Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 69 (2005) 73–85
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Changes in spectrin organisation in leukaemic and lymphoid cells upon chemotherapy

Patrycja M. Dubielecka[a], Bożena Jaźwiec[b], Stanisław Potoczek[b], Tomasz Wróbel[b], Joanna Miłoszewska[c], Olga Haus[b], Kazimierz Kuliczkowski[b], Aleksander F. Sikorski[a,d,*]

[a]*Laboratory of Cytobiochemistry, Institute of Biochemistry and Molecular Biology, University of Wrocław, S. Przybyszewskiego 63/77, 51-148 Wrocław, Poland*
[b]*Wrocław Medical University, Department of Hematology, L. Pasteura 4, 50-367 Wrocław, Poland*
[c]*The Maria Skłodowska–Curie Memorial Cancer Center, Institute of Oncology, Department of Cell Biology, ul. W.K.Roentgena 5, Warszawa, Poland*
[d]*Academic Centre for Biotechnology of Lipid Aggregates, S. Przybyszewskiego 63/77, 51-148 Wrocław, Poland.*

Received 5 March 2004; accepted 31 August 2004

Abstract

The aim of the present study was to investigate changes in spectrin and protein kinase C θ (PKC θ) organisation in human lymphoid and leukaemic cells undergoing chemotherapeutically induced apoptosis. An analysis of spectrin arrangement in human peripheral lymphoid (non-Hodgkin lymphoma) and leukaemic (acute lymphoblasic leukaemia) cells before and after chemotherapy revealed radical differences in the distribution of this protein. By using immunofluorescent technique, in lymphocytes isolated before chemotherapy, we found spectrin evenly distributed in the cytoplasm and the plasma membrane, while after the therapy changes in spectrin organisation occurred. Moreover, in lymphocytes after chemotherapy, extraction with buffer containing non-ionic detergent (Triton X-100) revealed presence of an insoluble fraction of spectrin. In normal or malignant cells before chemotherapy spectrin was totally soluble, however it should be mentioned that in total cell extracts and supernatants (but not in pellets) apoptotic fragments of spectrin (in addition to intact α and β chains) were also found. In malignant cells after chemotherapy changes in PKC θ organisation, similar to this observed in the case of spectrin, were shown by the immunofluorescence technique. In contrast, no differences in the distribution of other isoforms of protein kinase C: βI and βII, before and after chemotherapy, were found. Apoptotic phosphatidyloserine (PS) externalisation, as well as cell shrinkage, membrane protrusions and blebbing were observed in lymphocytes after chemotherapy and treatment with cytostatics in vitro. The overall results may suggest that spectrin redistribution/aggregation is the phenomenon involved in programmed cell death (PCD) of normal and neoplastic lymphocytes and lymphoblasts, however molecular basis of this phenomenon should be further investigated.

Keywords: Nonerythroid spectrin (fodrin); PKC θ; Chemotherapy; Apoptosis; ALL; nHL

1. Introduction

Spectrins play a crucial role in the structural integrity, morphology and organization of the cellular membranes. Spectrins are ubiquitous, multidomain actin-binding cytoskeletal proteins, which are composed of α- and β-subunits. These subunits are associated to form antiparallel heterodimers, which are assembled head–head to form 200 nm extended heterotetrameric filaments (for a review, see [1,2]). Several recent reports have implied that spectrins, in addition to their main structural function, i.e. providing mechanical support for the membrane bilayer, are also engaged in regulatory and signal transduction pathways in different cell types; fibroblasts, neurons, muscle cells, lymphocytes [3–6]. Among many domains responsible for interactions with membrane and membrane attachment proteins, spectrins possess two domains which are involved in regulatory and signalling pathways. SH3–Src protein tyrosine kinase homology domain, which is present in many proteins engaged in cell signalling and mediates

Abbreviations: PKC, protein kinase C; SMAC, supramolecular activation complex; NF-κB, nuclear factor κB; IS, immunological synapse; TCR, T-cell receptor; TGF β, transforming growth factor β; ELF, embryonic liver fodrin; PBMCs, peripheral blood mononuclear cells

* Corresponding author. Tel.: +48 71 3756 233; fax: +48 71 3756 208.
E-mail address: afsbc@ibmb.uni.wroc.pl (A.F. Sikorski).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.08.031

interactions with proline-rich stretches in a number of target proteins, is found mostly in α-spectrins (the only exception is βV spectrin identified in human tissues) [7]. PH (pleckstrin homology) domain is located to the COOH-terminal segment of some β-spectrin isoforms and was first resolved in pleckstrin (a major protein kinase C substrate) [8,9]. Observation of spectrin in various myeloid and lymphoid cell lines revealed appearance of two patterns of spectrin distribution: in most cell lines spectrin was evenly distributed in the cytoplasm, but in some lines the cells contained an aggregate of spectrin [10]. Additionally, treatment of these cells with phorbol myristate acetate (PMA) and mezerin caused disappearance of the aggregates. Afterwards, it was shown that treatment of lymphocyte hybridoma cell lines with the following antigenic stimuli: chicken ovalbumin, TCR-CD3 (T-cell receptor complex), concanavalin A or calcium ionopohore A231187, resulted in reversible disappearance of spectrin aggregates [11]. These findings initiated a number of experiments aiming to explain the function of spectrin and the possible role of its specific aggregation in lymphocytes [12–14]. One of the points which gave a new insight into the connection of the aggregation of spectrin with the activation of lymphocytes, was an indication of co-localisation (in untreated and activated cells) of spectrin and protein kinase C βII and θ, hsp70, and the receptor for activated C kinase-1 (RACK-1) [15]. Moreover, it was shown that treatment with phorbol ester (PMA), T receptor cross-linking and mild hyperthermia resulted in activation of lymphocytes and formation of cytoplasmic spectrin aggregates [15]. Recruitment of intracellular proteins to the plasma membrane is well known requirement for the initiation of signal transduction events, and participation of spectrin in this phenomenon may indicate its signalling function in lymphocytes. PKC θ, this Ca^{2+}-independent subfamily of serine/threonine specific protein kinase C, is expressed predominantly in haematopoietic cells and muscle [16]. PKC θ in resting T-cells resides in the cytosol, but upon activation translocates to the membrane-rich fraction, where it becomes active [17]. Translocation of PKC to the membrane site is observed upon activation of lymphocytes by pharmacological agents, which stimulate PKC independently of the cell surface receptors, as well as upon clustering of specific cell-surface receptors such as TCR. Interaction of a T lymphocyte with an antigen presenting cell (APC) results in the clustering of the T-cell antigen receptor and the assembling of a large signalling complex (supramolecular activation complex (SMAC)) (for review see [18]). Protein kinase C θ is the only known member of the PKC family to assume 'center stage' in the T-cell SMAC. Upon TCR stimulation PKC θ is rapidly recruited to the site of TCR clustering—central SMAC, where it transduces critical activation signals leading to IL-2 production. PKC θ is crucial to couple TCR stimulation to nuclear factor κB (NF-κB) upstream of the inhibitor of κB kinase (IKK) complex which is dispensable for TNFR-mediated activation of NF-κB. PKC θ is also crucial to generation of active AP-1 at a downstream of the ERK and JNK/MAP-kinase pathways. By being a part of TCR complex and NF-κB and AP-1 nuclear pathways of transactivation, PKC θ regulates secretion of IL-2 in peripheral T cells thus generating responses upon activation [19]. The junction between T lymphocyte and APC is called immunological synapse (IS) and consists of central cluster of TCR surrounded by a ring of the adhesion molecules. IS passes through few stages resulting the 'mature' IS. In the mature IS TCR/CD3, peptide-MHC, CD28 (accesory molecule) and its ligand CD80 and cytoplasmic signalling molecules (e.g., lck, fyn) and PKC θ, congregate in the centre of the interface. This central area is called central cluster (cSMAC). LFA-1, ICAM-1 and talin form a ring surrounding the cSMAC and is named peripheral SMAC (pSMAC). CD2 and its ligand CD48 surrounds pSMAC and finally CD43, a large mucin-like molecule is mostly excluded from the contact interface [20–23]. It is still not clear if there are any analogies between immunological synapses and spectrin-rich aggregates found by Repasky and coworkers [10–15] in chemically or physically activated myeloid and lymphoid cell lines. Hsp70, some of the SH2-containing effector molecules that associate with phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs), PKC θ and specific membrane associated RACKs proteins were found in spectrin-rich large aggregates [24,25]. Above facts implicate that occurrence of aggregation of spectrin and PKC θ in chemically and physically stimulated lymphocytes and formation of a large signalling complex at the site of TCR clustering imunological synapse may be related phenomena. A few recent reports point to the role of spectrin in key signalling pathways. It was shown [26] that disruption of embryonic liver fodrin (ELF, the shortest isoform of β-spectrin), leads to disruption of transforming growth factor-β (TGF-β) signalling by Smad proteins in mice. ELF is considered as an essential adaptor protein required for the key events in the propagation of TGF-β signalling. After stimulation with TGF-β, phosphorylated ELF may normally associate with endogenous Smad3 and the TGF-β receptor complex. This interaction is followed by its interaction with Smad4, leading to their translocation to the nucleus. The above information implies that spectrin may be involved in various signalling pathways, and apoptotic signalling pathways among them should also be considered.

Most of the recent studies concerning participation of spectrin in apoptosis concentrate on appearance of calpain and caspase generated breakdown products of spectrin, and have been based on the experiments on various cell lines treated with various apoptotic inducers [27–35]. In this study we attempt to characterise the distribution of spectrin in human malignant cells belonging to the B cell phenotypes during chemotherapy and to observe the accompanying changes in its extractibility by non-ionic detergent

solutions as well as PKC isoforms distribution. Our data strongly suggest that aggregation of spectrin and apoptosis are associated events. In some cases we found, except for normal, uncleaved α- and β-spectrin, its protease (calpains and caspases) generated fragments. We postulate that spectrin aggregation may be somehow associated with early apoptotic events such as loss of membrane lipid asymmetry (phosphatidyloserine, phosphatidylethanolamine) rather than with late apoptotic cleavage. Moreover, the observed rearrangement of PKC θ implies its relation to spectrin aggregation and participation of both events in regulatory and early steps of apoptosis.

2. Materials and methods

2.1. Patients

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples from previously untreated adult patients with acute leukaemia and a non-Hodgkin lymphoma subtype (acute leukaemia and lymphoma cases with B-cell immunophenotype were selected; T-cell lymphoma and Hodgkin's disease patients were excluded; see Table 1). Samples were collected before and after a 1-week cycle of ongoing cytostatical chemotherapy. The diagnosis was based on appropriate clinical and morphological features, and was confirmed by the expression of appropriate antigens on the malignant cells. The stage of the disease was defined according to the revised European–American lymphoma (REAL) classification. Clinical data concerning a detailed diagnosis and the applied chemotherapy are shown in Table 1.

2.2. Blood collection and PBMC preparation

Venous blood of healthy donors and patients (Table 1) before and after the first cycle of chemotherapy was collected on heparin as anticoagulant. Peripheral blood mononuclear cells were separated by density gradient centrifugation on Gradisol L (AQUA-MEDICA), and washed three times with phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free. To remove the remaining erythrocytes, PBMC were washed with hypotonic buffer and again with Ca^{2+} and Mg^{2+} free PBS.

2.3. Culture conditions

PBMCs were isolated from healthy subjects and from patients with acute lymphoblastic leukaemia and with follicular lymphoma (Table 1). These three populations were cultured in 1.0 ml plastic tubes (1×10^6 cells) in RPMI 1640 medium supplemented with heat-inactivated (56 °C, 30 min) fetal calf serum (FCS; 10%), 100 μg/ml gentamycin and 2 mM L-glutamine for 24 h at 37 °C in 5% CO_2. One part of leukaemic PBMC and lymphoid PBMC populations was cultured only in the medium, while the other part was cultured in the medium with an addition of cytostatics: fludarabine (1 μg/ml), mitoxantrone (0.5 μg/ml) and dexamethasone (0.5 μg/ml) separately and combined, for 24 h at 37 °C in 5% CO_2.

2.4. Antibodies

The anti-brain spectrin rabbit antibody used in this study for the immunofluorescence and Western blot (at the concentrations 1:250 and 1:1000, respectively), recognises both the α- and β-subunits of non-erythroid spectrin, and is not cross-reactive with spectrin in erythrocytes. This antibody was obtained in our laboratory using purified bovine brain whole spectrin molecules (both the α- and β-subunits).

The IgG against nPKC θ, cPKC βI and βII used in our experiments were affinity-purified rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc.).

The conjugated anti-rabbit IgG-biotin and fluorescein isothiocyanate (FITC)–streptavidine conjugates were purchased from DACO, Inc.

The anti-rabbit IgG (whole molecule)-alkaline phosphatase conjugate secondary antisera for the Western blot analysis were purchased from The Jackson Laboratory.

Table 1
Patient, diagnosis and chemotherapy applied

Number of patients	Diagnosis	Chemotherapy
1	Chronic lymphocytic leukaemia, CLL	Fludarabine, mitoxantrone, dexamethasone (FND)
2	Chronic lymphocytic leukaemia, CLL	Fludarabine, mitoxantrone, dexamethasone (FND)
3	Chronic lymphocytic leukaemia, CLL	Fludarabine, mitoxantrone, dexamethasone (FND)
4	Chronic lymphocytic leukaemia, CLL	Leukerane
5	Chronic lymphocytic leukaemia, CLL	Leukerane
6	Follicular lymphoma	Cyclofosfamide, doxorubicine, vincristine, prednisone (CHOP)
7	Follicular lymphoma	Cyclofosfamide, vincristine, prednisone (COP)
8	Follicular lymphoma	Cyclofosfamide, vincristine, prednisone (COP)
9	Follicular lymphoma	Cyclofosfamide, vincristine, prednisone (COP)
10	Acute lymphoblastic leukemia,	Prednisone
11	Acute lymphoblastic leukemia,	Prednisone
12	Acute lymphoblastic leukemia,	Prednisone
13	Lymphoplasmocytic lymphoma	Fludarabine, mitoxantrone, dexamethasone (FND)
14	Mantle cell lymphoma	Fludarabine, mitoxantrone, dexamethasone (FND)

2.5. Immunofluorescence

PBMC were first incubated with 1 mM EGTA in PBS for 10 min and then attached to coverslips by cytospin (800 × *g*, for 5 min) and dried for 12 h. Next, the cells were fixed in 2% formaldehyde, permeabilised with 0.5% Triton X-100 in PBS. To reduce non-specific binding, the coverslips were incubated in 1% fetal calf serum for 15 min. The cells were incubated for 30 min with the above-mentioned anti-brain spectrin (1:250), anti-PKC θ (1:200), βI (1:200) and βII (1:200) polyclonal rabbit antibodies. Next, the cells were incubated for 30 min with biotinylated anti-rabbit IgG (1:100) and then with a streptavidine–fluorescein isothiocyanate conjugate (1:100) for 15 min. Before each incubation, the cells were washed several times in PBS buffer. At the end, the cells were washed additionally in distilled water and mounted for analysis. Immunofluorescence results were analysed with an epi-fluorescence microscope (Zeiss); micrographs were taken using 400 ISO films (Fuji).

2.6. Non-ionic detergent extraction and the Western blot analysis

The PBMC were purified as described above and were incubated for 10 min at 0 °C in buffer containing 1% Triton X-100, 100 μg/ml PMSF and 1 mM EDTA, then cell lysates were centrifuged at 30,000 × *g* at 4 °C. The obtained supernatants and pellets as well as the controls (intact cell suspension in PBS) were treated with a sample buffer (125 mmol/L Tris–HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% β-mercaptoethanol, 10% gly-

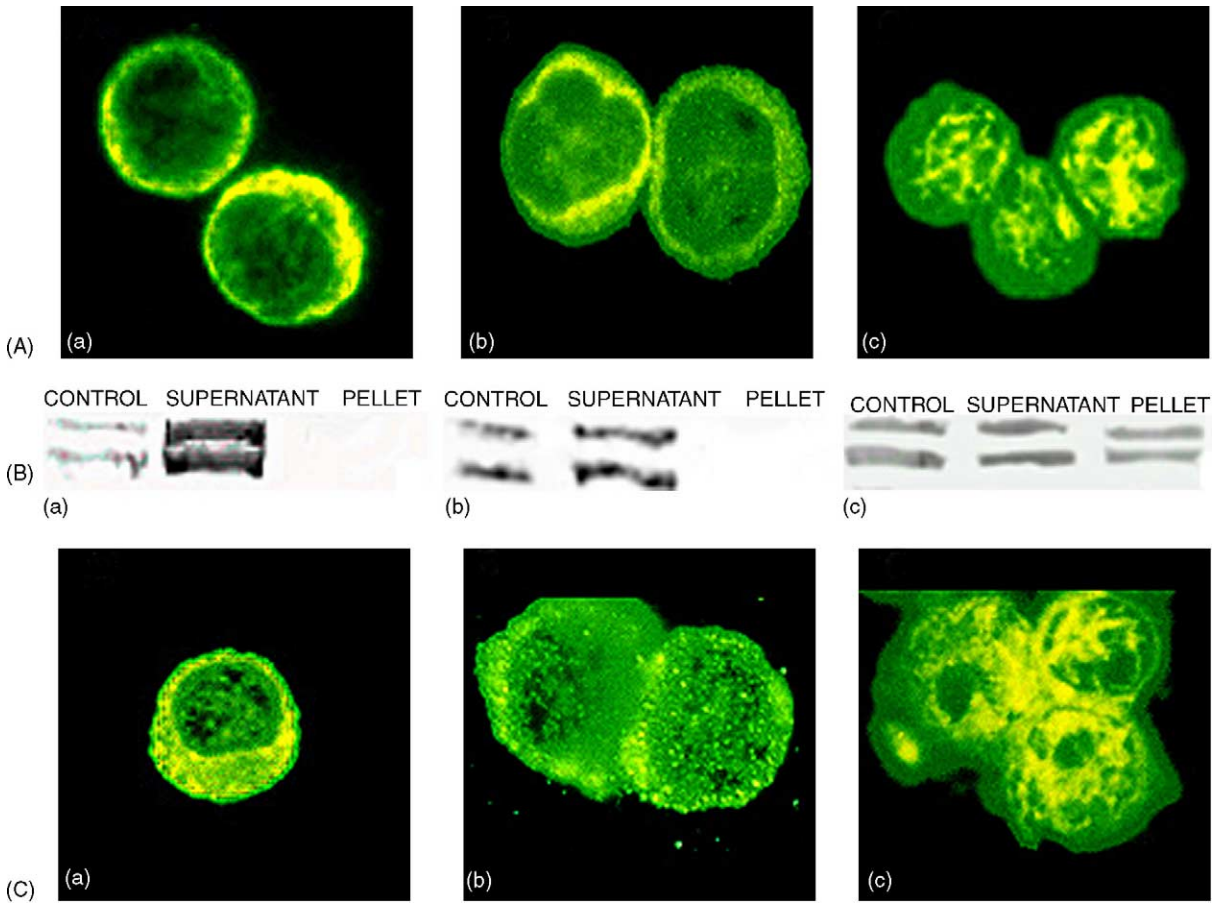

Fig. 1. Changes in spectrin and PKC θ organisation, but not PKC βI and βII, as well as cell shrinkage and membrane blebbing occur in malignant cells of non-Hodgkin lymphoma patients after the first cycle of chemotherapy. (A) Immunofluorescent pattern of spectrin distribution in normal (a) and malignant cells before (b) and after (c) chemotherapy; obtained by using anti-bovine spectrin antibody (1:250), biotinylated anti-rabbit IgG as a second antibody (1:100), and conjugates of streptavidine with FITC (1:100). Magnification 1000×. (B) A spectrin non-ionic detergent insoluble fraction occurs in malignant cells after chemotherapy (c). Normal (a) and malignant cells before (b) and after (c) chemotherapy were incubated for 10 min at 0 °C in buffer containing 1% Triton X-100, 100 μg/ml PMSF and 1 mM EDTA, then cell lysates were centrifuged at 30,000 × *g* at 4 °C. The obtained supernatants and pellets as well as the controls (intact cell suspension in PBS) were analysed by using the Western blot technique (see Section 2). (C) Immunofluorescent pattern of PKC θ distribution in normal (a) and malignant cells before (b) and after (c) chemotherapy; obtained by using anti-PKC θ antibody (1:200). The detection of the antigen–antibody complex was performed as in the legend to (A). (D) Immunofluorescent pattern of PKC βI and βII distribution in normal (a) and malignant cells before (b) and after (c) chemotherapy; obtained by using anti-PKC βI and anti-PKC βII antibodies (1:200), see legend to (A). Magnification 1000×. (E) Cell shrinkage, membrane protrusions and blebbing occur in malignant cells after chemotherapy (c). Normal lymphocytes (a) and malignant cells before (b) and after (c) chemotherapy were attached by cytospin (800 × *g*) to the cover slips and stained by standard May Grunwald Giemsa reagent. Magnification 1000×.

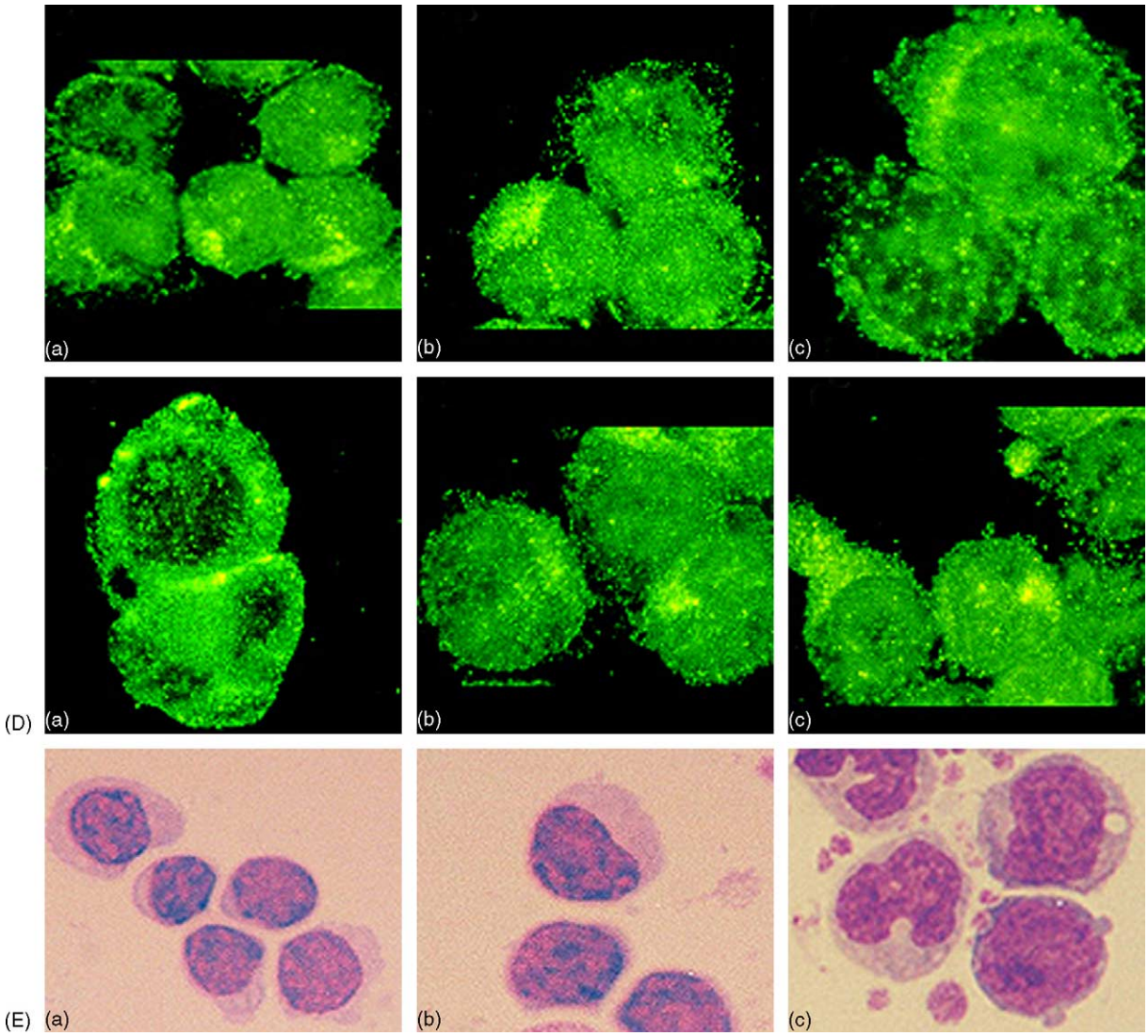

Fig. 1. (*Continued*).

cerol, 0.01% bromophenol blue), and heated at 100 °C for 7 min. The protein samples were subjected to 6% SDS-PAGE gel electrophoresis followed by electrotransfer onto a 0.22 μm pore nitrocellulose membrane in 0.2 M glycine–NaOH transfer buffer with an addition of 0.01% SDS. Filters were blocked for 2 h at room temperature with blocking buffer (10% FSC, 0.1% Tween 20, PBS), and subsequently incubated for 2 h at room temperature in blocking buffer containing anti-brain spectrin antiserum at a dilution of 1:1000. Membranes were then washed and incubated for 1.5 h at room temperature in blocking buffer containing a 1:10,000 alkaline phopsphatase labelled goat anti-rabbit antibody (The Jackson Laboratory) and extensively washed. The filters were incubated for 2 min in PBS, pH 9.5, with Mg^{2+} ions and buffer containing NBT and BCIP (Roche). Normal leukaemic and lymphoid cells, untreated with extraction buffer, were used as controls.

2.7. The apoptotic morphological changes assay

May Grünwald-Giemsa-stained cytospins of peripheral blood mononuclear cells were reviewed for apoptotic changes in morphology studies. At least 100 cells were assessed.

2.8. Detection of apoptosis by anexin V-FITC binding assays

Early stage apoptosis was assessed using the Annexin V-FITC apoptosis detection kit (Oncogen Research Products). PBMC were isolated from healthy subjects and from patients with acute lymphoblastic leukaemia and with follicular lymphoma (Table 1). These three populations were cultured with cytostatics (see Section 2.3 for details) and analysed according to the Annexin V-FITC apoptotis detection kit detailed protocol. A PAS flow cytometer

(Partec), equipped with the Flo Max 2.4 B software, emitting an excitation light at 488 nm from an argon ion laser was used to quantify Annexin V-FITC and propidium iodide signals. FITC signals were detected at 518 nm and propidium iodide at 620 nm.

3. Results

3.1. Spectrin and chemotherapeutically induced apoptosis

We analysed 11 non-Hodgkin lymphoma and 3 acute lymphoblastic leukaemia clinical cases. In all the cases we obtained the same results; in this report we present representative micrographs and figures. The objects of our study were mononuclear peripheral neoplastic cells, lymphoblasts and lymphoid lymphocytes, in which we analysed distribution of spectrin before and after chemotherapy, using the classical immunofluorscence and Western blot techniques. As a control we used (PBMC) isolated from healthy donors.

3.1.1. Changes in spectrin organisation in immature and mature malignant lymphocytes after the first cycle of cytostatical therapy: immunofluorescence studies

The purpose of our observations was a comparison of the immunofluorescence pattern of spectrin distribution in populations of PBMC isolated from healthy donors (control) and peripheral leukaemic and/or lymphoid cells before and after the first cycle of chemotherapy. Distribution of spectrin was assessed by immunofluorescence microscopy observations, using anti-bovine spectrin rabbit antibodies recognising both subunits of non-erythroid

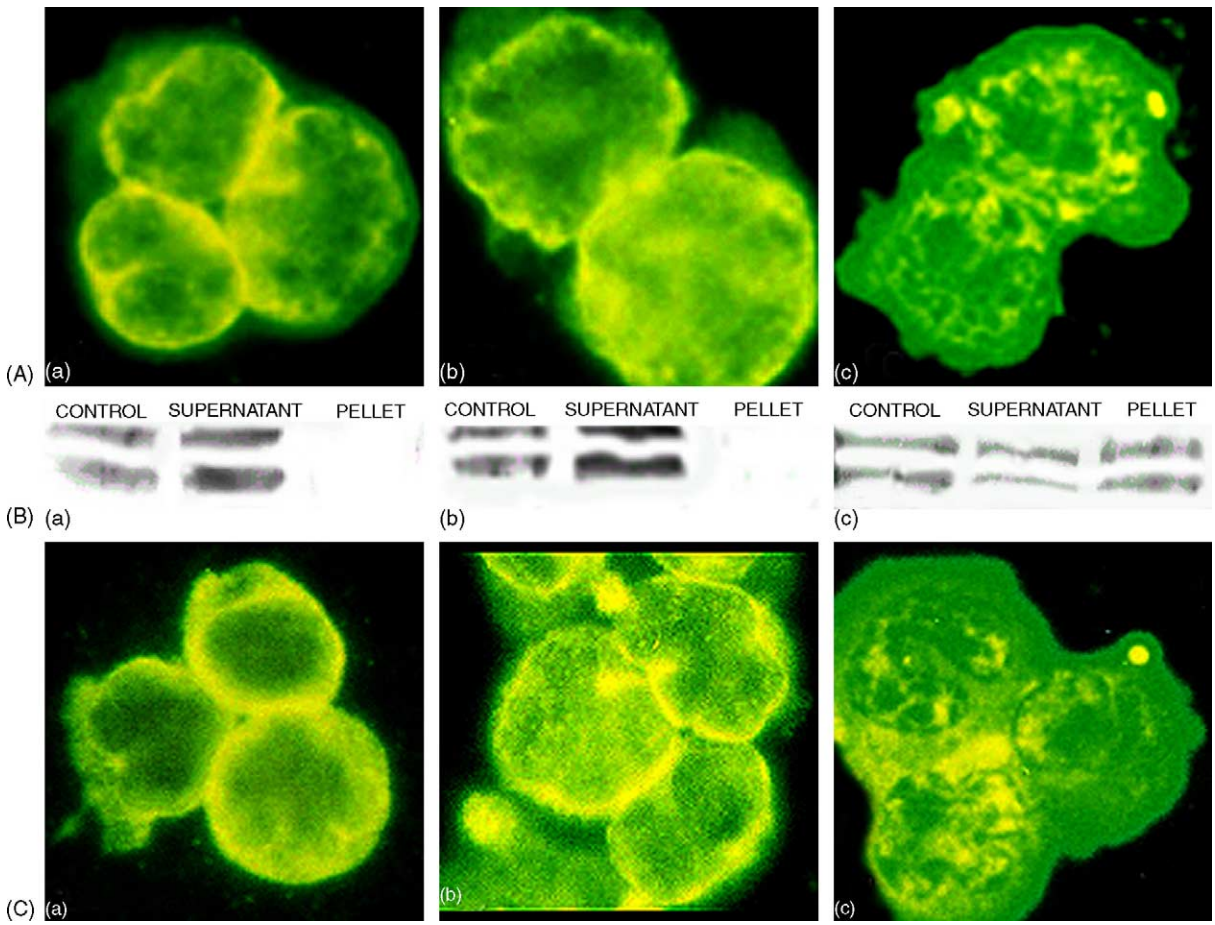

Fig. 2. Changes in spectrin and PKC θ organisation, but not PKC βI and βII, as well as cell shrinkage and membrane blebbing occur in leukaemic cells of acute lymphoblastic leukaemia patients after the first cycle of chemotherapy. (A) Immunofluorescent pattern of spectrin distribution in normal (a) and leukaemic cells before (b) and after (c) chemotherapy; obtained by using anti-bovine spectrin antibody (1:250), see legend to Fig. 1A. (B) Spectrin non-ionic detergent insoluble fraction occurs in leukaemic cells after chemotherapy (c). Normal (a) and leukaemic cells before (b) and after (c) chemotherapy were incubated for 10 min at 0 °C in buffer containing 1% Triton X-100, 100 μg/ml PMSF and 1 mM EDTA, then cell lysates were centrifuged at 30,000 × *g* at 4 °C. The obtained supernatants and pellets as well as the controls (intact cell suspension in PBS) were analysed by using the Western blot technique (see Section 2). (C) Immunofluorescent pattern of PKC θ distribution in normal (a) and leukaemic cells before (b) and after (c) chemotherapy); obtained by using anti-PKC θ antibody (1:200), see legend to Fig. 1A. (D) Immunofluorescent pattern of PKC βI and βII distribution in normal (a) and leukaemic cells before (b) and after (c) chemotherapy; obtained by using anti-PKC βI and anti-PKC βII antibodies (1:200), see legend to Fig. 1A. (E) Cell shrinkage, membrane protrusions and blebbing occur in leukaemic cells after chemotherapy (c). Normal lymphocytes (a) and leukaemic cells before (b) and after (c) chemotherapy were attached by cytospin (800 × *g*) to the cover slips and stained by standard May Grunwald Giemsa reagent. Magnification 1000×.

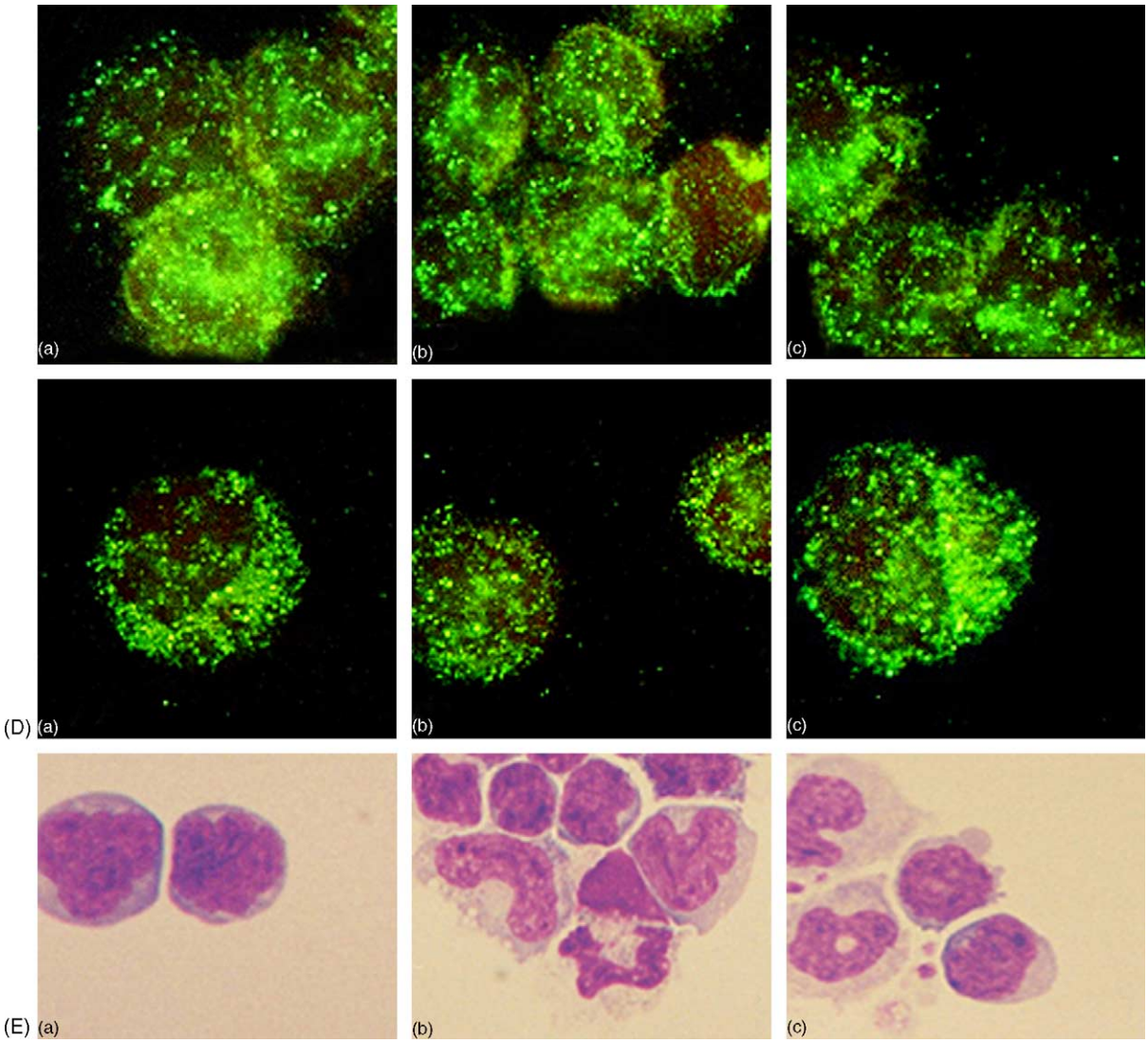

Fig. 2. (*Continued*).

spectrin (data not shown). The experiments revealed that spectrin in normal cells obtained from healthy donors (Fig. 1A(a) and Fig. 2A(a)) and in lymphoid and leukaemic cells isolated before the first cycle of chemotherapy (Fig. 1A(b) and Fig. 2A(b)) was evenly distributed in the cell cytoplasm and membrane. It should be noted however, that in some cases, cells in the culture, showed some aggregation at the periphery (Fig. 4A(b)), but the change which occurred upon treatment with cytostatics, was rather dramatic and could be mainly observed in the nuclear area. In the cells isolated after the first cycle of chemotherapy aggregation of spectrin occurred both in lymphoid (Fig. 1A(c)) and leukaemic (Fig. 2A(c)) cells. This specific aggregation of spectrin was observed after the first cycle of chemotherapy in each population of neoplastic cells of each ALL and nHL patient examined. This major change in spectrin organisation was well visible in the nuclear area. We also examined the distribution of spectrin in leukaemic and lymphoid cells after the second and following cycles of chemotherapy: changes in spectrin distribution in the analysed cells were sustained, however, the observed pattern of protein distribution was not so conclusive and the aggregates were successively dispersing (data not shown). This is most probably due to the removal of apoptotic cells from the circulation, by phagocytes, during the first stage of chemotherapy. To rule out any influence of extracellular Ca^{2+} ions on the observed aggregation of spectrin, the isolated lymphocytes were incubated with 10 mM EGTA before the preparations for microscopic observations were processed.

3.1.2. A fraction of spectrin insoluble in non-ionic detergent occurs in leukaemic and lymphoid cells after chemotherapy

We found that spectrin solubility in buffer containing non-ionic detergent varied before and after chemotherapy in immature and mature lymphocytes. PBMC from healthy donors (control) and leukaemic and lymphoid cells, iso-

lated before and after the first cycle of chemotherapy, were incubated in buffer containing 1% Triton X-100, 100 μg/ml PMSF, 1 mM EGTA, and centrifuged at 30,000 × *g* at 4 °C. After that treatment, the obtained supernatants and pellets, as well as the controls (untreated cells), were analysed using the classical Western blot technique. In normal (Fig. 1B(a) and Fig. 2B(a)) and in lymphoid and leukaemic cells before the first cycle of chemotherapy (Fig. 1B(b) and Fig. 2B(b)) spectrin was present only in the supernatants but absent in the pellets. However, in lymphoid and in leukaemic cells after chemotherapy (Fig. 1B(c) and Fig. 2B(c)) spectrin was present both in the pellets and in the supernatants.

3.1.3. Changes in PKC θ organisation in leukaemic and lymphoid cells after the first cycle of chemotherapy, while PKC βI and βII are evenly arranged in the cell: immunofluorescence observations

To determine distribution of PKC θ in normal and malignant lymphocytes before and after the first cycle of chemotherapy we compared, using the immunofluorescence technique, the distribution of PKC θ in lymphocytes isolated from healthy donors (control) with its distribution in leukaemic and lymphoid cells isolated before and after chemotherapy. In lymphoid and leukaemic cells isolated after chemotherapy (Fig. 1C(c) and Fig. 2C(c)) we found a characteristic aggregation of PKC θ, and the pattern of arrangement of this protein was very similar to that observed using anti-spectrin antibodies. In normal cells (Fig. 1C(a) and Fig. 2C(a)) as well as in lymphoid and leukaemic cells before chemotherapy (Fig. 1C(b) and Fig. 2C(b)) we observed PKC θ evenly distributed in the cell. The same behaviour of PKC θ in cells after therapeutic treatment was observed in each clinical ALL and nHL case.

To test possible changes of the arrangement of other protein kinase C family isoforms in leukaemic and lymphoid cells cytoplasm before and after chemotherapy, we examined, using the same immunofluorescence technique, distribution of two conventional PKC isoforms, βI and βII. In normal cells obtained from healthy donors (Fig. 1D(a) and Fig. 2D(a)) and in mature (Fig. 1D(b) and (c)) and immature (Fig. 2D(b) and (c)) malignant cells before and after the first cycle of chemotherapy, arrangement of PKC βI and βII generally did not change. We found both PKC βI and PKC βII evenly distributed in the cells, and no changes in protein distribution were observed in the cells isolated after chemotherapy. The lack of changes in PKC βI and βII was noticed in all the ALL and nHL cases.

3.1.4. Morphological apoptotic changes occur in immature and mature malignant cells after chemotherapy

Upon chemotherapy with cytostatics we observed expected changes in apoptotic pathway activation, and therefore apoptotic morphology of isolated PBMC. To determine the effect of chemotherapy on the morphology of peripheral leukaemic and lymphoid cells we used the classical May Grünwald-Giemsa staining technique. We compared the morphology of peripheral normal lymphocytes isolated from healthy donors and peripheral leukaemic and lymphoid cells isolated before and after chemotherapy. An analysis of morphological differences revealed appearance of typical apoptotic features in both lymphoid (Fig. 1E(c)) and leukaemic (Fig. 2E(c)) cells after therapy: changes of condensation of chromatin, membrane protrusions, blebbing and presence of small vesicles at the cells' proximity. In contrast, cell surfaces of normal cells (Fig. 1E(a) and Fig. 2E(a)) and lymphoid (Fig. 1E(b)) and leukaemic (Fig. 2E(b)) cells isolated before chemotherapy were smooth and free from protrusions and apoptotic bodies.

3.2. Spectrin and in vitro induced apoptosis

To determine whether the observed aggregations of spectrin and PKC θ are directly associated with chemotherapy we performed in vitro experiments. The objects were (i) PBMC isolated from healthy donors, (ii) peripheral leukaemic (ALL) cells, and (iii) peripheral lymphoid (nHL) cells. These three populations of cells were cultured for 24 h with an appropriate combination of fludarabine, mitoxantrone, dexamethasone (see Section 2) and analysed using the Annexin V-FITC detection kit (Oncogene) to confirm apoptotic PS externalisation, immunofluorescence with appropriate antibodies, extraction with non-ionic detergent and a Western blot analysis.

3.2.1. Changes in spectrin organisation in both immature leukaemic and mature lymphoid cells after in vitro 24-h incubation with fludarabine, mitoxantrone and dexamethasone: immunofluorescence observations

To determine spectrin arrangement in leukaemic and lymphoid cells cultured for 24 h with cytostatics we used the same classical immunofluorescence technique as described above. Populations of malignant cells obtained from peripheral blood of patients with ALL and from patients with follicular lymphoma (Table 1) were divided in two. One part of the population of leukaemic cells and one part of lymphoid lymphocytes were cultured in standard RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 100 μg/ml gentamycine-containing medium, while the other parts were cultured in the same medium with an addition of 0.1 μg/ml fludarabine, 0.25 μg/ml mitoxantrone and dexamethasone. As a control we used normal lymphocytes, isolated from healthy donors. In all the mentioned cell types cultured for 24 h in standard medium (Fig. 3A(a), (b) and Fig. 4A(a), (b)), we found spectrin evenly distributed in the cytoplasm. In lymphoid (Fig. 3A(c)) and in leukaemic (Fig. 4A(c)) cells incubated with drugs, spectrin aggregation occurred.

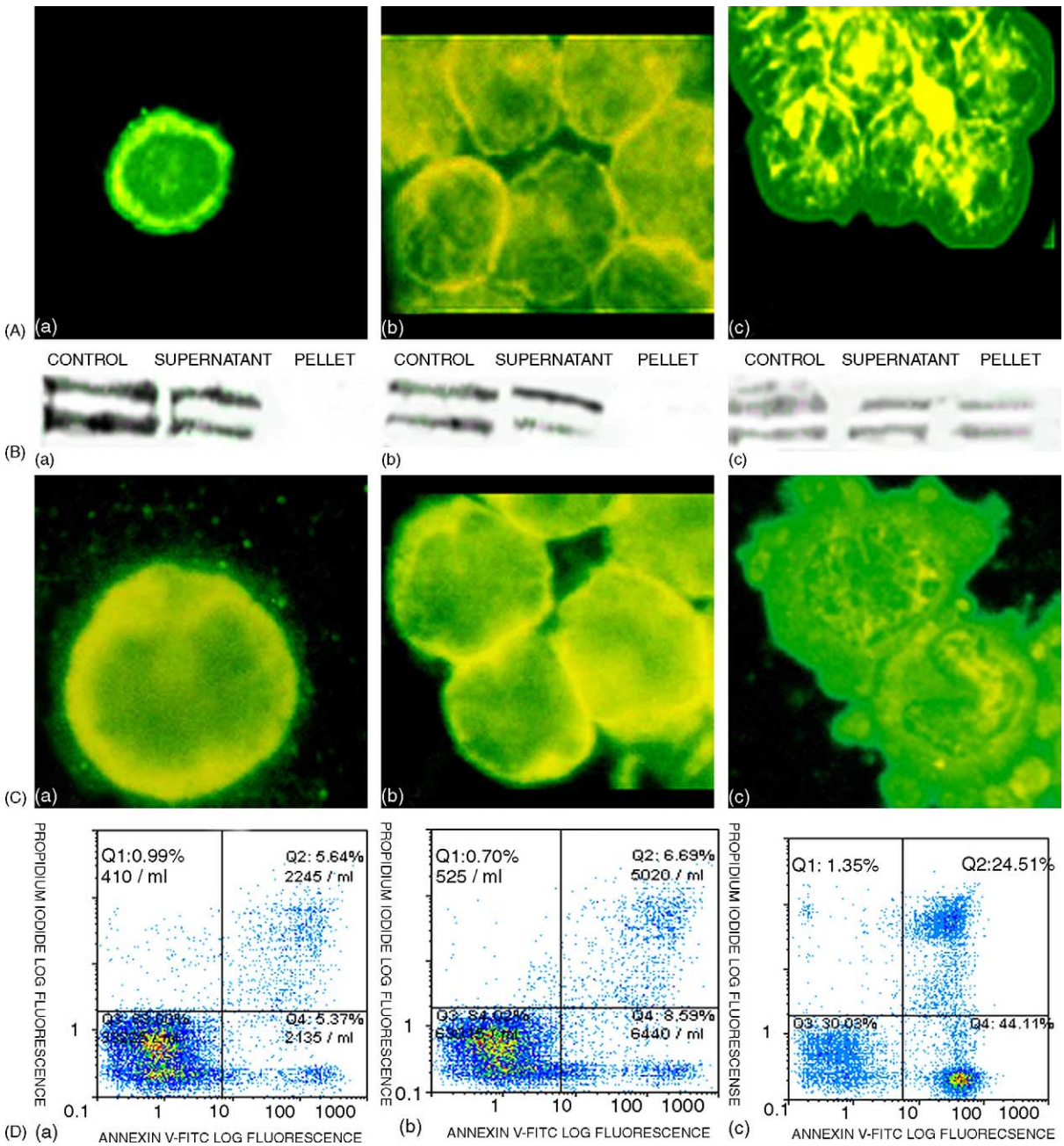

Fig. 3. Changes in spectrin and PKC θ organisation as well as cell shrinkage and membrane blebbing occur in nHL cells cultured with cytostatics. (A) Immunofluorescent pattern of spectrin distribution in normal (a) and malignant cells before (b) and after (c) incubation with fludarabine/mitoxantrone/dexamethasone for 24 h; obtained by using anti-bovine spectrin antibody (1:250), see legend to Fig. 1A. (B) spectrin non-ionic detergent insoluble fraction occurs in malignant cells after incubation with fludarabine/mitoxantrone/dexamethasone for 24 h (c). Normal (a) and malignant cells before (b) and after (c) chemotherapy were incubated for 10 min at 0 °C in buffer containing 1% Triton X-100, 100 μg/ml PMSF and 1 mM EDTA, then cell lysates were centrifuged at 30,000 × *g* at 4 °C. The obtained supernatants and pellets as well as the controls (intact cell suspension in PBS) were analysed by using the Western blot technique (see Section 2). (C) Immunofluorescent pattern of PKC θ distribution in normal (a) and malignant cells before (b) and after (c) chemotherapy; obtained by using anti-PKC θ antibody (1:200), see legend to Fig. 1A. (D) Apoptotic externalisation of PS occurred in malignant cells after incubation with cytostatics (fludarabine/mitoxantrone/dexamethasone) (c), while in normal (a) and malignant (b) cells cultured in standard medium low PS externalisation was observed. Annexin V-FITC and propidium iodide bindings were performed according to aporopriate protocols (Oncogene) and analysed by flow cytometry by using a Partec flow cytometer.

Moreover, we observed changes of arrangement of spectrin in cultured normal cells obtained from healthy donors: we found spectrin aggregated in normal lymphocytes cultured with cytostatics, although this aggregation was not so significant and undeniable as in neoplastic cells (data not shown).

3.2.2. A fraction of non-ionic detergent insoluble spectrin occurs in leukaemic and lymphoid cells after 24 h of incubation with fludarabine, mitoxantrone and dexamethasone: a Western blot analysis

To compare the results obtained from the analysis of in vivo ongoing chemotherapy (a non-ionic detergent

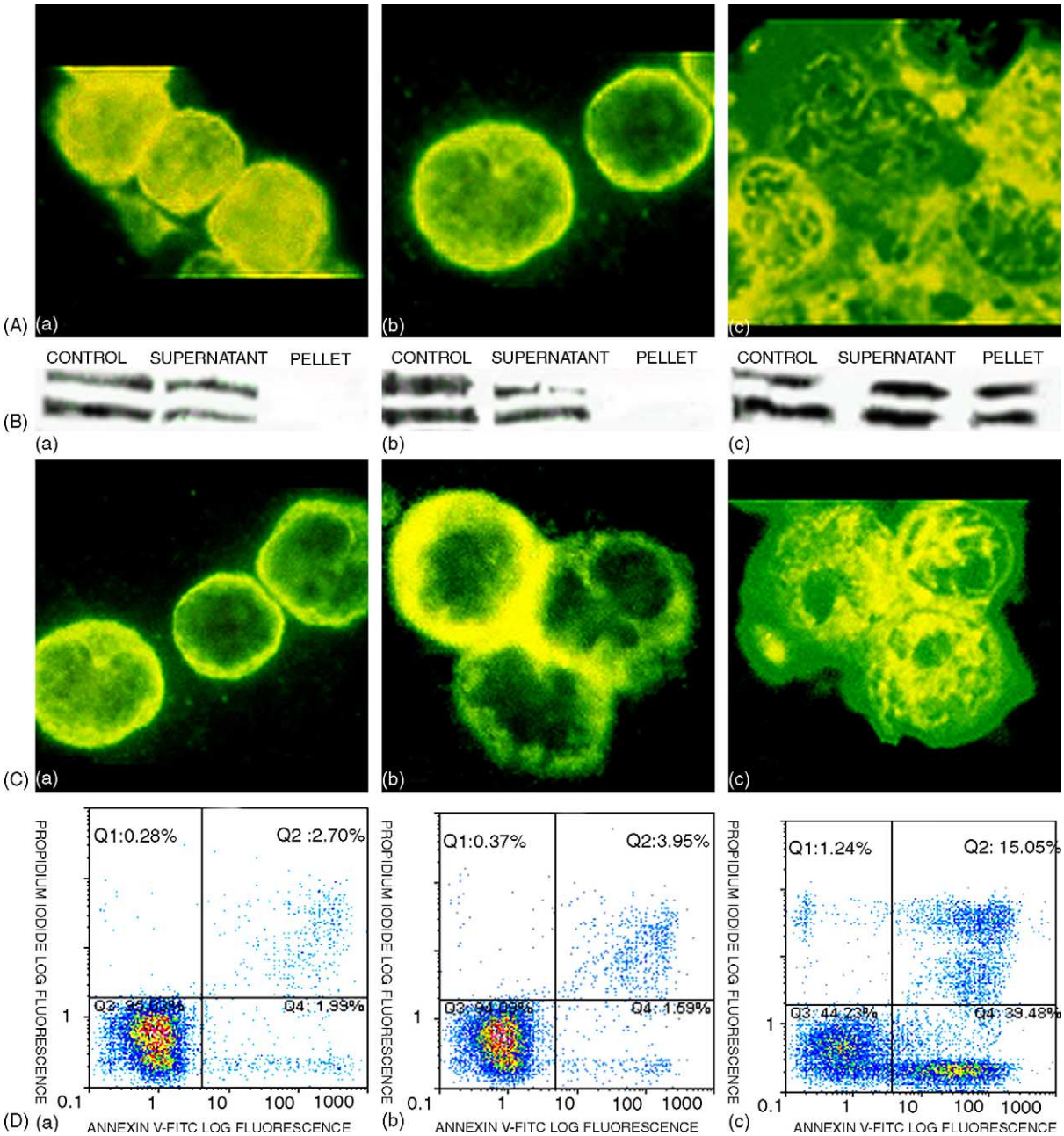

Fig. 4. Changes in spectrin and PKC θ organisation as well as cell shrinkage and membrane blebbing occur in leukaemic cells cultured with cytostatics. (A) Immunofluorescent pattern of spectrin distribution in normal (a) and leukaemic cells before (b) and after (c) incubation with fludarabine/mitoxantrone/dexamethasone for 24 h; obtained by using anti-bovine spectrin antibody (1:250), see legend to Fig. 1A. (B) Spectrin non-ionic detergent insoluble fraction occurs in leukaemic cells after incubation with fludarabine/mitoxantrone/dexamethasone for 24 h (c). Normal (a) and leukaemic cells before (b) and after (c) chemotherapy were incubated for 10 min at 0 °C in buffer containing 1% Triton X-100, 100 μg/ml PMSF and 1 mM EDTA, then cell lysates were centrifuged at 30,000 × *g* at 4 °C. The obtained supernatants and pellets as well as the controls (intact cell suspension in PBS) were analysed by using the Western blot technique (see Section 2). (C) Immunofluorescent pattern of PKC θ distribution in normal (a) and leukaemic cells before (b) and after (c) chemotherapy; obtained by using anti-PKC θ antibody (1:200), see legend to Fig. 1A. (D) Apoptotic externalisation of PS occurred in leukaemic cells after incubation with cytostatics (fludarabine/mitoxantrone/dexamethasone) (c), while in normal (a) and leukaemic (b) cells cultured in standard medium low PS externalisation was observed. Annexin V-FITC and propidium iodide bindings were performed according to appropriate protocols (Oncogene) and analysed by flow cytometry by using a Partec flow cytometer.

insoluble fraction of spectrin occurring after therapy in neoplastic cells could be observed after in vitro drug treatment), we performed analogous non-ionic detergent extraction and a Western blot analysis. Cells treated as above (Section 3.2.1) were treated with buffer containing 1% Triton X-100, and centrifuged at 30,000 × *g* at 4 °C. After centrifugation, the obtained supernatants and pellets as well as controls (untreated cells), were analysed using the Western blot technique. In normal cells (Fig. 3B(a) and Fig. 4B(a)), in lymphoid cells (Fig. 3B(b)) and in leukae-

mic lymphoblasts (Fig. 4B(b)) cultured in standard medium, we found only a non-ionic detergent soluble fraction of spectrin. In lymphoid cells (Fig. 3B(c)) as well as in leukaemic cells (Fig. 4B(c)), cultured in medium containing drugs, a detergent insoluble fraction appeared.

3.2.3. Changes in PKC θ organisation in both immature leukaemic and mature lymphoid cells after 24 h of incubation with fludarabine, mitoxantrone and dexamethasone: immunofluorescence observations

To find out whether and how localisation of PKC θ changes in normal and neoplastic cells after 24-h incubation with drugs, we used the immunofluorescence technique. In lymphoid (Fig. 3C(c)) and leukaemic (Fig. 4C(c)) cells cultured in standard medium with an addition of fludarabine, mitoxantrone and dexamethasone we observed changes in PKC θ organisation which were similar to these observed in the case of spectrin. In both mature (Fig. 3C(b)) and immature (Fig. 4C(b)) malignant cells cultured in medium without drugs, PKC θ was evenly arranged in the cells' cytoplasm, as was PKC θ distributed in the cytoplasm of normal cells (Fig. 3C(a) and Fig. 4C(a)).

3.2.4. Apoptosis occurs in both immature leukaemic and mature lymphoid cells after 24 h of incubation with fludarabine, mitoxantrone and dexamethasone: annexin V-FITC assays

In populations of normal lymphocytes (control), leukaemic and lymphoid cells cultured for 24 h in standard medium and in populations of these cells cultured with standard medium with an addition of fludarabine, mitoxantrone and dexamethasone, using the Annexin V-FITC apoptosis detection kit, we determined the percentages of cells undergoing apoptosis. We found that the majority of lymphoid (Fig. 3D(c)) and leukaemic (Fig. 4D(c)) cells after 24-h incubation with fludarabine, mitoxantrone and dexamethasone was apoptotic; however, propidium iodide staining also revealed the occurrence of some necrotic cells. In addition, we observed that the most potent apoptosis inductors were mitoxantrone and fludarabine (data not shown). Lymphoid (Fig. 3D(b)) and leukaemic (Fig. 3D(b)) cells cultured for 24 h in standard medium without the addition of the drugs resembled normal cells (Fig. 3D(a) and Fig. 4D(a)), in a normal cell cycle.

4. Discussion

We analysed spectrin distribution in neoplastic cells before and after chemotherapy in a group of patients (Table 1) with two major types of lymphoproliferative disorders: non-Hodgkin lymphoma and acute lymphoblastic leukaemia. We noticed characteristic change in nonerythroid spectrin organisation in both peripheral mature lymphoid lymphocytes and leukaemic lymphoblasts isolated after the first cycle of chemotherapy. The appearance of spectrin near the plasma membrane looked unchanged while the area over the nucleus looked overall brighter with the appearance of a dense network of spectrin. This phenomenon was observed in all the clinical cases analysed by us, usually after 1 week of ongoing chemotherapy where different cytostatics were used. This fact has not been described in the literature to date. The fact that change in spectrin organisation succeeds chemotherapy could indicate association of this phenomenon with apoptosis. Indeed, in neoplastic cells after chemotherapy we also observed aggregation of PKC θ—an essential protein for the activation of mature T cells, which might also lead to apoptosis and appearance of characteristic apoptotic membrane features. Beginning with the observation that spectrin assembles in one large polar aggregate, Repasky and coworkers [10–15] started a series of experiments aiming at the explanation of the physiological role of the characteristic behaviour of spectrin upon various activation agents. They found that besides spectrin, a number of other proteins also assembled in activated/stimulated (phorbol acetate myristate, mezerin, concanavalin A, ionofores, hyperthermia) lymphocytes. Moreover, they were mostly regulatory proteins: hsp 70, PKC βII, PKC θ, and this might lead to a hypothesis that when spectrin, hsp 70, PKC βII, and PKC θ aggregate together, the formed aggregate may be an important cellular regulatory factor. Although in our work we focused on different agents (chemotherapeutically used drugs), which are potent apoptosis inducers, and we analysed human peripheral, malignant lymphocytes before and after chemotherapy, we also found spectrin aggregated. However, instead of one large cytoplasmic aggregate we observed a rather characteristic, thick network of proteins (besides spectrin we also found PKC θ aggregated). We also examined the arrangement of other proteins: PKC βI and PKC βII, but we did not find changes in their distribution after chemotherapy. It should be noted that the observed changes of spectrin and PKC θ distribution accompanied mainly apoptotic changes, and those observed by Repasky and coworkers [10–15] accompanied activation of lymphoid cells in the culture. Most of the refered studies were performed on cells presenting T phenotype. However, two reports [36,37] focusing on B cell phenotype lines seem to conform the changes in spectrin organisation of T cell upon activation. We used the classical immunofluorecence staining; as a negative control we used rabbit IgG instead of the first antibodies (the results were negative, not shown), and as a positive control we used lymphocytes isolated from peripheral blood of healthy donors (Fig. 1A(a) and Fig. 4A(a)). In addition, in malignant cells after chemotherapy we found a non-ionic detergent insoluble fraction of specrin, while in normal and malignant lymphocytes before chemotherapy spectrin was totally soluble. In the same cells, treated with cytostatics, in which changes in spectrin and PKC θ organisation (observed by immunofluorescence) as well

as presence of a Triton X-100 insoluble fraction of spectrin was found, we observed cell shrinkage, membrane protrusions and blebbing. In vitro experiments with normal and malignant cells as well as experiments on lymphoid cell lines (unpublished results) indicated that changes in spectrin/kinase C θ organisation after treatment with cytostatics took place in the time-frame of several hours, therefore they occurred in circulating cells rather than in newborn cells. One, of course, cannot exclude that similar changes would occur in newly divided cells. Although in some cells from some patients we observed proteolysis of spectrin, an uncleaved fraction of protein predominated. In classically apoptotic cells, a caspase cleavage of α-spectrin to ~150 kDa fragments is believed to be important for disintegration of plasma membranes and formation of apoptotic vesicles [21–26]. However, it was recently shown that caspase-independent apoptosis pathways also exist [22,27–29], in particular in lymphocytes. Using various caspase inhibitors it was found that apoptosis continued to proceed in activated, mature T lymphocytes, and that despite the absence of caspase activity, dying lymphocytes retained the main cytoplasmic and membrane features of classical apoptosis: cell shrinkage, membrane blebbing, PS externalisation and dissipation of the mitochondrial inner transmembrane potential. However, maturation of apoptotic bodies was arrested [27]. Taking into account the experimental observations presented above, we can assume that the observed changes in spectrin and protein kinase C θ organisation may be connected with each other and connected with morphological apoptotic changes in normal and neoplastic lymphocytes upon chemotherapeutical treatment. Since these changes precede loss of asymmetry and are connected with protein kinase C θ aggregation, we hypothesise that spectrin may be involved in apoptotic signalling and/or early apoptotic events. Studies on defined cell lines (lymphoid and myeloid) should give a more precise insight into involvement of spectrin in early apoptosis.

Acknowledgement

Work supported by KBN grant 3P04C09725.

References

[1] Djinovic-Carugo K, Gautel M, Ylanne J, Young P. The spectrin repeat: a structural platform for cytoskeletal protein assemblies. FEBS Lett 2002;20:119–23.
[2] Bennett V, Baines AJ. Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol Rev 2001;81:1353–92.
[3] Ziemnicka-Kotula D, Xu J, Gu H, Potempska A, Kim KS, Jenkins EC, et al. Identification of a candidate human spectrin Src homology 3 domain-binding protein suggests a general mechanism of association of tyrosine kinases with the spectrin-based membrane skeleton. J Biol Chem 1998;273:13681–92.
[4] De Matteis MA, Morrow JS. Spectrin tethers and mesh in the biosynthetic pathway. J Cell Sci 2000;113:2331–43.
[5] Hu J, Ziemnicka D, Scalia J, Kotula L. Monoclonal antibodies to alpha I spectrin Src homology 3 domain associate with macropinocytic vesicles in nonerythroid cells. Brain Res 2001;898:171–7.
[6] Xu J, Ziemnicka D, Merz GS, Kotula L. Human spectrin Src homology 3 domain binding protein 1 regulates macropinocytosis in NIH 3T3 cells. J Cell Sci 2000;21:3805–14.
[7] Pawson T, Scott JD. Signalling through scaffold, anchoring, and adaptor proteins. Science 1997;278:2075–80.
[8] Mayer BJ, Ren R, Clark KL, Baltimore D. A putative modular domain present in diverse signalling proteins. Cell 1993;73:629–30.
[9] Haslam RJ, Koide HB, Hemmings BA. Pleckstrin domain homology. Nature 1993;363:309–10.
[10] Pauly JL, Bankert RB, Repasky EA. Immunofluorescent patterns of spectrin in lymphocyte cell lines. J Immunol 1986;136:246–53.
[11] Lee JK, Black JD, Repasky EA, Kubo RT, Bankert RB. Activation induces a rapid reorganisation of spectrin in lymphocytes. Cell 1988;55:807–16.
[12] Gregorio CC, Kubo RT, Bankert BR, Repasky EA. Translocation of spectrin and protein kinase C to a cytoplasmic aggregate upon lymphocyte activation. Proc Natl Acad Sci USA 1992;89:4947–51.
[13] Langner M, Repasky EA, Hui SW. Relationship between membrane lipid mobility and spectrin distribution in lymphocytes. FEBS Lett 1992;305:197–202.
[14] Di Y, Repasky EA, Subjeck JR. Distribution of HSP70, protein kinase C, and spectrin is altered in lymphocytes during a fever-like hyperthermia exposure. J Cell Physiol 1997;172:44–54.
[15] Wang XY, Ostberg JR, Repasky EA. Effect of fever like whole-body hyperthermia on lymphocyte spectrin distribution, protein kinase C activity, and uropod formation. J Immunol 1999;162:3378–89.
[16] Meller N, Altman A, Isakov N. New perspectives on PKC θ, a member of the novel subfamily of protein kinase C. Stem Cells 1998;16:178–92.
[17] Dienz O, Hehner SP, Droge W, Schmitz ML. Synergistic activation of NF-κB by functional cooperation between Vav and PKC θ in T lymphocytes. J Biol Chem 2000;275:24547–51.
[18] Dustin M, Chan A. Signalling takes shape in the immune system. Cell 2000;103:283–94.
[19] Merwe PA, Davis SJ, Shaw AS, Dustin ML. Cytoskeletal polarization and redistribution of cell-surface molecules during T-cell antigen recognition. Sem Immunol 2000;12:5–21.
[20] Janes PW, Ley SC, Magee AI, Kabouridis PS. The role of lipid rafts in T-cell antigen receptor (TCR) signalling. Semin Immunol 2000;12:23–34.
[21] Pizzo P, Viola A. Lymphocyte lipid rafts: structure and function. Curr Opin Immunol 2003;15:255–60.
[22] Arendt CW, Albrecht B, Soos TJ, Littman DR. Protein kinase C θ: signaling from the center of the T-cell synapse. Curr Opin Immunol 2002;14:323–30.
[23] Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, et al. The immunological synapse: a molecular machine controlling T-cell activation. Science 1999;285:221–7.
[24] Trushin SA, Pennington KN, Algecira-Schimnich A, Paya CV. Protein kinase C and calcineurin synergize to activate IκB kinase and NF-κB in T lymphocytes. J Biol Chem 1999;274:22923–31.
[25] Ghaffari-Tabrizi N, Bauer B, Villunger A, Baier-Bitterlich G, Altman A, Utermann G, et al. Protein kinase C θ, a selective upstream regulator of JNK/SAPK and IL-2 promoter activation in Jurkat T cells. Eur J Immunol 1999;29:132–42.
[26] Tang Y, Katuri V, Dillner A, Mishra B, Deng C, Mishra L. Disruption of transforming growth factor-β signalling in ELF-β spectrin-deficient mice. Science 2003;299:574–7.
[27] Brown TL, Patil S, Cianci CD, Morrow JS, Howe PH. Transforming growth factor β induces caspase 3-independent cleavage of αII-spectrin (α-fodrin) coincident with apoptosis. J Biol Chem 1999;274:23256–62.

[28] Cornelissen M, Vral A, Thierens H, De Ridder L. The effect of caspase-inhibitors on radiation induced apoptosis in human peripheral blood lymphocytes: an electron microscopic approach. Apoptosis 1999;4:449–54.
[29] Wang KK, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, et al. Simultaneous degradation of αII- and βII-spectrin by caspase 3 (CPP32) in apoptotic cells. J Biol Chem 1998;273: 22490–7.
[30] Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, et al. Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J Biol Chem 1995;270:6425–8.
[31] Lee A, Morrow JS, Fowler VM. Caspase remodelling of the spectrin membrane skeleton during lens development and aging. J Biol Chem 2001;276:20735–42.
[32] William ST, Smith AN, Cianci CD, Morrow JS, Brown TL. Identification of the primary caspase 3 cleavage site in alpha II-spectrin during apoptosis. Apoptosis 2003;8:353–61.
[33] Deas O, Dumon C, Mac Farlane M. Caspase-independent cell death induced by anti-CD2 and staurosporine in activated human peripheral T lymphocytes. J Immunol 1998;161:3375–83.
[34] Borner C, Monney L. Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ 1999;6:497–507.
[35] Bidere N, Senik A. Caspase-independent apoptotic pathways in T lymphocytes: a minireview. Apoptosis 2001;6:371–5.
[36] Evans SS, Wang WC, Gregorio CC, Han T, Repasky EA. Interferon-alpha alters spectrin organisation in normal and leukemic human B lymphocytes. Blood 1993;81:759–66.
[37] Masso-Welch PA, Black JD, Erikson J, Repasky EA. Polarized expression of immunoglobulin, spectrin, and protein kinase C beta II occurs in B cells from normal BALB/c, autoimmune lpr, and anti-ssDNA transgenic, tolerant mice. J Leukoc Biol 1999;66: 617–24.