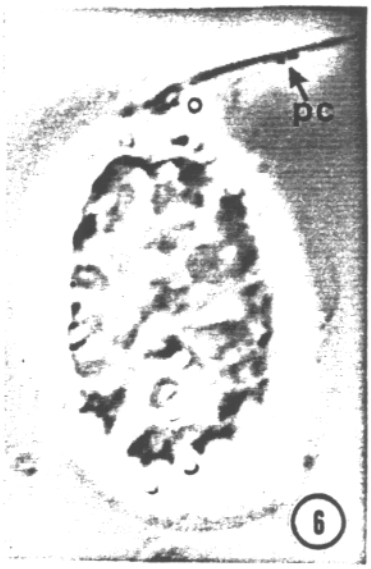



MB
N


OS
SP
SO
E
SP
E
SP
DP
DP
OS


PC


SP


PC