Thiopental-induced Apoptosis in Lymphocytes Is Independent of CD95 Activation

Marius Keel, M.D.,* Ladislav Mica, M.D.,† John Stover, M.D.,‡ Reto Stocker, M.D.,§ Otmar Trentz, M.D.,|| Luc Härtter, Ph.D.¶

Background: Barbiturate coma is used in patients with traumatic brain injury whenever increases in intracranial pressure remain unresponsive to less aggressive therapeutic regimens. However, barbiturate-mediated neuroprotection correlates with lymphopenia, which increases the risk of infection. The mechanisms by which barbiturates lead to lymphopenia remain to be determined.

Methods: Freshly isolated human lymphocytes and Jurkat cells were incubated with the barbiturate thiopental for 24 and 48 h. Apoptosis was measured by fluorescein isothiocyanate–Annexin and propidium iodide staining, rhodamine 123 staining, and the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method. Caspase-3 activity was detected by Western blot and substrate cleavage assay.

Results: Thiopental dose-dependently (5–500 μg/ml) increased apoptosis in Jurkat cells from basal levels (4.4 ± 1.9%) to 29.7 ± 2.8% after 24 h and 39.7 ± 3.2% after 48 h, whereas in lymphocytes, thiopental-induced necrosis was observed. Parallel to apoptosis, thiopental dose-dependently increased caspase-3–like activity in Jurkat cells. However, the pan-caspase inhibitor z-VAD-fmk (20 μM) only marginally reduced thiopental-induced (250 μg/ml) apoptosis in Jurkat cells (20.2 ± 2.5 to 17.2 ± 2.5%) and necrosis in lymphocytes (39.2 ± 7.5 to 30.7 ± 14%). In contrast, anti-CD95–induced apoptosis in Jurkat cells (27.0 ± 2.0%) was completely blocked by z-VAD-fmk (8.1 ± 1.8%). Neither expression of CD95 on Jurkat cells nor pretreatment with a neutralizing anti-CD95 antibody influenced thiopental-induced apoptosis, indicating that thiopental induces apoptosis independently of the CD95 system. The nuclear factor κB inhibitor gliotoxin accelerated both thiopental- and CD95-mediated apoptosis, indicating a mutual control mechanism of thiopental- and CD95-induced apoptosis.

Conclusions: Thiopental directly induces cell death in lymphocytes and Jurkat cells by a CD95-independent mechanism.

TRAUMATIC brain injury is the leading cause of death in injured patients, tightly through cerebral edema and secondary brain injury. Brain damage due to the increased intracranial pressure (ICP) can be avoided by applying different clinical strategies, such as controlled hyperventilation, mild hypothermia, and release of cerebrospinal fluid. In cases of increased ICP exceeding 30 mmHg, which was refractory to the therapeutic intervention, intravenous administration of barbiturate ("barbiturate coma") may become necessary. The beneficial effect of barbiturates in terms of decreasing increased ICP is thought to be due to decreased cerebral metabolism and blood flow. However, this therapy is often complicated by disturbed immunocompetence with reduction of lymphocytes followed by an increased infection rate. Lymphocytes are key players of the specific immune defense system and contribute to the immune response by cytokine release and activation of other immune cells. During homeostasis, the number of lymphocytes in the circulation and tissue is maintained at an equilibrium by the controlled cell death program, i.e., apoptosis. However the depressed activation of lymphocytes in patients under barbiturate treatment contributes to immunosuppression and increase of infections.

Apoptosis is a highly regulated mechanism involving several extracellular and intracellular factors. Binding of the soluble CD95 ligand to its plasma membrane receptor CD95 induces apoptosis and represents the best studied programmed cell death pathway in T-cell lymphocytes. Besides the CD95 system, different drugs are also able to induce T-cell apoptosis, depending partially on the CD95 pathway. Moreover, modulation of apoptosis by cytokines such as interleukin 2 or interferon γ has also been shown in T cells. In addition to the CD95 system, a group of intracellular proteases, called caspases, plays a prominent role in the regulation and execution of apoptosis. Among the various caspases, caspase-3 is considered the major executor of apoptosis, which cleaves several intracellular structural and cell-cycle-regulating proteins such as poly(adenosine diphosphate–ribose)polymerase. However, in recent years, evidence has accumulated showing alternative, e.g., caspase-independent forms of cell death in lymphocytes.

We have shown the serum concentrations of thiopental in patients undergoing thiopental treatment before. In this study, the effect of thiopental on lymphocyte survival was investigated using freshly isolated human lymphocytes from healthy volunteers or the human leukemic T-cell line Jurkat.

Recently, Loop et al. have shown that thiopental inhibits the activation of nuclear factor κB (NF-κB) in Jurkat cells. NF-κB is a member of the rel/-gen family of transcription factors and is thought to be involved in mediating cell survival but also regulates the transcription of genes involved in inflammatory and acute stress responses. Here we show that thiopental induces apoptosis/necrosis and caspase-3 activation in lymphocytes.
by mechanisms independent of the CD95 system, possibly through inhibition of NF-κB.

Materials and Methods

Special Reagents
The following reagents were obtained from commercial sources: propidium iodide (PI) and gliotoxin (Sigma Chemical Co., St. Louis, MO); Annexin-V-fluorescein isothiocyanate (FITC), terminal deoxyribo nucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) kit (Boehringer-Mannheim, Mannheim, Germany), pan-caspase inhibitor z-VAD-fmk (Bachem AG, Bubendorf, Switzerland); caspase-3 fluorogenic substrate z-DEVD-afc and rhodamine 123 (Molecular Probes, Inc. Eugene, OR); agonistic anti-CD95 antibody (CH11; Immunotech, Coulter, Marseille, France); antagonistic anti-CD95 antibody (SM 1/23; Alexis, Grünberg, Germany); FITC-labeled anti-CD3 antibody (UCHT1; R&D Systems, Wiesbaden-Nordenstadt, Germany); phycoerythrin-labeled anti-CD95 antibody (33455X; Becton Dickinson, Basel, Switzerland); and thiopental (Pentotal, Lot. No. 57879TF; Abbott AG, Baar, Switzerland).

Human Lymphocytes
Human lymphocytes were isolated after Ficoll density centrifugation of peripheral venous blood from healthy volunteers. The interface containing monocytes and lymphocytes was washed twice and resuspended in medium RPMI-1640 (Gibco BRL, Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (Gibco BRL) and L-Glutamax (Gibco BRL). After 2 h of incubation in 24-well cell culture plates (Costar Co.) at 37°C in a humidified atmosphere (5% CO₂), nonadherent cells (>95% lymphocytes) were collected, washed, and resuspended in the same medium at a density of 1 × 10⁶/ml. Purity was measured by flow cytometry (FACS Calibur; Becton Dickinson) after staining with an FITC-CD3 antibody and was found to be greater than 95%.

Jurkat Cells
Jurkat T cells (10⁶ cells/ml) were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Gibco BRL) and 1.5 mm L-Glutamax in 24-well cell culture plates (Costar Co.) at 37°C in a humidified atmosphere (5% CO₂).

Experimental Protocol
Cells were preincubated for 1 h with or without antagonistic anti-CD95 (SM 1/23) antibody (500 ng/ml), z-VAD-fmk (20 μM), or gliotoxin (0.001–0.01 μg/ml) before stimulation with or without various concentrations of thiopental (5–500 μg/ml) or agonistic anti-CD95 (CH11) antibody (20 or 100 ng/ml) for times indicated. The cell loss in Jurkat cultures was less than 5%, irrespective of the experimental design or the added proteins using trypan blue exclusion and microscopic cell counting.

Cytofluorometric Measurement of Apoptosis and Necrosis
Determination of apoptosis and secondary necrosis uses the high affinity of Annexin-V for phosphatidylserine, which is exposed on the cell surface of apoptotic cells, and of PI to DNA strands. Jurkat cells (1 × 10⁶/ml) were washed with phosphate-buffered saline, resuspended in binding buffer (10 mm HEPES-NaOH, pH 7.4, 140 mm NaCl, 2.5 mm CaCl₂) and incubated with 0.25 mg/ml FITC–Annexin-V and 50 μg/ml PI. The mixture was kept on ice for 5 min, and cell fluorescence was measured by two-parameter flow cytometry (FACS Calibur). When green fluorescence (FITC) was plotted against red fluorescence (PI) in a dot plot, three distinct cell populations could be detected: viable cells (FITC negative, PI negative), apoptotic cells (FITC positive, PI negative), and secondary necrotic cells (FITC positive, PI positive). A minimum of 10,000 events (within a gate in the forward and side scatter window) was counted per sample, and data were reported as the percentage of apoptotic cells (Annexin-V–FITC positive, PI negative) and secondary necrotic cells (Annexin-V–FITC positive, PI positive).

Detection of Apoptosis by TUNEL Staining of Cytospin Slides
For the detection of apoptosis after the experiment, cytospin preparations of freshly isolated human lymphocytes or Jurkat cells (1 × 10⁵) were treated with a commercially available kit for the TUNEL technique (Boehringer Mannheim) according to the manufacturer’s guidelines. Briefly, air-dried cytospin preparations were fixed with 4% paraformaldehyde solution, rinsed with phosphate-buffered saline, and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min on ice. The slides were rinsed again with phosphate-buffered saline and incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase, FITC-labeled nucleotides) for 1 h in a humidified chamber. The slides were mounted in 50% glycerol–phosphate-buffered saline and examined on a fluorescence microscope (Leica Dialux 22 EB; Leica, Wetzlar, Germany). Bright homogeneously FITC-stained nuclei were considered as typical apoptotic features. Up to 500 cells were counted per slide. Data are reported as the percentage of apoptotic cells of all cells stained with 4′,6-diamidino-2-phenylindole following standard protocol.
Detection of Mitochondrial Membrane Breakdown in Jurkat Cells

Apoptosis of mitochondria was measured in Jurkat cells (1 × 10⁶/ml) stained with rhodamine 123 (0.2 μM) for 1 h at 37°C. After a washing step, fluorescence was measured in the cytometer (FACS Calibur). The mitochondrial membrane breakdown in the cells is represented by a shift of mean fluorescence to the left (decrease in channel number). Data are given as percent of shifted cells from 10,000 cells measured within a forward and side scatter gate.

Analysis of CD95 Expression on Jurkat Cells

Expression of CD95 on Jurkat cells was measured by flow cytometry (FACS Calibur) with a phycoerythrin-anti-human-CD95 (33455X) antibody. After 24 h incubation Jurkat cells (10⁶/ml) were stained with 0.25 mg/ml phycoerythrin-labeled anti-CD95 antibody for 1 h on ice before fluorescence was measured in FACS. A minimum of 10,000 events was counted per sample, and data were reported as the mean channel fluorescence.

Caspase-3-like Activity Measurement and Western Blot

Caspase-3-like activity was measured in cellular extracts from Jurkat cell samples. After incubation, cells were lysed by freeze-thaw procedure in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 0.1% Triton-X 100, 1 mM Pefablock® with addition of peptatin, leupeptin, and aprotinin [1 μg/ml each]) and subsequently centrifuged (15 min, 14,000g, 4°C), and the supernatant was stored at −80°C until further use. The fluorometric cleavage assay for caspase-3-like activity (DEVD-afc; Calbiochem, Nottingham, United Kingdom) was performed in microtiter plates (Greiner, Nuer-tingen, Germany) according to the method described by Thornberry. Using the fluorometric plate reader Victor-2 (Wallac Instruments, Turku, Finland), with the excitation wavelength set at 385 nm and an emission wavelength of 505 nm. The protein concentrations of the respective samples were measured with a commercially available kit (Pierce Assay; Pierce, Cramlington, United Kingdom), and caspase-3-like activity was calculated as microunits per milligram protein with 1 μM af biotin being equal to the cleavage of 1 pmol 7-amino-4-triflurometh-ylcoumarin per milligram protein and minute.

In addition, caspase-3 activation in Jurkat cells was measured by Western blot. Equal amounts of whole cell lysate (5 μl/lane, corresponding to 5 × 10⁵ cells) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% polyacrylamide gels in a Mini Protein II chamber (BioRad, Hercules, CA). Proteins were subsequently electrotransferred onto a nylon membrane (Immobilon P; Millipore, Bedford, MA), and membranes were blocked overnight at 4°C in tris-buffered saline supplemented with 0.1% Tween-20 and 0.2% bovine serum albumin (Sigma). Caspase-3 protein was detected using a specific antibody against caspase-3 from Santa Cruz (Santa Cruz, CA) and a peroxidase coupled secondary antibody (Dako, Glostrup, Denmark). Specific binding was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom) on x-ray film (Kodak, Rochester, NY) following the manufacturer’s recommendations. The molecular weight of the protein bands were determined by use of pre-stained low-molecular-weight markers (Sigma) on the same gel.

Statistics

Data are given as mean ± SD. The dose-dependent effect of thiopental was analyzed by one-way analysis of variance and post hoc testing (Tukey); in other experiments, mean values were compared using the Student two-tailed t test for independent means or the Mann-Whitney U test. Differences were regarded as significant if P was less than 0.05.

Results

Thiopental Dose-dependently Induces Cell Death in Human Lymphocytes

Thiopental in concentrations from 5 to 500 μg/ml dose-dependently increased apoptosis and necrosis in isolated human lymphocytes (10⁵ cells/ml). The proportion of necrotic (Annexin-V-positive, PI-positive) cells was higher than that of apoptotic (Annexin-V-positive, PI-negative) cells. Apoptosis increased only marginally, whereas necrosis increased from 3.0 ± 1.3% to 38.1 ± 16.9% after 24 h and up to 48.4 ± 19.9% after 48 h (fig. 1). Analysis of variance (P < 0.05) and post hoc tests (Tukey) revealed significant differences of necrotic cells at thiopental concentrations above 250 μg/ml (P < 0.05) compared with medium alone.

Effect of Thiopental on Apoptosis of Jurkat Cells

Spontaneous apoptosis (Annexin-V positive, PI negative) of cultured Jurkat cells (10⁶ cells/ml) was 4.4 ± 1.9% after 24 h and 6.1 ± 2.0% after 48 h (fig. 2), whereas 3.0 ± 1.0 and 4.0 ± 1.0% necrotic (Annexin-V-positive, PI-positive) cells were detected after 24 and 48 h, respectively (fig. 2). Incubation with 5–500 μg/ml thiopental dose-dependently increased apoptosis up to 29.7 ± 2.8% after 24 h and 39.7 ± 3.2% after 48 h and secondary necrosis up to 21 and 29%, respectively (fig. 2). In contrast to lymphocytes, Jurkat cells exhibited a lower proportion of secondary necrotic (Annexin-V-positive, PI-positive) cells compared with apoptotic (Annexin-V-positive, PI-negative) cells. The amount of pure necrotic (Annexin-V-negative, PI-positive) cells was 1.7 ± 2.3% after 24 h and 2.0 ± 2.0% after 48 h and was independent of thiopental treatment.

Thiopental-induced apoptosis was also seen in cyto-
spin slides stained by the TUNEL method. Here, the percent of TUNEL-positive cells after 24 h in freshly isolated lymphocytes and Jurkat cells (3.2/11006 2.6 and 5.5/11006 3.2%, respectively) increased dose dependently after incubation with thiopental up to 17.7/11006 4.9 and 20.1/11006 8.2%, respectively (table 1).

Expression of CD95 after Thiopental Treatment in Jurkat Cells

To investigate whether thiopental-induced apoptosis was mediated through CD95 expression and subsequent autocrine and/or paracrine induction of cell death, expression of CD95 on Jurkat cells was measured in FACS after incubation with thiopental (5–500/11006 g/ml). However, expression of CD95 on the cell surface of Jurkat cells (6.9 ± 0.6 mean fluorescence) was not altered significantly 4 h after incubation with 250/11006 g/ml thiopental (8.6 ± 0.9 mean fluorescence) (table 2). To further test whether the CD95 system is involved in thiopental-induced apoptosis, Jurkat cells were preincubated with an antagonistic anti-CD95 (SM 1/23) antibody (500 ng/ml) before stimulation with thiopental (250/11006 g/ml) or an agonistic anti-CD95 (CH11) antibody (100 ng/ml). Anti-CD95 induced apoptosis (16.7 ± 0.4%) could be completely blocked (9.7 ± 2.8%) similar to necrosis (60.0 ± 7.0 vs. 5.1 ± 3.3%, respectively) by thiopental. Anti-CD95-induced apoptosis could be completely blocked (9.7 ± 2.8%) similar to necrosis (60.0 ± 7.0 vs. 5.1 ± 3.3%, respectively) by thiopental.

Table 1. Incubation with Thiopental and TUNEL Staining of Lymphocytes and Jurkat Cells

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<td>* 6.3 ± 3.2†</td>
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Lymphocytes (1 × 10⁶/ml) were stimulated with or without thiopental for 24 h and apoptosis determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining. Jurkat cells (1 × 10⁶/ml) were preincubated 1 h with or without z-VAD-fmk (20 µM) before stimulation with or without thiopental for 24 h. Data is given in percent of TUNEL-positive cells. Data are from five independent experiments and are presented as mean ± SD.

Analysis of variance (Tukey test): * P < 0.05 thiopental vs. medium; † P < 0.05 z-VAD-fmk vs. medium.

ND = not determined.

Fig. 1. Thiopental induces apoptosis in human lymphocytes. Isolated cells (1 × 10⁶/ml) were stained with fluorescein isothiocyanate–Annexin-V and propidium iodine after incubation with increasing concentrations of thiopental (5–500 µg/ml) for 24 h (black bars) or 48 h (gray bars). Data are reported as the percentage of apoptotic (Annexin-V–positive, propidium iodine–negative) cells (A) or secondary necrotic (Annexin-V–positive, propidium iodine–positive) cells (B). Results represent the mean ± SD of eight separate experiments. Analysis of variance (Tukey test): * P < 0.05 with versus without thiopental.

Fig. 2. Thiopental induces apoptosis and secondary necrosis of Jurkat cells. Cells (1 × 10⁶/ml) were stained with fluorescein isothiocyanate–Annexin-V and propidium iodine after incubation with increasing concentrations of thiopental (5–500 µg/ml) for 24 h (black bars) or 48 h (gray bars). Data are reported as the percentage of apoptotic (Annexin-V–positive, propidium iodine–negative) cells (A) or secondary necrotic (Annexin-V–positive, propidium iodine–positive) cells (B). Results represent the mean ± SD of eight separate experiments. Analysis of variance (Tukey test): * P < 0.05 with versus without thiopental.
preincubation with antagonistic anti-CD95 antibody (SM 1/23), whereas no effect was observed in thiopental-induced apoptosis (15.3 ± 0.7 vs. 16.0 ± 0.6%, respectively) or necrosis (37.8 ± 13.1 vs. 38.2 ± 10.0%) (fig. 3). This indicates that thiopental-induced apoptosis and necrosis is independent of the CD95 system.

**Effect of Thiopental on Caspase-3–like Activity**

To test whether caspase-3 was involved in thiopental-induced apoptosis, caspase-3–like activity was measured in Jurkat cells after incubation with increasing doses of thiopental (5–500 μg/ml). Thiopental dose-dependently and in parallel to apoptosis significantly \( P < 0.05 \) increased caspase-3–like activity from basal level (88.0 ± 14.4 U) to 364.0 ± 95.1 U after incubation with 500 μg/ml thiopental for 24 h (fig. 4). The activation of caspase-3 was confirmed in Western blot. Whole cell lysates from Jurkat cells incubated for 24 h with medium or thiopental (5–500 μg/ml) were separated by electrophoresis and Western blot stained with caspase-3–specific antibodies. Activation of caspase-3 is characterized by a reduction of the pro–caspase-3 band. As depicted in figure 5, thiopental dose-dependently reduces pro–caspase-3 band, indicating activation of caspase-3 by thiopental (fig. 5). Densitometric analysis of Western blots where the medium control was set at 100% yielded values for incubation with 5, 50, 100, 250, and 500 μg/ml thiopental.

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Table 2. Effect of Thiopental on CD95 Expression on Jurkat Cells

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<td>500</td>
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Jurkat cells (1 × 10^6/ml) were treated with or without thiopental for 24 h. Expression of CD95 on the cell surface was detected by phycoerythrin-labeled anti-human CD95 antibody. Data are from three independent experiments and are presented as mean ± SD.

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**Fig. 3. Thiopental is independent of CD95-induced apoptosis.** Jurkat cells (1 × 10^6/ml) were preincubated with (shaded bars) or without (white bars) an inhibitory anti-CD95 antibody (SM1/23; 500 ng/ml) 1 h before stimulation with thiopental (250 μg/ml) and/or an agonistic anti-CD95 antibody (CH11; 100 ng/ml) for 24 h. Data are reported as the percentage of apoptotic (Annexin-V–positive, propidium iodine–negative) cells (A) or secondary necrotic (Annexin-V–positive, propidium iodine–positive) cells (B). Results represent the mean ± SD of five separate experiments. Student t test: * \( P < 0.05 \) with versus without thiopental; † \( P < 0.05 \) with versus without anti-CD95; # \( P < 0.05 \) with versus without SM1/23.

**Fig. 4. Caspase-3 activation in Jurkat cells by thiopental.** Jurkat cells (1 × 10^6/ml) were incubated with increasing concentrations of thiopental (5–500 μg/ml) for 24 h, and caspase-3 activity was measured in the cell lysate by DEVD-afc cleavage. Results represent the mean ± SD of five separate experiments. Analysis of variance (Tukey test): * \( P < 0.05 \) with versus without thiopental.

**Fig. 5. Decrease of pro–caspase-3 in Jurkat cells by thiopental.** Jurkat cells (1 × 10^6/ml) were incubated with increasing concentrations of thiopental (5–500 μg/ml) for 24 h and the decrease of pro–caspase-3 (detected by molecular size) detected in Western blot after electrophoresis. Equal protein, corresponding to 5 × 10^5 cells, of whole cell lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel and immunostained with a caspase-3 antibody and respective horseradish peroxidase-labeled secondary antibodies and visualized by enhanced chemiluminescence. One of five separate experiments shown. The amount of pro–caspase-3 protein was quantified by densitometry, and the relative density of bands is given as mean ± SD (% from the medium-only treated cells value (set at 100%) of the five Western blots.
Inhibition of Caspases and Thiopental-induced Apoptosis

The involvement of caspase-3 was further studied by preincubating Jurkat cells (10^6 cells/ml) and human lymphocytes (10^6 cells/ml) with the pan-caspase inhibitor z-VAD-fmk (20 μM), and apoptosis was measured after 24 and 48 h. Thiopental (250 μg/ml)-induced apoptosis after 24 h of Jurkat cells (20.2 ± 2.5%) was reduced only insignificantly by z-VAD-fmk (17.2 ± 2.5%), whereas secondary necrosis (17.0 ± 3.0%) was significantly reduced (6.6 ± 1.2%) (fig. 6). In contrast, CD95-induced apoptosis (27.0 ± 2.0%) was completely (P < 0.05) blocked (8.1 ± 1.8%). These result are also supported by TUNEL-stained cells (table 1) showing a reduction of thiopental-stimulated TUNEL-positive cells (15.1 ± 3.7%) after preincubation with z-VAD-fmk (6.8 ± 2.0%).

Similar results were seen after 48 h in Jurkat cells (fig. 6). In isolated lymphocytes, no effect of z-VAD-fmk was seen on thiopental-induced apoptosis and necrosis after 24 and 48 h (fig. 7).

CD95-induced caspase-3-like activity (551.0 ± 56.9 μU), as well as thiopental (250 μg/ml)-induced activity (232.0 ± 33.6 μU) in Jurkat cells after 24 h was completely blocked by z-VAD-fmk (7.0 ± 3.0 and 8.5 ± 3.6 μU, respectively) (fig. 8), confirming the effectiveness of z-VAD-fmk.

Activation of Mitochondrial Apoptosis by Thiopental

It has been shown that thiopental leads to activation of mitochondria. Therefore, Jurkat cells (10^6 cells/ml) were incubated with increasing doses of thiopental (5–500 μg/ml) for 24 and 48 h, and cells were stained afterward with rhodamine 123 for detection of mitochondrial membrane breakdown. The mitochondrial membrane breakdown is represented by a shift of mean fluorescence (decrease in channel number) in the cells.
Parallel to apoptosis, thiopental dose-dependently increased the amount of rhodamine-shifted cells (13.9 ± 3.9 to 60.2 ± 10.9%) after 24 and 48 h (82.6 ± 13.4%) of all cells counted (fig. 9). Incubation with agonistic CD95 antibody increased rhodamine-negative cells to 94.1 ± 2.7% after 24 h and 99.0 ± 0.4% after 48 h (data not shown).

**Inhibition of NF-κB and Thiopental-induced Apoptosis**

Recently, activation of NF-κB by thiopental has been shown in Jurkat cells. Therefore, gliotoxin, a specific inhibitor of NF-κB, was used as a pharmacologic tool to investigate the involvement of NF-κB in the regulation of thiopental-induced apoptosis. Because of the high rate of cell death after incubation with agonistic CD95 antibody, after 24 h, the concentration of agonistic CD95 antibody was decreased from 100 ng/ml to 20 ng/ml, and the incubation time was reduced to 16 h in this experiment. Jurkat cells (10^6 cells/ml) were incubated for 16 h with increasing doses of gliotoxin (0.001–0.01 g/ml) or agonistic CD95 antibody (250 µg/ml) and/or anti-CD95 antibody (CH11; 20 ng/ml) for 16 h after pretreatment with medium (black bars) or 0.001 µg/ml (light gray bars) or 0.01 µg/ml (dark gray bars) gliotoxin. The cells were stained with fluorescein isothiocyanate–Annexin-V and propidium iodine and analyzed in flow cytometry. Data are reported as the sum of apoptotic (Annexin-V-positive, propidium iodine-negative) and secondary necrotic (Annexin-V-positive, propidium iodine-positive) cells. Results represent the mean ± SD of five separate experiments. Student t test: * P < 0.05 with versus without thiopental or anti-CD95; # P < 0.05 with versus without gliotoxin.

**Discussion**

In patients with severe traumatic brain injury, the barbiturate coma can be the final resort in the therapy of...
life-threatening increases of the ICP, whenever other therapeutic regimens do not reduce ICP. However, this therapy is often complicated by disturbed immunocompetence with leukopenia and an increased infection rate. It could be speculated that thiopental would have proapoptotic activity on immune cells. This suggestion is supported by the observation that intravenous administration of barbiturate depresses lymphocyte activation.

The presented results demonstrate the proapoptotic activity of thiopental on Jurkat T cells and human lymphocytes. In previous studies, drug-induced apoptosis of immune cells was focused on cancer therapy. Villunger et al. showed that drug-induced apoptosis is associated with enhanced CD95 ligand expression but occurs independently of CD95 signaling in human acute lymphatic leukemia T cells. In contrast, Krammer and coworkers postulated CD95/CD95 ligand signaling as the central effector pathway of drug-induced apoptosis. Although thiopental enhanced the CD95-induced apoptosis in Jurkat cells, the facts that thiopental did not alter CD95 expression on Jurkat cells and thiopental-induced Jurkat cell apoptosis could not be prevented by pretreatment with antagonistic anti-CD95 antibody points to a CD95-independent pathway of thiopental-induced lymphocyte apoptosis.

In a recent study, it was shown that opioids, commonly used as potent analgesics, induced CD95 expression on human lymphocytes and promoted CD95-mediated apoptosis. This effect was blocked by naloxone, an antagonist of the opioid receptors, which are also expressed on lymphocytes. In the same line, Yin et al. showed that stress modulates the immune system with an increase of T-cell apoptosis through CD95-mediated apoptosis dependent on endogenous opioids. These data were in accord with previous results from Oka et al. who demonstrated that circulating lymphocytes in the early perioperative period are susceptible to CD95-mediated apoptosis, which may cause depletions of circulating lymphocytes after surgery. However, in the current study, no influence of naloxone was observed on the thiopental-induced apoptosis (data not shown). Therefore, in contrast to opioids, thiopental acts via a different mechanism.

The observation that caspase-3 activity increased in parallel to apoptotic rate after incubation with thiopental suggested a pivotal role of caspase-3 in thiopental-induced apoptosis. However, the pretreatment with the pan-caspase inhibitor z-VAD-fmk did not completely inhibit the apoptotic effect by thiopental, whereas the anti-CD95-induced apoptosis was completely blocked by z-VAD-fmk. The current dogma that caspase activity is essential for execution of apoptosis has been under scrutiny recently, because certain apoptotic characteristics such as cell shrinkage, membrane blebbing, and nuclear condensation were found to be insensitive toward caspase inhibitors. This is confirmed through the results of this study that effective inhibition of the complete caspase cascade through the pan-caspase inhibitor z-VAD-fmk did not completely reduce thiopental-induced Jurkat cell apoptosis and lymphocyte necrosis. This caspase independence and the differences seen between Jurkat cells and freshly isolated lymphocytes in the mode of cell death and induction of necrosis in our experiments have been described before. Therefore, our results indicate that further factors must be involved in the regulation and execution of thiopental-induced cell death, i.e., apoptosis and necrosis. Today, apoptosis and necrosis are seen as the two extremes of a wide spectrum of cell demise including caspase-independent cell death, with a multitude of factors involved.

Recently other proteases, namely calpains, cathepsins, or the proteasome have been discussed as alternative factors of cellular apoptosis. As further alternative concept of apoptotic execution, a loss in mitochondrial membrane barrier function is discussed in the literature. This is supported by recent findings showing an exaggerated generation of oxygen radicals after incubation with 20 mmol/ml thiopental. One increased production of oxygen radicals by thiopental could enhance lymphocyte commitment to apoptosis and is also in accord with our observation of decreased rhodamine staining after thiopental incubation. However, further investigations are necessary to clarify this mechanism.

Recently, it has been shown that thiopental inhibits activation of NF-κB in lymphocytes and Jurkat cells via inhibition of the calmodulin–calcineurin complex pathway. We could confirm these findings by gliotoxin inhibition of NF-κB, which enhanced the spontaneous apoptosis and the proapoptotic effect of thiopental in Jurkat cells. This shows that NF-κB plays a central role not only in regulation of drug-induced apoptosis but also in the survival of lymphocytes during homeostasis, possibly by transcription of survival genes. It has been shown in another study that NF-κB controls the transcriptional activity of a gene, which induces the synthesis of survival proteins. Furthermore, in a number of immune cells, NF-κB activation has been shown to play a pivotal role in regulating the genes for inflammatory cytokines, which inhibit the apoptotic process, probably by activation of NF-κB in a positive-feedback loop. Recently, Humar et al. showed that thiopental and other barbiturates indirectly suppress the activation of the transcription factor activator protein 1. However, in contrast to other closely related barbiturates, thiopental does not act through activation of mitogen-activated protein kinases and, through this, might contribute to the severe immuno-
suppression during thiopental coma in patients with traumatic brain injury. We are aware of the preliminary nature of our observations, and the exact mechanisms of thiopental-induced cell death in lymphocytes need further investigation. Elucidating the mechanism of thiopental-induced cell death might help to improve thiopental therapy and prevent lymphocyte apoptosis and risk of nosocomial infections in thiopental-treated patients.

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