Scanning Microscopy

Volume 10 | Number 3 Article 15

5-24-1996

Apoptosis and Red Blood Cell Echinocytosis: Common Features

Alexei B. Chukhlovin
N. N. Petrov Research Institute of Oncology, vna@camt.spb.su

Scanning Microscopy, Vol. 10, No. 3, 1996 (pages 795-804) 1051-6794/96$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

APOPTOSIS AND RED BLOOD CELL ECHINOCYTOSIS: COMMON FEATURES

Alexei B. Chukhlovin

Bone Marrow Transplantation Department, N.N. Petrov Research Institute of Oncology, Leningradskaya St., 68 Pesochnyi-2, 189646, St. Petersburg, Russia

Telephone number: (7-812) 235-9571 / FAX number: (7-812) 235-0334 / E.mail: vna@camt.spb.su

(Received for publication February 7, 1996 and in revised form May 24, 1996)

Abstract

Apoptosis of nucleated blood cells induced by oxidants and/or reactive oxygen species is accompanied by the typical membrane pathology. Meanwhile, red blood cell (RBC) membrane is a popular object for studying appropriate cytotoxic effects. Scanning electron microscopy provides a reliable tool for detecting the oxidative changes in RBC shape and size. Transition of normal discoid erythrocytes to crenated forms (echinocytes) is often induced by the same factors which cause apoptosis of blood cells, e.g., ionizing radiation and other reactive oxygen intermediate-inducing agents, exogenous oxidants, *in vitro* aging conditions, cytosolic calcium increase, etc. Moreover, the biochemical membrane alterations in oxidant-induced echinocytosis is strongly reminiscent of the changes associated with apoptosis, e.g., cell shrinkage, lipid oxidation, energy depletion and loss of transmembrane lipid asymmetry. Hence, characteristic changes in cell shape in oxidant-treated RBCs are of value for interpreting the membrane alterations occuring in leukocyte apoptosis.

Key Words: Apoptosis, leukocytes, echinocytosis, erythrocytes, shape, cell membrane, cytoskeleton, oxidant effects, scanning electron microscopy.

Introduction

Apoptosis is a common mode of programmed cell death (PCD) in various nucleated cell populations. Early steps of apoptosis in blood cell populations are switched and/or modulated by specific "cell suicide" genes (Szumiel, 1994; reviewed by Wyllie, 1994).

Several pathways of apoptosis triggering are discussed. Most of them include early involvement of plasma membranes (Allan, 1992; McConkey *et al.*, 1994; Szumiel, 1994). Alterations of distinct surface markers are observed much earlier than apoptosis-specific DNA fragmentation (Kubasova *et al.*, 1981; Zherbin and Chukhlovin, 1984; Chukhlovin, 1996). Some inhibitors, e.g., Cd and Zn ions, postpone apoptosis-associated DNA decay (Zherbin *et al.*, 1986; Cohen *et al.*, 1992). Certain features of PCD are revealed even in the absence of nuclei, e.g., when studying the cytoplasts obtained from oligodendrocytes (Jacobson *et al.*, 1994).

Sufficient decrease in surface area, i.e., cell shrinkage or pyknosis, occurs in apoptotic lymphoid cells, in parallel with DNA decay (Klassen *et al.*, 1993). Cell membrane blebbing and shedding of microvesicles are also observed during apoptosis (Liepins and Bustamante, 1994). However, it is difficult to analyze the intrinsic changes of membranes, induced by different apoptosis-causing agents. The anucleate blood erythrocytes may be considered an appropriate alternative model system for such studies.

Variability and Regulation of Erythrocyte Shape

The erythrocyte membrane is an extensively studied mammalian plasma membrane with respect to metabolic functions and mechanisms of cell shape alterations (reviewed by Schroit and Zwaal, 1991; Zachowski, 1993). Normal mammalian erythrocytes are present in a variety of discoid forms (Fig. 1). Pathological changes in red blood cell (RBC) shape and size have been well established and classified, especially since the introduction of scanning electron microscopy (SEM) (Bessis, 1973). Red blood cell deformities such as echinocytosis (RBC

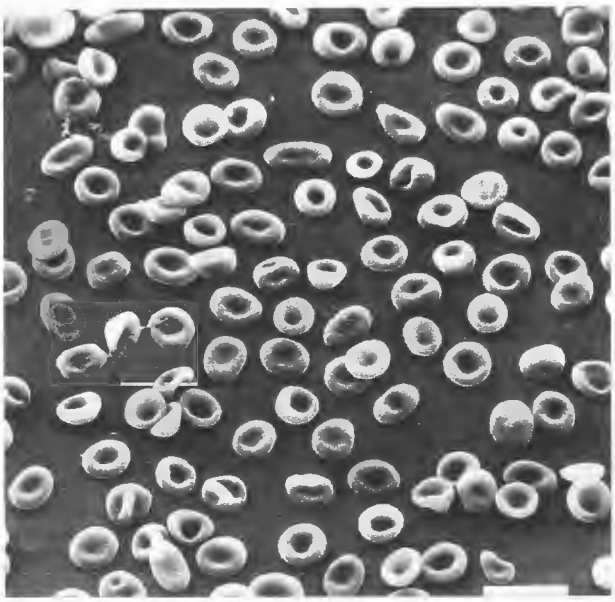

Figure 1. Scanning electron micrograph of normal, predominantly discoid erythrocytes from rat peripheral blood. Bar = 10 μm.

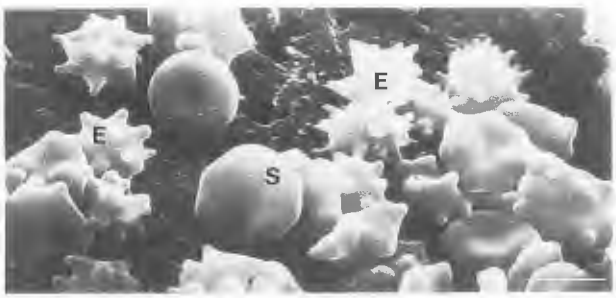

Figure 2. Scanning electron micrograph of sphero (S)/ echinocytosis (E) of murine erythrocytes exposed for 60 minutes to 1 mM of phenylhydrazine. Bar = 3 μm. (Adapted from Ciccoli *et al.*, 1994).

---

shrinkage and crenation), stomatocytosis and spherocytosis are shown to be quite different in their origins and pathogenetic mechanisms.

Echinocytosis, or RBC crenation, are the primary focus of the present review. Echinocytic RBCs are not uncommon in peripheral blood of normal individuals (Simpson, 1989). Such cells initially acquire microspicules, followed by notable shrinkage (Fig. 2). This non-specific trait is common in a variety of blood diseases and non-hematological disorders (Castoldi and Beutler, 1988; Agroyannis *et al.*, 1995). Exogenous cytotoxic agents, e.g., oxidative chemicals and ionizing irradiation, cause different RBC deformities, including echino- and/or spheroechinocytosis (Figs. 2, 3 and 4).

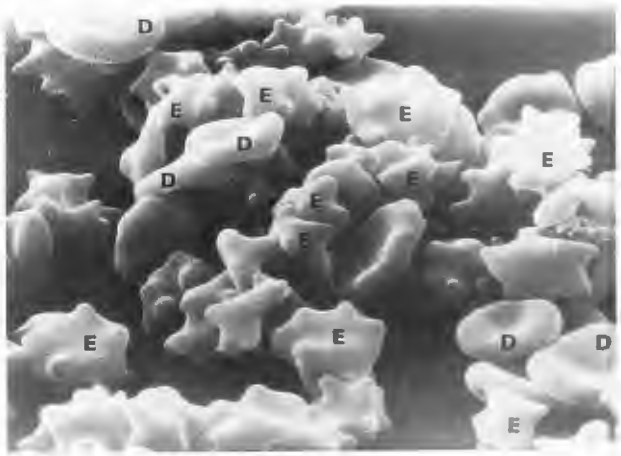

Figure 3. Red blood cells from irradiated (8 Gy) hamsters at 5 days post-treatment demonstrates echinocytes (E) and occasional discocytes (D). SEM; photo width = 27 μm. (From Thompson and Johnstone, 1987).

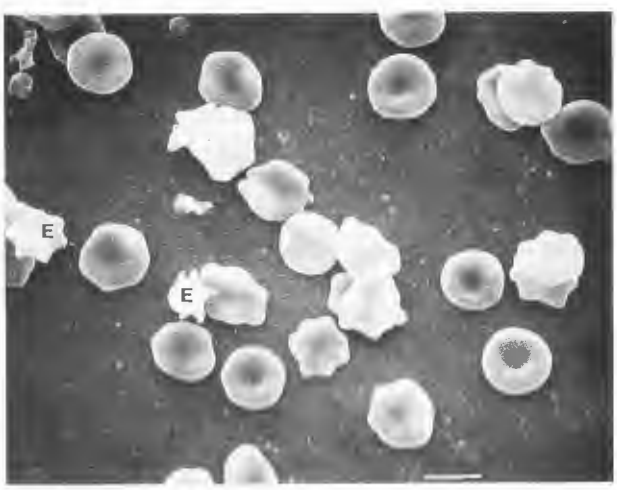

Figure 4. Scanning electron micrograph of echinocytic RBCs (E) from irradiated (1.0 Gy) rats at 7 days after treatment. Bar = 5 μm.

The normal shape of RBCs is determined by many specific interactions between cytoskeletal proteins and the membrane lipid bilayer (Fig. 5). Normally, the network of actin-spectrin-ankyrin complexes is bridged to specific transmembrane proteins (band 3, glycophorin C). In addition, band 4.1 protein provides binding between cytoskeletal actin and phosphatidylserine (PS) molecules exposed at the inner membrane leaflet (Bennett, 1989; Bennett and Gilligan, 1993). Any imbalance in this framework may cause specific changes of RBC size and shape (Mohandas, 1992).

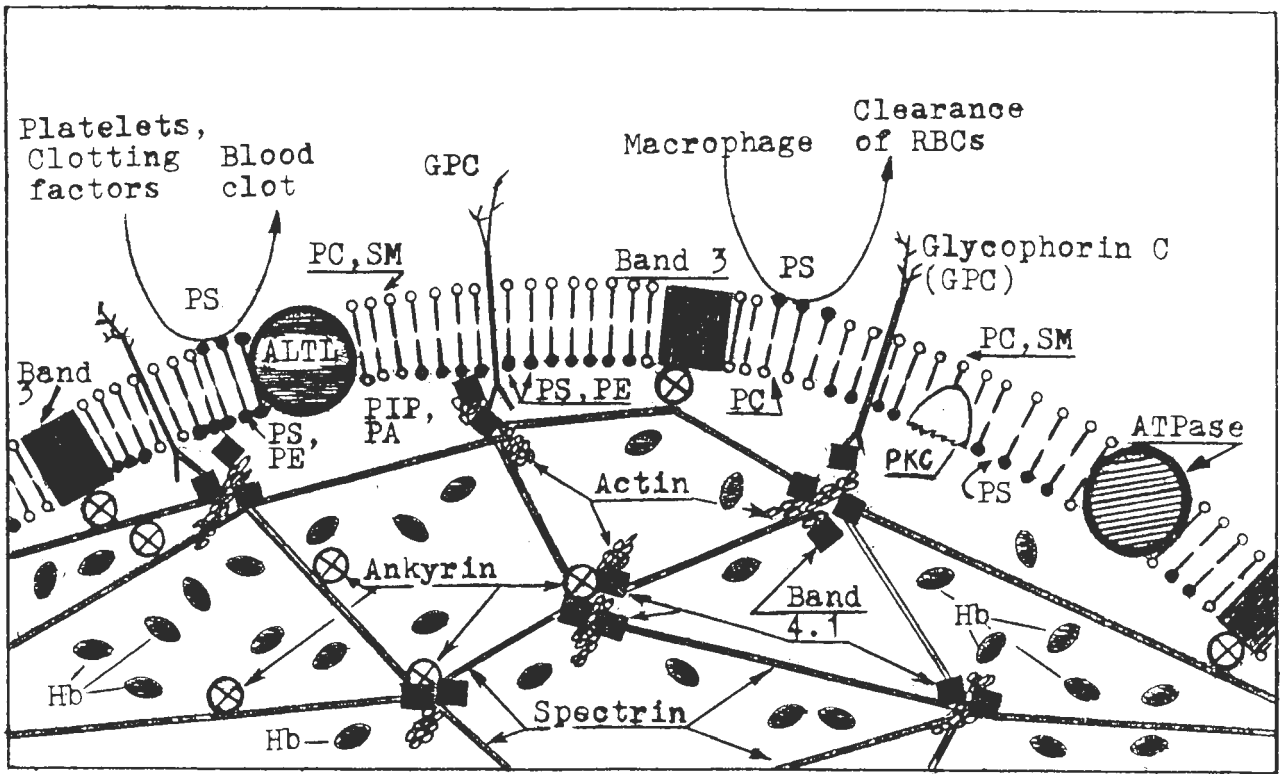

Figure 5. Cell shape-determining interactions of erythrocyte plasma membrane and cytoskeleton. PS: phosphatidylserine; PE: phosphatidylethanolamine; PA: phosphatydic acid; ALTL: aminolipid translocase; PKC: protein kinase C; PIP: phosphatidylinositol phosphate; Hb: hemoglobin; SM: sphingomyelin. (Adapted from Bennett, 1989; Zachowski, 1993).

Deficiency or inactivation of specific cytoskeletal proteins prevent optimal membrane/cytoskeleton contacts, as shown in hereditary sphero- and elliptocytosis (Liu *et al.*, 1990; Mohandas, 1992). In HbS (hemoglobin S, or sickle-cell anemia) disease, the auto-oxidation of membrane proteins, e.g., band 3, may cause the disturbed assembly of cytoskeletal network, thus contributing to sickling (Platt and Falcone, 1995).

PS molecules inside the RBC membrane provide specific bonds with cytoskeletal proteins (Bennett, 1989). Thus, the shape of erythrocytes largely depends on the predominance of PS and phosphatidylethanolamine (PE) at the inner leaflet of plasma membrane, whereas phosphatidylcholine (PC) and sphingomyelin molecules are primarily exposed in the outer lipid monolayer. This difference is referred to as transverse lipid asymmetry of cell membranes (as reviewed by Zachowski, 1993). The internal levels of PS are permanently maintained by its transfer from outside by a specific carrier enzyme, aminolipid translocase. PS transport is energy-dependant, as it is coupled to membrane adenosine triphosphatase (ATPase) activity (Vermeulen *et al.*, 1995).

The prevalence of PC and/or PS in the external membrane leaflet causes transition of RBCs from discocytic to echinocytic shape (expansion of outer leaflet), while overexposure of PS in the inner leaflet induces stomatocytic cell deformation (Zachowski, 1993). Various toxic agents and non-physiological metabolic conditions may alter this normal lipid exchange, thus producing rapid transformation of discoid erythrocytes to echino- or stomatocytic forms (Reinhart and Chien, 1987; Reinhart *et al.*, 1989).

Echinocytosis and Apoptosis are Induced by Similar Factors

Apoptosis of blood and immune cells is often mediated by reactive oxygen intermediates (ROI), which severely affect cell membranes. This evidence is derived from several sources: (1) high susceptibility of lymphoid cells to membrane-damaging oxidants and ROI-generating agents (Buttke and Sandstrom, 1994); (2) endogenous ROI generation at the early stages of cellular death (Kroemer *et al.*, 1995); and (3) apoptosis-preventing effects of antioxidants (Ramakrishnan *et al.*, 1993).

As shown in Table 1, apoptosis of blood karyocytes and echinocytic transformation of RBCs may be caused by similar cytotoxic agents.

Table 1. Examples of RBC echinocytosis and apoptosis of nucleated cells produced by common cytotoxic factors.

	Red Cell Echinocytosis		Apoptosis	
Inducing agent	**Objects, species**	**Reference**	**Objects, species**	**Reference**
Ionizing radiation	*In vivo*, mammalian RBCs	Thompson and Johnstone, 1987	*In vitro*, lymphocytes	Ashwell *et al.*, 1986
		Zharskaya and Chukhlovin, 1996	*In vitro*, cell lines	Radford *et al.*, 1994
Hydrogen peroxide	*In vitro*, human RBCs	Brunauer *et al.*, 1994	Haemopoietic cell lines	Buttke and Sandstrom, 1994
Cytotoxic cell-born oxidants	*In vitro*, feline RBCs	Weiss *et al.*, 1992	*In vitro*, lymphocytes	Buttke and Sandstrom, 1994
			In vitro, tumor cells	Liepins and Bustamante, 1994
Accelerated *in vitro* "aging"	*In vitro*, human RBCs	Reinhart and Chien, 1987 Gedde *et al.*, 1995	*In vitro*, rat thymocytes	Zherbin and Chukhlovin, 1984
			In vitro, human neutrophils	Savill *et al.*, 1989
Antibodies to surface antigens	*In vitro*, human RBCs	Rahamim *et al.*, 1990	*In vitro*, human lymphocytes	Buttke and Sandstrom, 1994

Table 2. Common membrane-affecting alterations associated with "oxidative" echinocytosis and apoptosis.

	Red Cell Echinocytosis		Apoptosis	
Alterations, registered	**Objects, species**	**Reference**	**Objects, species**	**Reference**
Decreased cell volume (shrinkage)	Human RBC, various species	Reinhart and Chien, 1987	Lymphoid cells	Klassen *et al.*, 1993 Szumiel, 1994
Endogenous generation of ROI	Human RBC, oxidative stress	Ciccoli *et al.*, 1994 Delikonstantinos *et al.*, 1995	Several cellular models	Kroemer *et al.*, 1995
Metabolic depletion	Aging human RBC	Gedde *et al.*, 1995	Lymphoid cells	Carson *et al.*, 1986
Calcium overload	*In vitro*, human RBCs	Dreher *et al.*, 1980	*In vitro*, thymocytes	McConkey *et al.*, 1994
Protein kinase C activation	Human RBCs, oxidative stress	Zachowski, 1993	Lymphoid cells, Myeloid cells	Ojeda *et al.*, 1991 Pongracz *et al.*, 1994
Loss of membrane lipid asymmetry	Human RBCs, oxidative stress	Zachowski, 1993 Lin *et al.*, 1994	Lymphoid cells, oxidative agents	Fadok *et al.*, 1992 Koopman *et al.*, 1994

Both types of cell pathology can be induced by ionizing radiation and hydrogen peroxide. Their damaging effects may result from the secondary oxygen intermediates affecting plasma membranes. Moreover, some known oxidants, i.e., ozone and phenylhydrazine, are also capable of producing echinocytosis of RBCs *in vitro* and *in vivo* (Larkin *et al.*, 1978; Ciccoli *et al.*, 1994).

Similarity of Membrane Changes in Echinocytosis and Apoptosis

Apoptosis of white blood cells initiate via several alternative routes such as those involving metabolic depletion, effects of increased intracellular calcium or protein kinase C (PKC) activation. (McConkey *et al.*, 1994). Similar mechanisms can be detected for echinocytic transformation of RBCs (see Table 2).

Mechanisms of Molecular Damage in Echinocytosis

Metabolic depletion and loss of lipid asymmetry

Echinocytic transformation of RBCs, including oxidant-induced crenation, is generally accompanied by marked ATP depletion (Reinhart and Chien, 1987). A pronounced inhibition of PS transport to inner side of membrane is observed under these conditions, thus causing loss of transmembrane lipid asymmetry (Schroit and Zwaal, 1991). This feature is inherent to echinocytosis induced by oxidants and some other toxic agents (Zachowski, 1993). Appropriate PS translocase enzyme may be inhibited due to non-specific cross-linking of SH groups in membrane proteins of oxidized cells (Deuticke *et al.*, 1992; Daleke *et al.*, 1994) or by ATP deficiency itself (Schroit and Zwaal, 1991).

Upon strong oxidative treatment, echinocytic deformation may be accompanied by scrambling of lipid layers, followed by the release of PS into the cytosol of RBCs (Brunauer *et al.*, 1994).

Membrane lipid peroxidation

The mechanisms of echinocytic transformation may be understood from the point of possible ROI generation. For example, ionizing radiation produces increased lipid peroxidation in RBC from irradiated animals, however, several hours after treatment (Kergonou *et al.*, 1986).

A sufficient NO synthesis activity is the important trait of RBC metabolism, similarly to some other cell types. Therefore, oxidant treatment of erythrocytes induces endogenous synthesis of NO and peroxynitrite, thus amplifying the process of intracellular peroxidation (Delikonstantinos *et al.*, 1995).

Oxidative or ROI-inducing effects of irradiation, chemical agents, or RBC aging are followed by increased production of malonic dialdehyde. This compound can form adducts with PE and PS, as shown by Jain (1988).

A possible role of membrane protein oxidation was also proposed for the development of echinocytosis and other membrane deformities (Truong *et al.*, 1986; Deuticke *et al.*, 1992).

Activation of protein kinases and cytoskeleton disfunction

Upregulation of PKC is a well studied mechanism which may accompany changes of red cell shape. This enzyme is active in presence of sufficient PS amounts (Daleke *et al.*, 1994; Pongracz *et al.*, 1994). PKC is activated by the products of lipid hydrolysis. For instance, in the case of red cell oxidation, Zachowski (1993) has suggested a metabolic cascade which suggests cleavage of including hydrolysis of PC by phospholipase C, while the *de novo* formed diacylglycerol is causing PKC activation. Indeed, the *in vivo* irradiated erythrocytes exhibit sufficient PC decay (Badginian *et al.*, 1995). The resulting lysolecithin is a potent inducer of echinocytic transformation (Chasis and Schrier, 1989).

PKC activation may potentially cause increased phosphorylation of some membrane proteins, e.g., band 4.1, thus inducing dissociation of skeleton/membrane complexes (Danilov *et al.*, 1990). Meanwhile, ATP depletion results in dephosphorylation of phosphatidylinositol, thus causing deficient anchorage of spectrin and band 4.1 to the inner lipid leaflet (Bjork and Backman, 1994; Gedde *et al.*, 1995).

Echinocytic deformation of erythrocytes is indeed characterized by clear separation of lipids from skeletal structures at the sites of RBC spiculation (Liu *et al.*, 1989). The authors propose this morphologic change results from an imbalance of membrane lipid and cytoskeleton surface areas. A similar dissociation between cell skeleton and membranes was revealed for the sickled red cells (Liu *et al.*, 1991). The latter data are interpreted as a result of HbS polymerization through gaps in cytoskeletal network, the latter being locally retracted. One may presume that oxidative damage of RBCs may be also accomplished by a cross-linking, e.g., of hemoglobin and spectrin, thus causing changes in the affected erythrocytes' ability to deform (McKenney *et al.*, 1990).

Effects of increased cytosolic calcium

Normal erythrocytes contain only small amounts of free calcium. Intrusion of RBCs with micromolar amounts of Ca induced the so-called Gardos-effect, i.e., rapid echinocytic transformation, loss of potassium, dehydration and volume decrease (Dreher *et al.*, 1980; Gedde *et al.*, 1995). Several mechanisms are proposed to explain the calcium-induced echinocytosis: (1) altered transverse asymmetry of membrane phospholipids caused by the inhibition of aminophospholipid translocase

(Williamson *et al.*, 1992; Zachowski, 1993); (2) activation of PKC, thus producing dissociation of the bonds between cytoskeletal proteins 4.1 and 3 (Danilov *et al.*, 1990); (3) decrease in membrane phosphatidylinositole bi- and monophosphates (Folk and Strunecka, 1990; Gedde *et al.*, 1995), which again affects the membrane-cytoskeleton contacts. Although these factors may be important, Lin *et al.* (1994) have shown that normal transport of PS to the inner membrane layer proved to be the only biochemical feature correlating with the reversal of Ca-crenated RBCs to their discoid shape.

Some others mechanisms of Ca-induced cytoskeletal changes may involve intracellular proteolysis and/or transglutaminase-mediated cross-linking, thus destroying the normal interactions between band 3, band 4.1 and spectrin (reviewed by Reinhart and Chien, 1987; Lin *et al.*, 1994).

Membrane Pathology Associated with Apoptosis

Metabolic alterations and loss of lipid asymmetry

The problems of metabolic depletion in apoptosis have been discussed for decades. At the early stages of apoptosis, some degree of metabolic activation is observed, however, accomplished by the uncoupling of oxidative phosphorylation, thus correlating with auto-oxidation of mitochondrial membranes (as reviewed by Kroemer *et al.*, 1995). A general decrease in energy-producing potential occurs at later times (Carson *et al.*, 1986), thus resembling appropriate changes associated with erythrocytes aging (Gedde *et al.*, 1995).

In contrast to the erythrocyte model, only limited data bear on the loss of transverse lipid symmetry in apoptotic lymphocytes. Koopman *et al.* (1994), by means of a specific annexin probe, have shown an increased surface exposure of PS occuring during the stage of nuclear chromatin decay, indicating the loss of aminolipid preponderance at the inner leaflet of the cell membrane. Fadok *et al.* (1992) reported a similar increase of PS exposure on apoptotic lymphoid cells and proposed that such cells are selectively recognized by the phagocytes.

Lipid peroxidation

The oxidative damage to cell membranes is a well known fact, coupled to the ROI-induced alterations (Köteles, 1986). Many authors have found that enhanced lipid peroxidation accompanies radiation-induced apoptosis of lymphoid cells, and this pathology is inhibited by lipid-specific antioxidant agents (Ramakrishnan *et al.*, 1993). Peroxidation of membrane phospholipids and proteins is regarded as an intrinsic feature of lymphoid cell damage caused by a variety of oxidative agents (Buttke and Sandstrom, 1994).

Activation of protein kinases

Increased activity of PKC during radiation-induced apoptosis of normal lymphoid cells is well documented. However, the role of this event is not clear (Ojeda *et al.*, 1991). Post-irradiation DNA fragmentation in malignant lymphoblasts is also preceded by very rapid membrane alterations involving sphingomyelin decay and activation of lck protein kinase, followed by switching of membrane-associated PKC (Waddick *et al.*, 1993). This catabolic cascade seems to be homologous to those events leading to PKC activation in red blood cells (Zachowski, 1993).

Changes induced by increased cytosolic calcium

The increase of cytosolic calcium seems to accompany apoptosis induced by multiple cytotoxic agents (McConkey *et al.*, 1994). For example, an early elevation of free Ca in irradiated thymocytes precedes nuclear chromatin fragmentation (Allan, 1992).

Free Ca ions are able to activate a variety of catabolic enzymes, including DNA endonuclease, catalyzing the decay of nuclear chromatin (McConkey *et al.*, 1994). For irradiated lymphoid cell lines, the mechanism of Ca-induced phospholipase C activation is suggested, thus causing initial degradation of membrane bilayer. Other effects of intracellular Ca elevation presume activation of PKC (Pongrasz *et al.*, 1994), as shown in some cases of apoptosis.

Conclusions

(1). Transformation of normal discoid erythrocytes to crenated forms (echinocytes) is often induced by factors which also cause apoptosis of nucleated blood cells (e.g., ionizing radiation, chemical oxidants, *in vitro* aging, cytosolic calcium overload, etc.).

(2). The membrane pathology in oxidant-induced echinocytosis is strongly reminiscent of the changes associated with apoptosis (e.g., cell shrinkage, lipid peroxidation, energy depletion and loss of transmembrane lipid asymmetry).

(3). Hence, an understanding of membrane and cytoskeleton pathology of oxidant-crenated RBCs may be helpful for interpreting the mechanisms of apoptosis.

References

Agroyannis B, Dalamangas A, Tzanatos H, Fortounas C, Kopelias I, Koutsikos D (1995) Relation between spiculated red cells and platelets in uraemic patients. Br J Haematol **89**: 686-687.

Allan DJ (1992) Radiation-induced apoptosis: Its role in a MADCaT (mitosis-apoptosis-differentiation-calcium toxicity) scheme of cytotoxicity mechanisms. Int J

Radiat Biol **62**: 145-152.

Ashwell JD, Schwartz RH, Mitchell JB, Russo A (1986) Effect of gamma radiation on resting B-lymphocytes. J Immunol **136**: 3649-3656.

Badginian SA, Kazarian PA, Akopov SE, Saarian AV (1995) Changés in structure and functional state of the membrane of erythrocytes after ionizing irradiation. Radiobiol Radiaz Ecol **35**: 364-369 (in Russian).

Bennett V (1989) The spectrin-actin junction of erythrocyte membrane skeletons. Biochim Biophys Acta **988**: 107-121.

Bennett V, Gilligan DM (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Ann Rev Cell Biol **9**: 27-66.

Bessis M (1973) Living Blood Cells and Their Ultrastructure. Springer-Verlag, Berlin, Germany. pp. 183-284.

Bjork J, Backman L (1994) Sodium selenite as modulator of red cell shape. Biochim Biophys Acta **1191**: 141-146.

Brunauer LS, Moxness MS, Huestis WH (1994) Hydrogen peroxide oxidation induces the transfer of phospholipids from the membrane into cytosol of human erythrocytes. Biochemistry **33**: 4527-4532.

Buttke TM, Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. Immunol Today **15**: 7-10.

Carson DA, Seto S, Wasson DB, Carrera CJ (1986) DNA strand breaks, NAD metabolism, and programmed cell death. Exp Cell Res **164**: 273-281.

Castoldi GL, Beutler E (1988) Erythrocytes. In: Atlas of Blood Cells. Vol. 1. 2nd ed. Zucker-Franklin D, Grossi C, Marmont A (eds.). Fischer, Stuttgart, Germany. pp. 49-156.

Chasis JA, Schrier SL (1989) Membrane deformability and the capacity for shape change in the erythrocyte. Blood **74**: 2562-2568.

Chukhlovin AB (1996) Coupling of membrane pathology and DNA damage in irradiated lymphoid cell populations. In: NATO ASI Symposia Series, Subseries H, Cell Biology, Vol. 94. Springer-Verlag, Berlin. pp. 399-404.

Ciccoli L, Signorini C, Alessandrini C, Ferrali M, Comporti M (1994) Iron release, lipid peroxidation and morphological alterations of erythrocytes exposed to acrolein and phenylhydrazine. Exp Mol Pathol **60**: 108-118.

Cohen GM, Sun X-M, Snowden RT, Dinsdale D, Skilleter DN (1992) Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J **286**: 331-334.

Daleke DL, Huestis WH, Newton AC (1994) Protein kinase C as a measure of transbilayer phosphatidylserine asymmetry. Anal Biochem **217**: 33-40.

Danilov YN, Fennell R, Ling E, Cohen CM (1990) Selective modulation of band 4.1 binding to erythrocyte membranes by protein kinase C. J Biol Chem **265**: 2556-2562.

Delikonstantinos G, Villiotou V, Stavrides JC, Salemes N, Gogas J (1995) Nitric oxide and peroxynitrite production by human erythrocytes: A causative factor of toxic anemia in breast cancer patients. Anticancer Res **15**: 1435-1446.

Deuticke B, Klonk S, Haest WM (1992) Erythrocyte membrane barrier function: Role of bilayer polarity and skeletal proteins. Biochem Soc Trans **20**: 769-773.

Dreher KL, Eaton JW, Breslawec KP, Berger E, Blackshear PL, White JG (1980) Calcium-induced erythrocyte rigidity. The roles of cellular metabolism, hydration and ionic balance. Am J Pathol **101**: 543-555.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol **148**: 2207-2216.

Folk P, Strunecka A (1990) Calcium affects phosphoinositide turnover in human erythrocytes. Gen Physiol Biophys **9**: 281-290.

Gedde M, Yang E, Huestis WH (1995) Shape response of human erythrocytes to altered cell pH. Blood **86**: 1595-1599.

Jacobson MD, Burnen JF, Raff MC (1994) Programmed cell death and Bcl-2 protection in the absence of nucleus. EMBO J **13**: 1899-1910.

Jain S (1988) Evidence for membrane lipid peroxidation during the *in vivo* aging of human erythrocytes. Biochim Biophys Acta **937**: 205-210.

Kergonou JF, Thiriot C, Braquet M, Ducousso R, Rocquet G (1986) Influence of whole-body gamma-irradiation upon rat erythrocytes: Lipid peroxidation and osmotic fragility. Biochimie **68**: 311-318.

Klassen NV, Walker PR, Ross CK, Cygler J, Lach B (1993) Two-stage cell shrinkage and the OER of radiation-induced apoptosis of rat thymocytes. Int J Radiat Biol **64**: 571-581.

Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, van Oers MHJ (1994) Annexin V for flow cytometry detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood **84**: 1415-1420.

Köteles GJ (1986) Cell membranes in radiation injury. Isotopenpraxis **10**: 361-368.

Kroemer G, Petit P, Zamzami N, Vayssiere JL, Mignotte B (1995) The biochemistry of programmed cell death. FASEB J **9**: 1277-1287.

Kubasova T, Köteles GJ, Varga LP (1981) Surface alteration of mammalian cells upon ionizing radiation as detected by lectin-binding techniques. Int J Radiat Biol **40**: 175-194.

Larkin EC, Kimzey SL, Siler K (1978) Response of rat erythrocytes to ozone exposure. J Appl Physiol **45**: 893-898.

Liepins A, Bustamante O (1994) Cell injury and apoptosis. Scanning Microsc **8**: 631-643.

Lin S, Yang E, Huestis WH (1994) Relationship of phospholipid distribution to shape change in Ca-crenated and recovered human erythrocytes. Biochemistry **33**: 7337-7344.

Liu SC, Derick LH, Duquette MA, Palek J (1989) Separation of the lipid bilayer from the membrane skeleton during discocyte-echinocyte transformation of human erythrocyte ghosts. Eur J Cell Biol **49**: 358-365.

Liu SC, Derick LH, Agre P, Palek J (1990) Alteration of the erythrocyte membrane skeletal ultrastructure in hereditary spherocytosis, hereditary elliptocytosis, and pyropoikilocytosis. Blood **76**: 198-205.

Liu SC, Derick LH, Zhai S, Palek J (1991) Uncoupling of the spectrin-based skeleton from the lipid bilayer in sickle red cells. Science **252**: 574-576.

McConkey DJ, Jondal M, Orrenius S (1994) The regulation of apoptosis in thymocytes. Biochem Soc Trans **22**: 606-610.

McKenney J, Valeri CR, Mohandas N, Fortier N, Giorgio A, Snyder LM (1990) Decreased *in vivo* survival of hydrogen peroxide-damaged baboon red blood cells. Blood **76**: 206-211.

Mohandas N (1992) Molecular basis for red cell membrane viscoelastic properties. Biochem Soc Trans **20**: 776-782.

Ojeda F, Andrade J, Maldonado C, Guarda MI, Folch H (1991) Radiation-induced surface IgG modulation: Protein kinase C involvement. Int J Radiat Biol **59**: 53-58.

Platt OS, Falcone JF (1995) Membrane protein interactions in sickle red blood cells: Evidence for abnormal protein 3 function. Blood **86**: 1992-1998.

Pongracz J, Johnson GD, Crocker J, Burnett D, Lord JM (1994) The role of protein kinase C in myeloid cell apoptosis. Biochem Soc Trans **22**: 593-597.

Radford IR, Murphy TK, Radley JM, Ellis SL (1994) Radiation response of mouse lymphoid and myeloid cell lines. II. Apoptotic death is shown by all cell lines examined. Int J Radiat Biol **65**: 217-227.

Rahamim E, Kahane A, Sharon R (1990) Electron microscopy of red blood cells affected by autoimmunity-inducing drugs. Vox Sang **58**: 292-299.

Ramakrishnan N, McClain DE, Catravas GN (1993) Membranes as sensitive targets in thymocyte apoptosis. Int J Radiat Biol **63**: 693-701.

Reinhart WH, Chien S (1987) Echinocyte-stomatocyte transformation and shape control of human red blood cells: Morphological aspects. Am J Haematol **24**: 1-14.

Reinhart WH, Singh A, Straub PW (1989) Red blood cell aggregation and sedimentation: The role of cell shape. Br J Haematol **73**: 551-556.

Savill JS, Wyllie AH, Henson JF, Walport MJ, Henson PM, Haslett C (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest **83**: 865-875.

Schroit AJ, Zwaal RFA (1991) Transbilayer movement of phospholipids in red cell and platelet membrane. Biochim Biophys Acta **1071**: 313-329.

Simpson LO (1989) Blood from healthy animals and humans contains nondiscocytic erythrocytes. Br J Haematol **73**: 561-564.

Szumiel I (1994) Ionizing radiation-induced cell death. Int J Radiat Biol **66**: 329-341.

Thompson JC, Johnstone AC (1987) The effects of gamma-irradiation on haemopoiesis and red blood cell destruction in hamsters infected with Leptospira interrogans serovar ballum. Br J Exp Path **68**: 389-398.

Truong HT, Ferrell JF, Huestis WH (1986) Sulfhydryl reducing agents and shape regulation in human erythrocytes. Blood **67**: 214-221.

Vermeulen WP, Briede JJ, Bunt G, Op den Kamp JA, Kraaijenhagen H, Roelofsen B (1995) Enhanced Mg-ATPase activity in ghosts from HS erythrocytes and in normal ghosts stripped of membrane skeletal proteins may reflect enhanced aminophospholipid translocase activity. Br J Haematol **90**: 56-64.

Waddick KG, Chae HJP, Tuel-Ahlgren L, Jarvis LJ, Dibirdik I, Myers D, Uckun FM (1993) Engagement of CD 19 receptor on human B-lineage leukemia cells activates lck tyrosine kinase and facilitates radiation-induced apoptosis. Radiat Res **136**: 313-319.

Weiss DJ, Aird B, Murtaugh MP (1992) Neutrophil-induced immunoglobulin binding to erythrocytes involves proteolytic and oxidative injury. J Leukoc Biol **51**: 19-23.

Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF (1992) Ca induces transbilayer redistribution of all major phospholipids in human erythrocytes. Biochemistry **31**: 6355-6360.

Wyllie AH (1994) Death gets a brake. Nature **369**: 272-273.

Zachowski A (1993) Phospholipids in animal eukaryotic membranes: Transverse asymmetry and movement. Biochem J **294**: 1-14.

Zharskaya VD, Chukhlovin AB (1996) *In vivo* post-radiation echinocytosis of rat red blood cells as detected by scanning electron microscopy. Scanning Microsc **10**: 279-284.

Zherbin EA, Chukhlovin AB (1984) Possible association of membrane and nuclear changes in gamma-irradiated rat thymocytes. Int J Radiat Biol **45**: 179-183.

Zherbin EA, Chukhlovin AB, Köteles GJ, Kubasova TA, Vashchenko VI, Hanson KP (1986) Effects of *in vitro* cadmium ions on some membrane and nuclear parameters of normal and irradiated thymic lymphoid cells. Arch Toxicol **59**: 21-25.

Discussion with Reviewers

R.L. Warters: Apoptosis can be induced in nucleated blood cells by treatment with either sphingomyelinase C, which hydrolyses sphingomyelin to ceramide, or with ceramide itself. Thus, one pathway for radiation-induced apoptosis has been suggested to be the activation of sphingomyelinase. Does treatment with either sphingomyelinase C or ceramide induce "apoptosis-like" changes in RBCs?
Author: To my knowledge, such effects upon RBCs were not investigated. Appropriate trials with erythrocytes concerned, mainly, hydrolysis of the membrane phosphatidylcholine (PC) by phospholipase C, followed by the activation of PKC by resulting diacylglycerol and RBC crenation induced by the products of PC cleavage (Zachowski, 1993). Similar effects of ceramide or sphingomyelinase upon RBCs, if revealed, may provide additional homologies between apoptosis and echinocytosis.

J.E. Trosko: Assuming the hypothesis that the morphological changes seen in these ROI-treated red blood cells are equivalent to those associated with apoptosis in other nucleated cells, what biochemical parameter would be the most important potential target for triggering this process?
Author: A loss of membrane asymmetry would be of special interest, due to its early expression and pathological significance. For example, a degree of phosphatidylserine exposure at the outer leaf of blood cells membrane could be specifically assessed by means of annexin V binding to the cells under study (as reviewed by Kroemer *et al.*, 1995).

J.E. Trosko: Since many tumor-promoting chemicals, such as phorbol esters, block apoptosis in nucleated cells, activate PKC, and induce oxidative stress, how might this be used to test your ideas?
Author: The effects of phorbol esters seem to be quite different for various cell populations. These compounds may induce apoptosis or inhibit it, depending on the cell model and toxic agents under trials (as reviewed by McConkey *et al.*, 1994; Obeid and Hannun, 1995). Red blood cells may be also tested with phorbol esters, with the aim of evaluating probable membrane oxidative damage. Comparisons would be made, e.g., to erythroblasts or avian nucleated RBCs.

J.E. Trosko: Retinoids and dexamethasone can induce apoptosis. Would you predict that it could mimic the changes seen in the transition of discoid forms to crenated forms in the red blood cells?
Author: In my opinion, their effects could be specially tested and considered. In the present review, the effects of proven oxidative agents (H_2O_2, irradiation, etc.) were discussed. For glucocorticoids and retinoids, specific cell receptors are well known to mediate apoptosis in different cell models, thus suggesting alternative modes of membrane damage.

F. Ojeda: Could you change the title of your paper, because the term "oxidative apoptosis" is unclear?
Author: Indeed, the term "oxidative" can be omitted from the title, since this definition does not correspond to common classifications of apoptosis. Oxidant-induced initiation of lethal events is shown for many types of apoptosis (Buttke and Sandstrom, 1994). However, oxidative damage is not too evident for some other models, e.g., calcium + ionophore treatment, or corticosteroid-induced death of lymphoid cells (McConkey *et al.*, 1994). Hence, while omitting the term "oxidative" from the title, we try, mainly, to consider the possible consequences of oxidative damage for the triggering of apoptosis, such as appropriate homologies to RBC crenation (echinocytosis).

G.J. Köteles: As well known, apoptosis appears to be an active process of cellular self-destruction involving a series of cellular events including membrane changes upon oxidative stress, and requires active cell participation, macromolecular synthesis and enzyme activation. The listed examples about apoptotic "markers" of erythrocyte membrane are not unambiguous facts. For example, some agents, inducing changes of cytoplasmic calcium concentration, frequently do not cause apoptosis. There are data that protein kinase C activation blocks apoptosis; other ones suggest that this process promotes apoptosis as reviewed recently by Lucas and Sanchez-Margalet (1995). Can you provide some unambiguous data which are characteristic of apoptosis process?
Author: Indeed, apoptosis-associated intracellular calcium increase, as well as PKC activation are limited only to certain cytotoxic treatments, and their expression is strongly determined by the nature of target cells. However, the involvement of both metabolic events is proposed for lymphoid cells subjected to ionizing irradiation and some other agents (Ojeda *et al.*, 1991; McConkey *et al.*, 1994). For parallel studies of oxidative membrane pathology in RBCs and nucleated blood cells, some "apoptosis-specific" tests are needed. These tests are now based, mainly, upon detection of DNA decay, and thus, not acceptable for studies of anucleate

RBCs. However, erythrocytes are very well studied with respect to physiology and pathology of cell membranes. Therefore, definite changes of membrane ultrastructure and metabolism of RBCs treated by apoptosis-inducing agents may be useful in providing additional markers for apoptosis detection in nucleated blood cells, e.g., plasma membrane vesiculation and shedding, phosphatidylserine exposure, etc.

Additional References

Obeid L, Hannun YA (1995) Ceramide: A stress and mediator of growth suppression and apoptosis. J Cell Biochem **58**: 191-198.

Lucas M, Sanchez-Margalet V (1995) Protein kinase C involvement in apoptosis. Gen Pharmacol **26**: 881-887.