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Cholesterol induced variations of membrane dynamics related to the induction of apoptosis in mouse thymocytes

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Abstract.
Purpose: To evaluate the involvement of cholesterol induced variations of membrane dynamics in mouse thymocyte apoptosis. *Materials and methods*: Membranes of thymocytes of RK mice were enriched with cholesterol using methyl-β-cyclodextrins as carriers. Spontaneous apoptosis was compared with apoptosis induced either by X-irradiation, by treatment with dexamethasone (DEX), and by phorbol-12-myristate-13-acetate (PMA). Apoptotic cells were quantified by means of flow cytofluorometry. *Results:* Small amounts of incorporated cholesterol enhance the cellular sensitivity for spontaneous apoptosis whereas larger amounts of incorporated cholesterol protect against spontaneous apoptosis and apoptosis induced by irradiation, DEX, or PMA. *Conclusions:* Cholesterol exerts specific rigidity effects on lipid membranes which have been shown to be involved in thymocyte apoptosis. The general effect of higher concentrations of cholesterol protecting against apoptosis hints towards a central protective mechanism. This study believes that either cholesterol paralyses great parts of the cell metabolism or that the apoptotic chain reaction is interrupted at a central point due to protection of membrane lipid regions from oxidative stress.

Abbreviations
AO is acridine orange, Bis-pyrene is 1,3-bis-(1-pyrenyl)propane ($C_{35}H_{24}$), DEX is dexamethasone (9-α-fluoro-16-α-methyl-prednisolone), DPH is 1-6-diphenyl-1,3,5-hexatriene, LPO is lipid peroxidation, PKC is protein kinase-C, PMA is phorbol-12-myristrate-13-acetate, p-(D)HPA is p-(di)hydrophenylic acid, ROS is reactive oxygen species.

1. Introduction

In 1972, Kerr *et al.* introduced the Greek word *apoptosis* to describe the morphological changes occurring during active cellular suicide or programmed cell death (PCD). Since then, many investigators have focused their attention on thymocytes not only because of their apoptotic sensitivity but also because of their immunological relevance (Kroemer 1995, Duke *et al.* 1996, Hale *et al.* 1996). Apoptosis may or may not require macromolecular synthesis depending on the initiation type of apoptosis or the cell type used (Godar *et al.* 1995). Recently, the terms 'pre-programmed' (no macromolecular synthesis required post-insult) and 'programmed cell death' have been suggested to describe the distinct biochemical mechanisms leading to apoptosis (Godar 1996). The term *necrosis* on the other hand describes a passive form of cell death. During necrosis, severe injury of a physical or chemical nature leads to uncontrolled swelling and rupture of cellular membranes by which active enzymatic compounds are released and can provoke inflammatory responses.

During the apoptotic process, cells can form apoptotic bodies (small membrane coated vesicles enclosing active cellular components), probably involving the micro-filamental apparatus (Cotter *et al.* 1992). At a late stage of the apoptotic process, many cell types show chromatin fragmentation into subunits of about 180–200 bp, electrophoretically visible as 'DNA laddering' (Chapman *et al.* 1995). DNA fragmentation is the result of an activated endonuclease activity and was used as general biochemical evidence for apoptosis until it was reported that in some cell types, nuclear DNA fragmentation is not required (Schulze-Osthoff *et al.* 1994).

Induction of apoptosis in T cells can be accomplished by a variety of different stimuli. Exposure to or withdrawal of specific biomolecules, insufficient nutrition medium or mild injuries of physical or chemical nature (cold or heat shock (Kruman *et al.* 1992, Migliorati *et al.* 1992), ionizing radiation (Sellins and Cohen 1987), or oxidising reagents (Matsuzawa *et al.* 1997)) may cause cellular apoptosis.

Ojeda *et al.* (1994) presume inter-relationships between membrane fluidity deviations and apoptosis. Ionizing radiation exerts dose rate dependent rigidity effects in liposomes (Konings 1987), mediated by lipid peroxidation (LPO) processes. Protein rich microsomal membranes and thymocyte ghosts showed fluidity effects and in natural membrane specimens both effects have been reported, depending on the cell type (Kölling *et al.* 1994).

The present work examines if exogenously induced changes of cell membrane fluidity in freshly extracted mouse thymocytes correlate with variations of the cellular apoptotic sensitivity. Since cholesterol is

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known to cause rigidity of membranes (Yeagle 1985), this study used an efficient and simple method to incorporate well defined quantities of cholesterol into membranes of microsomes and entire cells, and further investigated its effect on spontaneous apoptosis as well as on apoptosis induced by X-irradiation, by the glucocorticoid receptor agonist dexamethasone (DEX), and by the protein kinase-C (PKC) activator phorbol-12-myristate-13-acetate (PMA).

2. Materials and methods

2.1. *Microsome preparation*

Microsomal fractions from fresh pig livers were prepared by differential centrifugation according to Lu (1976). Microsomes (29.22 ± 0.44 mg protein/ml suspension, determined according to Peterson (1977)) were suspended in 10 mmol dm^{-3} potassium phosphate buffer (pH 7.4) and stored at −80°C. Phospholipid content was estimated according to Kaschny (1989) (protein/phospholipid: 2:1 {w/w}). Cholesterol content (see *cholesterol determination*) was determined to be 6.1 ± 0.3 mol% (moles cholesterol/moles phospholipids × 100).

2.2. *Membrane fluidity measurements in cholesterol enriched microsomes*

The fluidity sensitive fluorescent dyes pyrene and bis-pyrene (1,3-bis-(1-pyrenyl)propane, Molecular Probes, Eugene, Or, USA) were dissolved in ethanol (Merck, Darmstadt, Germany). DPH was dissolved in DMF (Janssen, Geel, Belgium). Equivalents from stock solutions were added to 2 ml of microsomal suspensions (lipid concentration = 0.1 mg/ml), in order to achieve a molar ratio of 100:2 ({lipid}:{dye}) for pyrene, 10 000:1 for bis-pyrene, and 400:1 for DPH. Two or ten times the original amount of cholesterol in microsomes was added to the suspensions using methyl-β-cyclodextrins (Sigma, Deisenhofen, Germany) as carriers. Under gentle stirring, probes and controls were heated to 40°C for 30 min in order to optimize the diffusion controlled flux of single cholesterol molecules from the hydrophobic cavities of the β-cyclodextrins to the thermodynamically non-saturated acceptor membranes of the microsomes. After cooling the probes to 25°C, the fluidity signals were determined in quartz curvettes of 1 cm^2 cross section with a Kontron SFM 25 spectrofluorometer (Kontron, Eching, Germany) equipped with a L-format anisotropy inset and a Julabo HC thermostat (Julabo Labortechnik, Seelbach, Germany). Pyrene and bis-pyrene were excited at 336 nm. Emission intensities were recorded at 395 nm for monomer emission (M) and at 475 nm for excimer emission (E). E/M values from pyrene were interpreted as index fluidity of the membranes according to Birks (1970). For bis-pyrene, E/M interpretation followed Cheng *et al.* (1994).

The fluorescence anisotropy of DPH was calculated according to Lakowicz (1984):

$$r = \frac{I_{II} - G \times I_{\perp}}{I_{II} + 2 \times G \times I_{\perp}}$$

where r is the steady static fluorescence anisotropy and I_{II} and $I_{\perp}$ are the fluorescence intensities at 425 nm parallel and perpendicular to the polarization of the excitation light (358 nm) and G is the 'Grating factor'.

2.3. *Cell suspension*

Thymocytes were obtained from 6 to 8 week old RK mice bred in the Institute of Immunology of the University of Valdivia. Animals were killed by an ether overdose. The thymus was extracted and teased in cold buffer (RPMI-1640 solution, (Sigma) supplemented with 10 mmol dm^{-3} Hepes H3375 (Sigma) and 2 g/l sodiumhydrogencarbonate (Merck), 4°C). The tissue was cut into small pieces with scissors and washed through a fine metal gauze. Additional filtering through thin layers of cotton removed remaining cell clusters. As far as possible, all steps were performed under cooling in an ice water bath.

2.4. *Enrichment of cells with cholesterol and α-tocopherol*

Cholesterol loaded methyl-β-cyclodextrins or cholesterol free β-cyclodextrins (Sigma) were dissolved in the cell suspension buffer. Different quantities of these stock solutions (see legend of figure 3), or 33.47 ± 2.46 mg of dl-α-tocopherol (Merck, Art. No. 8283) were added to 15×10^6 cells in a glass tube and diluted to a final volume of 3 ml. Following a 2 h incubation at 37°C under gentle stirring (5% CO_2 atmosphere), cells were washed three times. Afterwards, cells were filtered through a thin layer of cotton. Cholesterol content in cells incubated with cholesterol loaded β-cyclodextrin suspensions was determined with an enzymatic fluorescence assay (see below).

2.5. *Cholesterol determination*

For direct cholesterol quantification in microsomes and in cells, a microenzymatic fluorescence assay (Gray *et al.* 1995) was used.

2.5.1. *In microsomes.* 100 μl assay mixture and 10 μl of 10% Triton X-100 (Sigma) was added to sample volumes of 5–20 μl. Phosphate buffer was added to a final assay volume of 200 μl.

2.5.2. *In cells.* 15×10^6 cells were centrifuged for 3 min at 200 g, the supernatant was removed and cells resuspended in 100 μl of 0.1 mol dm^{-3} phosphate buffer (pH 7.4) additionally containing 1% (v/v) Triton X-100 and 150 mmol dm^{-3} NaCl. Afterwards, the cell suspensions were sonified carefully with a titanium tip in order to further destroy the intact membrane systems which protect the cholesterol molecule from access of the cholesterol oxidase enzyme of the assay. Finally, 100 μl of the fluorescence assay mixture containing p-HPA was added.

After an incubation time of 8 h at 37°C, 1.8 ml distilled water were added to the probes and fluorescence intensities of p-DHPA were monitored at 406 nm following excitation at 320 nm with a Kontron SFM 25 spectrofluorometer.

2.6. *Induction of apoptosis*

2.6.1. *X-irradiation.* A Philips X-ray machine type PW 1140 operated at 3 mA and 80 kV was used to induce apoptosis in thymocytes with a dose of 3 Gy (0.3 Gy/min). 2 ml of cell suspensions containing 10^6 cells/ml were irradiated in cooled glass tubes (0°C).

2.6.2. *Chemical inducers.* 5×10^{-7} M DEX (Sigma) or 20 ng PMA (Sigma) were added to 2×10^6 cells and adjusted to 2 ml with RPMI-cell buffer.

After the induction, 20 μl of antibiotic serum (125 U/ml penicillin G and 90 U/ml streptomycinsulphate) and 66 μl BSA solution (0.6%) were added to 2 ml of the induced and the control cell suspensions. After incubation at 37°C for 5 h (5% CO_2 atmosphere), apoptotic cells were determined.

2.7. *DNA labelling technique with acridine orange (AO)*

Following Darzynkiewicz *et al.* (1979), 10^5 cells in a 100 μl suspension were mixed with 200 μl of acid detergent (0.1% Triton X-100, 0.08 mol dm^{-3} HCL and 150 mmol dm^{-3} NaCl). After 30 s, 600 μl of the AO staining solution were added (100 ml contained 1.2 mg AO (Sigma), 29 mg ethylene-diamine-tetra-acetic acid (EDTA), 150 mmol dm^{-3} NaCl, 0.1 mol dm^{-3} $C_6H_8O_7\times H_2O$, and 0.2 mol dm^{-3} Na_2HPO_4). All steps were performed in a 0°C ice water bath. After 10 min, the DNA content of the nuclei was determined by flow cytometry.

2.8. *Determination and quantification of apoptosis*

DNA content per nucleus was determined using the fluorescence signal of each individual cell in the green spectral range (515–575 nm, AO bound to DNA) with a flow cytofluorometry unit of our own design (Ojeda *et al.* 1992a). Excitation was performed by a 50 mW laser at 488 nm and 5000 cells were counted for each experiment. The statistical evaluation of the signals (AO fluorescence intensity, side scattering intensity at 90°, forward scattering intensity) was performed with home-made software. Cells with normal DNA content were counted 'healthy' (cells in G_0/G_1-phase, relative fluorescence intensity between 600 and 900 units, see figure 1 and white or black columns in figure 2), and cells with less than normal DNA were considered as apoptotic (relative fluorescent intensity between 0 and 600 units, light grey and striped columns in figure 2). Units higher than 1000 marked cells in the G_2-/M-phase (figure 2, dark grey columns).

In order to show DNA fragmentation, DNA ladder tests were performed by agarose gel electrophoresis according to Davis and Thomas (1980).

Healthy and apoptotic cells were distinguished

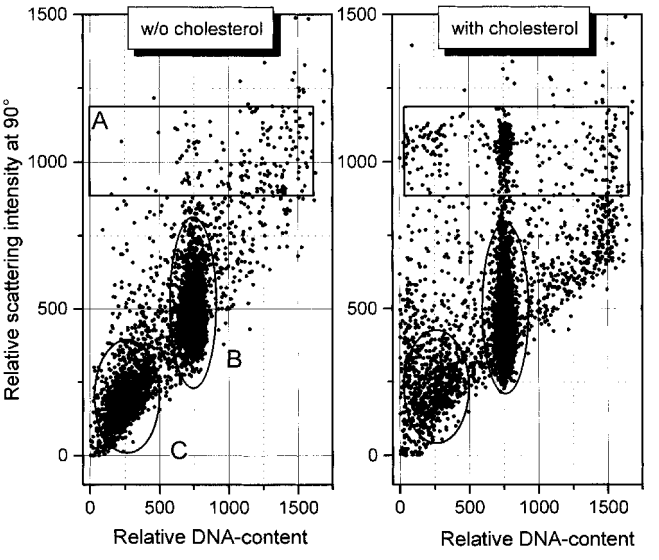

Figure 1. Inhibition of spontaneous apoptosis by cholesterol obtained from flow cytometry. Both bivariant histograms show relative DNA content (acridine orange fluorescence in the green fluorescent range) and 90° scattering of the excitation laser light (λ_{ex} = 488 nm) from 5000 thymocytes. The left plot is obtained from untreated cells, the right one from cells which had been incubated with 1.38×10^{-8} mol cholesterol/10^6 cells. Both cell samples had been incubated for 5 h at 37°C before the measurement. Circle B surrounds cells with normal DNA content in the G_0/G_1-phase. Circle C shows apoptotic cells with little remaining DNA. Sqaure A marks a region with high side scattering values.

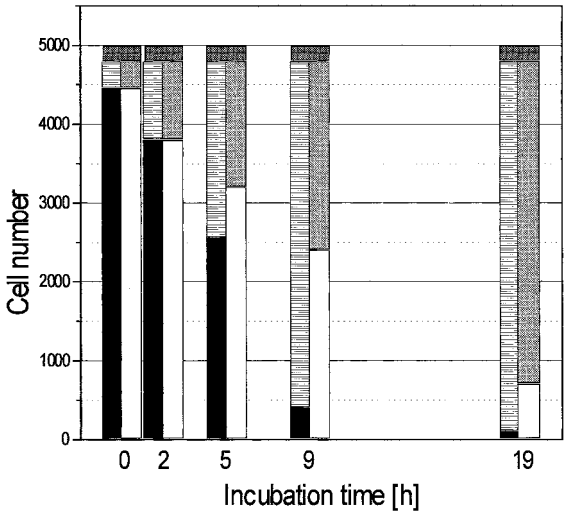

Figure 2. Time dependence of spontaneous and radiation induced apoptosis. Flowcytometric analysis was performed after 0, 2, 5, 9 and 19 h. White and light grey columns represent the numbers of normal cells (G_0-/G_1-phase) and of spontaneous apoptotic cells, respectively. Black columns (normal cells) and striped columns (apoptotic cells) represent cells irradiated with 3 Gy (0.3 Gy/min). Dark grey columns represent the number of cells in the G_2/M-phase which never altered significantly. An error of $n^{1/2}$ must be taken into account for n cells counted.

from necrotic ones according to Warters 1992: Trypan blue was added to cell samples in a microscopic cell counting chamber. After 2 min, trypan blue invaded cells (necrotic) and cells with membranes resisting trypan blue invasion (healthy or apoptotic) were counted to perform statistical evaluation at a basis of 100 cells.

3. Results

Fluidities of cholesterol enriched microsomal membranes were measured with the fluorescent probes pyrene, bis-pyrene and DPH. As shown in table 1, the relative fluidity of microsomal membranes decreased to about 90%, after incubation with twice the original molar cholesterol concentration of the microsomes, and to about 75% with a ten fold concentration. From calibration experiments performed with the dyes in egg yolk liposomes of defined cholesterol content (Härtel *et al.* 1998), the corresponding molar cholesterol content in the microsomal membranes was calculated. In microsomes (6.1 ± 0.3 mol% of cholesterol), a mean rise of 9.8 ± 2.4 mol% was found when incubated in a medium with twice the original cholesterol concentration, and of 23.4 ± 5.9 mol% in medium containing a ten fold increase.

The p-HPA fluorescence assay revealed that virtually all cholesterol molecules were incorporated into the microsomes when incubated with a two fold increase of its concentration (Härtel *et al.* 1998). Saturation at 390 ± 10% was detected when more than four times the original cholesterol concentration was added. The direct micro-enzymatic assay and the fluidity sensitive method showed a comparable incorporation of cholesterol into the membranes.

The p-HPA assay detected $1.5 \pm 2 \times 10^{-10}$ mol cholesterol in 10^6 thymocytes. Cells incubated with 1.38×10^{-9} mol cholesterol/10^6 cells increased the cholesterol concentration to 250 ± 145%, cells incubated with 1.38×10^{-8} mol cholesterol/10^6 cells to 311 ± 127%, and cells incubated with 1.38×10^{-7} mol cholesterol/10^6 cells to 355 ± 168% (data not plotted).

Two characteristic phenomena can be observed in cytograms when thymocytes are incubated with high concentrations of cholesterol. Less cells appear in the apoptotic region (figure 1, circle C) and more cells in the high side-scattering region (figure 1, square A).

Figure 2 shows the time course on which untreated and irradiated cells become apoptotic. The apoptotic rate of untreated cells (called 'spontaneously occurring apoptosis') is much slower than that of irradiated cells. This difference of apoptotic rates is even more pronounced for cells treated with DEX, but slightly

Table 1. Cholesterol induced fluidity changes in microsomal membranes as detected by the fluorophores pyrene, bis-pyrene, and DPH.

	Control microsomes: cholesterol content = 6.1 ± 0.3 mol% (100 × {cholesterol}/{phospholipid})	Microsomes plus two times their original cholesterol content in β-cyclodextrins.	Microsomes plus ten times their original cholesterol content in β-cyclodextrins.
	Relative fluidity ± sd {%}	Relative fluidity ± sd {%}	Relative fluidity ± sd {%}
Pyrene	100 ± 0,32	90,32 ± 0,65	74,19 ± 0,65
Bis-Pyrene	100 ± 2,73	91,06 ± 0,76	79,39 ± 0,61
DPH	100 ± 4,02	87,19 ± 6,53	—

100% is set for the relative fluidity values of the untreated microsomal membranes. Further values were measured after cholesterol incorporation as described in Section 2. Results shown are means of 3–4 independent experiments with standard deviations.

smaller for cells exposed to PMA (compare figure 3 for values after 5 h). The number of cells in the G_2/M-phase was not influenced significantly by time, by treatment, by enrichment with cholesterol or by treatment with pure β-cyclodextrins (data not shown). In all further experiments cell numbers were evaluated 5 h after treatment, since at that time, apoptosis was still in progress and the apoptotic rate difference is sufficiently distinct to be recognized.

The 100% reference value used in figure 3 corresponds to the spontaneous apoptotic rate of untreated cells 5 h after their preparation (e.g. the 5 h light grey column in figure 2). DEX treatment exerted the strongest additional apoptotic induction, followed by irradiation, and PMA (figure 3). Cholesterol loaded β-cyclodextrins exert significant effects on the apoptotic sensitivity of the cells. The effects strongly depend on the amount of cholesterol incorporated

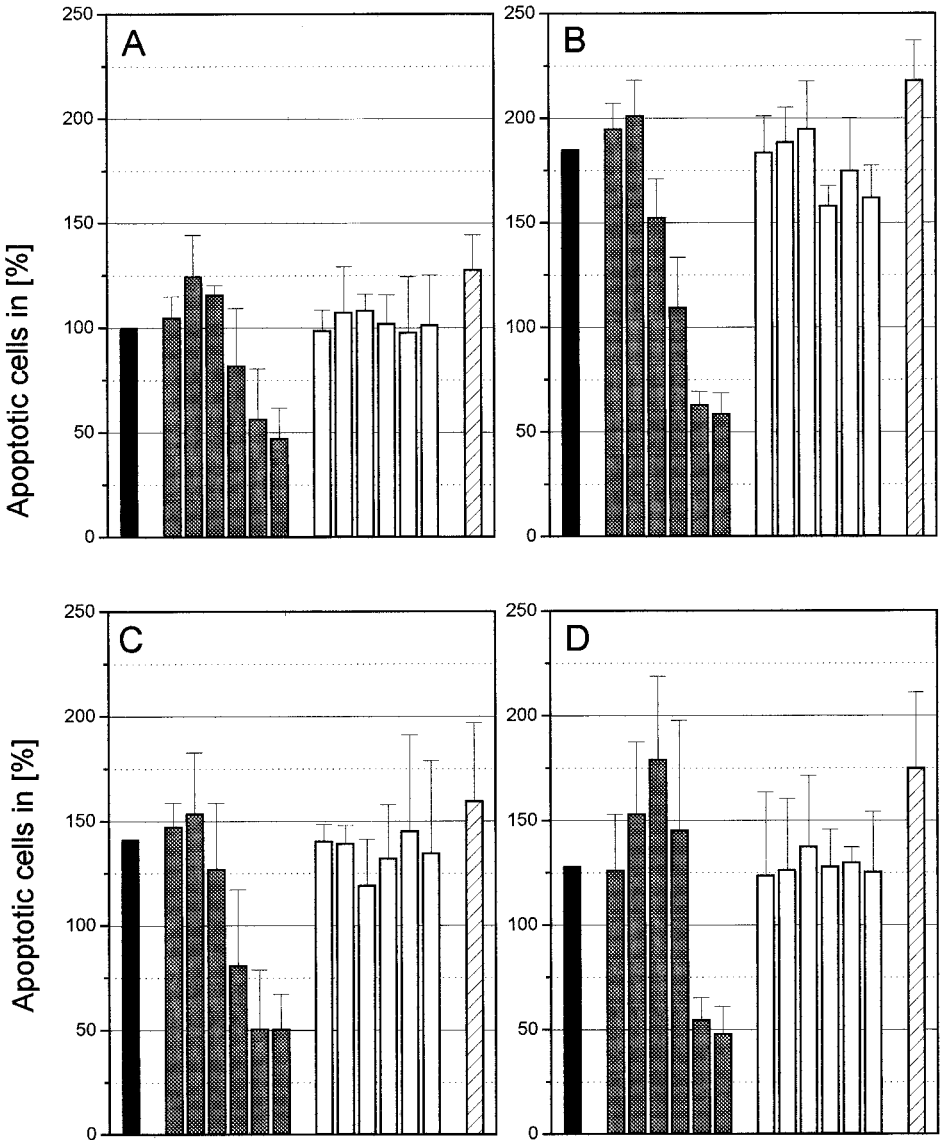

Figure 3. The effects of cholesterol incorporation, α-tocopherol, and treatment with pure β-cyclodextrins on the apoptotic rate of murine thymocytes. Flow cytometric quantification of the apoptotic rates were performed 5 h after: {A} no primary treatment, exposure to DEX {B}, to irradiation {C}, or to PMA {D}. 100% apoptosis is set for the 'spontaneous apoptotic rate' of untreated cells (black columns, {A}). (*black columns*: cells without additional cholesterol, α-tocopherol, or β-cyclodextrins, *grey columns (from left to right)*: cells incubated with 0.1, 1, 2.5, 5, 7.5 and 10 (x 1.38×10^{-8}mol/10^6 cells) cholesterol in β-cyclodextrins, *white columns (from left to right)*: cells incubated with the corresponding amounts of pure β-cyclodextrins. *Striped columns*: cells incubated with 33.47 ± 2.46mg of α-tocopherol/15×10^6 cells). Results shown are means of 3–9 independent experiments with their standard deviations.

into the membranes. While low cholesterol incorporation enhances the apoptotic rate of the cells, high incorporation decreases the apoptotic rate. High concentrations of cholesterol 'protect' not only treated cells from apoptosis, but also untreated cells (figure 3A). α-Tocopherol acts like low cholesterol incorporations. Pure β-cyclodextrins do not cause any particular effects.

Figure 4, which is another representation of figure 3, summarizes the effects of cholesterol on the apoptotic rate. The 'apoptosis preventing' (or 'protective') effect of higher amounts of cholesterol clearly is a general feature for all cells, further treated or not. The 'apoptosis inducing' effect of cholesterol depends on the type of treatment. PMA treated cells show the highest apoptosis enhancing effect, followed by the untreated cells, the irradiated cells and finally the cells treated with DEX.

In the trypan blue exclusion test, cells from all samples did not allow trypan blue to invade the cells. This means that no necrotic or late apoptotic cells were present.

The DNA ladder by agarose gel electrophoresis showed clear DNA fragmentation for DEX treated cells. In contrast, freshly extracted cells or cells treated with a high amount of cholesterol showed no fragmentation (data not shown).

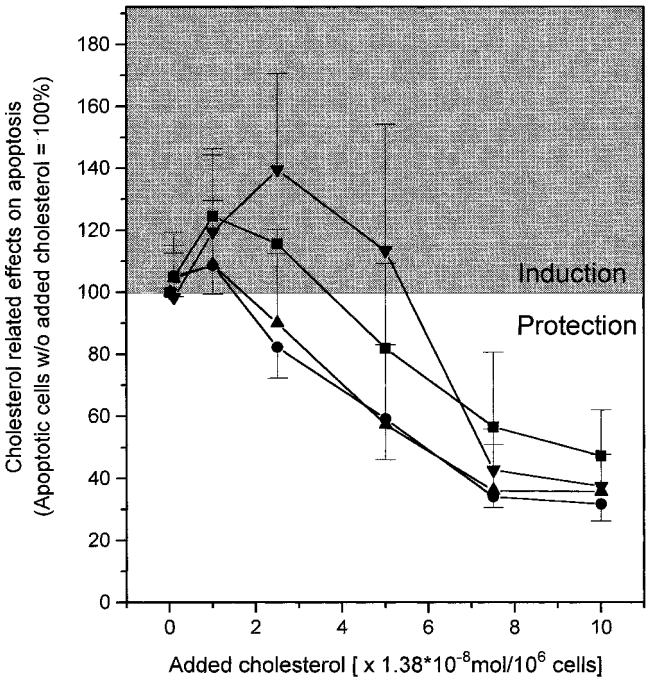

Figure 4. Synopsis of cholesterol related effects from figure 3. All cells without cholesterol or β-cyclodextrin treatment were normalized to 100%. Cells without treatment (■), irradiated cells (▲), PMA treated cells (▼) and DEX treated cells (●). Results shown are means of 3 to 9 independent experiments with their standard deviations.

4. Discussion

The micro enzymatic fluorescence assay and the fluidity sensitive membrane dyes congruently prove that the water soluble cholesterol carrier, methyl-β-cyclodextrin, incorporates cholesterol into microsomal membranes. Easy application, good incorporation results and negligible cytotoxic effects favour this method for experiments with living cells (Awad *et al.* 1997, Härtel *et al.* 1998). Since rigidity effects of cholesterol on the acyl chain region of liposomal and microsomal membranes are known, it is also expected that thymocyte membranes become rigid with increasing cholesterol content, as it has been reported for Chinese hamster ovary CHV-K1, and lymphoblastic RPMI 1788 cell lines (Berroud *et al.* 1996).

This study's results are partly in line with the findings of Posokhof *et al.* (1992). In untreated rat thymocytes they also found that small amounts of additional cholesterol enhanced the apoptotic rate, but that a further increase of cholesterol resulted in an inhibition of spontaneous and of radiation-induced apoptosis. Their effects were similar in irradiated, normal cells to those reported here. However, in this study the results yielded a much stronger inhibition of apoptosis in untreated cells of high cholesterol content. In contrast they found no increase of the apoptotic rate in irradiated cells after small amounts of cholesterol had been added. They suggested that cholesterol directly protects the cellular membranes from radiation produced reactive oxygen species (ROS). Since the results from this study show that cholesterol qualitatively exerts the same inhomogeneous effects for all treatments, the hypothesis from Posokhof *et al.* is doubted to be a general one. We postulate a common protective mechanism which acts after PKC activation since all apoptotic stimuli used can be suppressed by the PKC inhibitor H7 (Kizaki *et al.* 1989, Ojeda *et al.* 1992b). This hypothesis is supported by Ramakrishnan *et al.* (1993) who found that the radical scavenger trolox (a water soluble α-tocopherol analogue) blocked Ca^{2+} influx and apoptosis in mouse thymocytes even after irradiation or DEX treatment.

Cholesterol provides LPO protective properties, although it does not actively bind ROS like trolox, NAC (N-Acetylcystein), BABTA ($C_4O_2S_2H_{10}$), and other radical scavengers, reported to inhibit apoptosis (Fernandez *et al.* 1995, Slater 1995). Because the chemical structure, the size, and the polarity of trolox, NAC, and BABTA are very different, a common protective mechanism other than ROS scavenging is unlikely. Since cholesterol protects from LPO by increasing the packing density of mem-

branes, oxidative stress on other cellular components is further increased. Oxidative stress on proteins, RNA, or DNA is also discussed to be essential for apoptosis (McConey 1994). Following the hypothesis that cholesterol increases oxidative stress on these compounds, a steady increase of apoptotic responses should have been found with increasing membrane cholesterol content but was not. If an anti-oxidative mechanism was the common key for the prevention of apoptosis by cholesterol and the radical scavenger, prevention of LPO is the only common mechanism that can be thought of, since cholesterol (strongly hydrophobic) is restricted in its movement to the membranes.

It seems noteworthy that the inversion from 'slightly apoptosis enhancing' to 'full protection' occurs within a very narrow range of cholesterol concentrations (figure 4). Taking into account the amounts of cholesterol actually incorporated into the cellular membranes, it results that the inversion occurs within a change of about 10% of membrane cholesterol content. Cholesterol can induce a variety of different sub-phases in lipid bilayers (Lange *et al.* 1980, McMullen *et al.* 1994). Parasassi *et al.* (1995) reported formation of molecular microdomains in membranes of different PC composition at several cholesterol concentrations. They showed that variations of the cholesterol content of about 15 mol% result in about the same effects on the membrane dynamics and the polarity inside the membranes as temperature changes of about 20°C. A comparable effect in cellular membranes could significantly alter membrane signal transduction properties.

A small sub-group of thymocytes enriched with cholesterol exposed discretely modified cellular morphologies, indicated by enhanced side scattering properties (figure 1, square A). The number of cells in this group is too small to be correlated with the inhibitory effects of cholesterol on apoptosis, but might be a hint towards phase transition like events and/or enhanced micro domain formation driven by cholesterol.

This paper's results reveal that α-tocopherol treatment increases apoptotic sensitivity to a degree comparable to that of small amounts of incorporated cholesterol. Cholesterol and α-tocopherol are both very hydrophobic molecules of comparable size and structure. So, similar effects on the dynamical properties of the lipid matrix can be expected. We must also expect low incorporation yields of the hydrophobic α-tocopherol into the cellular membranes, which would explain why the scavenger properties observed for trolox by Ramakrishnan *et al.* (1993) do not regulate apoptosis in this study's experiments. Independent mechanisms for the involvement of α-tocopherol and cholesterol on apoptosis seem unlikely. It is suggested here that the apoptotic rate increase in those thymocytes, which had been incubated with small amounts of cholesterol, has its origin in an imbalance of a second messenger system regulating the homeoviscosity of the cellular membranes (Ojeda *et al.* 1994).

For radiation induced apoptosis, two principal pathways are presently discussed. One model takes into account direct DNA damage, followed by the expression of the tumour suppressor gene p53 and activation of the PKC (Clarke and Purdie 1993, Lowe *et al.* 1993). The other model implies an initial damage of cellular membranes by LPO and activation of PKC (Ojeda *et al.* 1994). This paper's hypothesis that cholesterol inhibits apoptosis by protecting cellular membranes from LPO after PKC activation is not contradictory to either model and may operate in the high cholesterol concentration range. The suggestion here that rigidity (or possibly also fluidity) effects imbalance a second messenger system, which is responsible for apoptotic simulation, is based on the assumption of a common inducing mechanism with model two since radiation induced LPO also alter membrane dynamics (Konings 1987, Kölling *et al.* 1994). This effect may dominate the low cholesterol concentration range.

The results in this study favour the assumption of two distinct cholesterol related mechanisms acting on the induction of apoptosis in mouse thymocytes. A competition of both mechanisms can explain the common cholesterol concentration dependence of the apoptotic rates (figure 4).

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