INTRODUCTION

Extracorporeal photopheresis (ECP) is a leukapheresis-based immunomodulatory therapy by which white blood cells isolated from patient’s blood are extracorporeally exposed to 8-methoxypsoralen (8-MOP) and UVA prior to reinfusion to the patient. In 1988 ECP has been approved for the treatment of cutaneous T-cell lymphoma (CTCL) by Food and Drug Administration (FDA). This treatment provides significant disease remission and prolongation of life in patients with CTCL. In addition, ECP has been effective in the prevention and reversal of solid organ transplant rejection and graft-vs-host disease (GvHD), systemic sclerosis (SSc) and others (1-6). The underlying mechanisms by which ECP acts on immunity are not well understood. However induction of apoptosis of different cell subpopulations, increased immunogenicity and clearance of apoptotic cells, participation of T regulatory cells and modulation of cytokine release such as TNF α, IL-10, IL-4 are suggested to play a vital role in therapeutic action of ECP (7-10).

In this study we investigated 8-MOP, chlorpromazine (CPZ) and 4,6,4’-trimethylangelicin (TMA) because of differences in their ability to induce immune suppression in rats in vivo. It has been proven that contact hypersensitivity (CHS) could be effectively inhibited by transfer of leukocytes irradiated with UVA in the presence of 8-MOP and CPZ, while TMA and UVA had no effect (11-13). Our previous study showed that all three selected drugs induce apoptosis upon UVA irradiation in Jurkat cells, and rat leukocytes that were similarly treated as the samples for animal model study (14). Interestingly, using 10 times lower (clinical) UVA doses, we showed that TMA was the most effective drug to induce apoptosis in Jurkat cells (15). Furthermore, we demonstrated that all three photosensitizers combined with the UVA irradiation were able to inhibit the release of TNF-alpha from U-937 cells in vitro following UVA irradiation (14, 16).

Apoptosis also called a suicidal cell death is a very important physiological phenomenon. It may not only clear the tissue from damaged cells, but also induce inhibition of immunological response. Extracorporeal photopheresis induced apoptosis of lymphoid cells and mitochondria may play a key role in apoptotic cell death induced by PUVA (psoralen plus UVA light) therapy (7, 10, 17, 18). It was shown, that freshly
isolated monocytes could interact with apoptotic cells resulting in increased production of anti-inflammatory cytokines (19, 20). It was also reported, that peripheral blood lymphocytes subjected to extracorporeal phototherapy increased production of IL-10 and IL-1Ra by freshly isolated PBMCs and monocytes (8, 9).

In this study we compared the activity of 8-MOP, TMA, and CPZ at therapeutically relevant concentrations to induce, upon photoactivation with UVA, at doses up to 10 kJ/m², apoptosis in normal human peripheral blood lymphocytes. We also analyzed changes of mitochondrial potential and susceptibility of different sub-populations of lymphocytes to photo-treatment. In addition we investigated interactions of freshly isolated monocytes with lymphocytes subjected to the treatment with UVA irradiation and photosensitizers.

MATERIALS AND METHODS

Isolation of human lymphocytes and monocytes

Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation from heparin-treated blood of healthy donors. The laboratory has been approved by the ethical committee of the Jagiellonian University. The cells were suspended in Hank's balanced salt solution supplemented with 1% BSA and subjected to countercurrent centrifugal elutriation (Beckman JE-6B elutriation system equipped with a 5-ml Sanderson separation chamber) as previously described (21). Lymphocytes were washed once with a medium, RPMI 1640 (Gibco BRL, Life Technologies TM, Germany) and kept on ice until used. Monocytes were suspended in cold RPMI 1640 (Gibco BRL, Life Technologies TM, Germany) supplemented with 10% FCS (Biochrom, Germany), 2 mM L-glutamine (Sigma) and 50 µg/ml gentamycin (Sigma). Lymphocytes when electronically gated on the basis of forward vs. side scatter were at least 96% pure. The monocytes were 90% positive when labeled with CD14 antibodies.

Photochemotherapy

According to our previous published study (15) about 3x10⁶ lymphocytes in 1 ml PBS were incubated with 8-MOP (50-300 ng/mL; 0.23-1.4 nmol/mL; Aldrich, Germany) and the final ethanol concentration in samples was 1%. The samples were irradiated with UVA in glass vials (φ2.7x10⁻² m, height 1x10⁻² m) or 12-well plates using Philips HB 311 lamps (emission between 300-420 nm, max 354 nm) or left in darkness. The final UVA intensity was 1 J/cm² and was measured by an UVX 36 digital radiometer (Ultra-Violet Products, California, USA). Next, all samples were washed with PBS and incubated in culture medium up to 36 h following UVA exposure. Each experiment was repeated three to five times.

Cell culture conditions

Both lymphocytes alone and in co-culture with monocytes were incubated in RPMI 1640 (Gibco BRL, Life Technologies TM, Germany) supplemented with 10% FCS (Biochrom, Germany), 2 mM L-glutamine (Sigma) and 50 µg/ml gentamycin (Sigma) at 37°C with 5% CO₂. Lymphocytes after photochemotherapy were cultured on 12-well plates (TPP, Switzerland), incubation of monocytes and lymphocytes (at 1:1 ratio) cultured together, was performed in Falcon tubes (BD Bioscience).

Annexin-V-FITC/PI staining of lymphocytes

Annexin-V-FITC/PI staining was carried out as described in the protocol of Bender MedSystem, Austria. In short, human lymphocytes were washed with Hapes buffer and stained with annexin-V-FITC. Next, PI (5 µg/mL) was added and the measurement was carried out again to detect cell death. Analysis was performed on a FACScan flow cytometer (BD) and a percentage of viable and apoptotic cells was determined.

TUNEL assay

TUNEL assay was done as we described earlier using an apoptosis detection system, fluorescein kit, Promega, USA (15, 25). Cells were fixed with 4% formaldehyde, washed with PBS, than incubated overnight with methanol at 4°C. After washing samples were stained with fluorescein isothiocyanate (FITC)/dUTP, using terminal deoxynucleotidyl transferase (TdT) and propidium iodide (PI), and analyzed by the FACScan flow cytometer using the CellQuest software to obtain the percentages of cells exhibiting DNA fragmentation (dUTP+/PI+).

Cell cycle analysis

Lymphocytes were spun down and fixed with 70% ice cold ethanol and stored overnight at 4°C. Subsequently cells were washed with PBS and suspended to the density of 1x10⁶/ml. 1 ml aliquots was incubated with RNA-ase (100 U/ml; Fluka) 30 min at 37°C, then for additional 30 min with PI (20 µg/ml, Sigma) and analyzed on the FACScan flow cytometer.

Staining with red CMXRos

Lymphocytes mitochondrial potential was analyzed using cell-permeable 8(4′-chloromethyl) phenyl-2, 3, 5, 6, 11, 12, 14, 15-octahydro-1H, 4H, 10H, 13H-diquinolizino-8H-xanthylium chloride (Red CMXRos, Molecular Probes, Leiden, The Netherlands) what correlates with cellular viability and apoptosis (23, 24). Lymphocytes were incubated with Red CMXRos (40 mM) at 37°C for 15 min than samples were washed once and resuspended with RPMI medium and analyzed using the FACScan.

Staining of lymphocytes with CD3, CD16 and CD56

5x10⁴ cells were suspended in 100 µl RPMI (Gibco) and incubated with 10 µl of antibodies for 20 min on ice. The following monoclonal antibodies were used: phycoerythrin PE-conjugated anti-CD3 (Daco, Denmark), anti-human- CD19 (Immunotech, Marseille), anti-human- CD56 (Becton- Dickinson, San Jose, CA, USA). The cells were washed with 2 mL PBS then labeled with annexin-V-FITC.

Analysis of monocyte-lymphocyte interactions

The lymphocytes were isolated one day before monocytes and stained with PKH26, red cell membrane dye (Sigma). It is a lipophilic dye that stably inserts into the cell membrane (25). Labeling procedure was done according to manufacturer's instruction. Washed cells were resuspended in supplied Diluent C and immediately combined with an equal volume of PKH26 in the same diluent. Final staining conditions were 1x10⁵ cells/ml in 2x10⁴ M PKH26. After 4 min at room temperature an equal volume of FBS (BioWest) was added to stop staining reaction. Staining cells were washed three times and resuspended in a complete medium. Next they were treated with optimal doses of 8-MOP, TMA or CPZ as described above and treated with UVA to induce cell apoptosis. After 24 h of incubation lymphocytes
were washed with culture medium (RPMI supplemented with FCS, gentamycin and glutamine as described), counted and mixed with freshly isolated monocytes (1:1 ratio) in Falcon tubes (BD Bioscience). After 2 h of incubation in 37°C with 5% CO2 conditions cells were mixed and analysed using flow cytometry. To determine percentage of PKH 26 labelled lymphocytes from gate set on population of monocytes which was determined on base of their FSC/SSC parameters. Monocytes gate was the same for all samples and 10000 monocytes were collected in each. The fact that monocytes and lymphocytes were derived from different donors had no influence on the results since the recognition of apoptotic cells is comparable in case of the same and different donors (19, 26).

RESULTS

UVA activated 8-MOP, TMA, and CPZ induce apoptosis of lymphocytes

AnnexinV binding at 24 h after exposure was used to investigate the dose dependency of apoptosis (Fig. 1A-C). The effect was dose dependent for all photosensitizers tested, and reached about 50-65% AnnexinV positive lymphocytes after irradiation with UVA in the presence of 200 ng/mL of CPZ (0.56 nmol/mL) or 8-MOP (0.93 nmol/mL), and 50 ng/mL (0.22 nmol/mL) of TMA. Those concentrations were chosen for further study. UVA alone induced a slight response in lymphocytes (20-28% AnnexinV positive cells). Photosensitizers in the dark, and 1% of ethanol had no detectable effect.

Kinetic studies show an increase in the percentage of annexin V binding cells after phototreatment. Peak levels of annexin V binding were reached within 24 hours with a subsequent decline of the binding (Fig. 1D). Using TUNEL assay and DNA-content analysis we demonstrated that 24 h after exposure to irradiation with UVA in the presence of 8-MOP, CPZ or TMA caused DNA-fragmentation in normal, human lymphocytes (Fig 2). At the concentrations used the percentage of positively staining cells was highest with TMA+UVA (67% in lymphocytes).

Decrease of mitochondrial potential of lymphocytes 24 h after photo-treatment

During mitochondrial potential damage occurs that causes a decrease of the mitochondrial membrane potential. We detected such an effect using a Chloromethyl-X-Rosamine dye (23, 24). Recently Red CMXRos staining was used to monitor the changes of mitochondrial membrane potential during the process of apoptosis induced by phagocytosis of S. aureus. It was found that cells undergoing apoptosis have decreased ability to bind CMXRos dye (27). We decided to determine, how photopheresis treatment of lymphocytes would influence this phenomenon. As it is shown in Fig. 3, mitochondrial potential significantly decreases 24 h after photo-treatment with CPZ (0.56 nmol/mL), TMA (0.22 nmol/mL) and 8-MOP (0.93 nmol/mL) in contrast to lymphocytes incubated with selected photosensitizers in the dark, or irradiated with UVA alone. The effect is dependent on the dose of UVA, those cells that received higher dose of UVA had higher percentage of apoptotic cells. These results correlate with AnnexinV-FITC staining (unshown).

Induction of apoptosis in subpopulations of lymphocytes 24 h after photo-treatment

Since freshly isolated PBL contain different sub-populations of lymphocytes, we wanted to determine if there was any difference in the susceptibility of their main subpopulations: lymphocytes T, B and NK cells to apoptosis. The study was performed 24 h after phototreatment. Cells were double-stained with AnnexinV-FITC and antibodies against human CD3, CD19 or CD56, which are considered a marker proteins for lymphocytes T, B and NK respectively. Results showed on Fig. 4 indicate that irradiation of
the cells in the presence of TMA, 8-MOP and CPZ induce apoptosis of CD3+ and CD56+ subpopulations of lymphocytes (Fig. 4A-C). Apoptosis is also induced to some extent in CD19+ cells, however the difference is not statistically significant in comparison to control samples, incubated with photosensitizers but not irradiated with UVA (Fig. 4B). These data suggest that CD3+ and CD56+ are similarly susceptible to apoptosis induced by photoactivation of the sensizers, whereas CD19+ cells are more resistant to apoptosis induced by 8-MOP, TMA and CPZ.

Interaction of monocytes with apoptotic lymphocytes

In the same time two independent groups reported that monocytes may produce IL-10 and other antiinflammatory cytokines upon the culture with apoptotic cells (19, 20). Additionally, Bzowska et al. (19) showed that physical contact between monocytes and apoptotic cells is necessary for the production of IL-10. In the light of these informations we decided to check if there was an interaction between monocytes and apoptotic lymphocytes. We stained lymphocytes prior to apoptosis induction with PKH26, a red dye that stains cell membrane. After overnight culture following phototreatment, cells were washed and than mixed with freshly isolated monocytes for 2 h of incubation. Subsequently cells were collected with flow cytometer, and percentage of monocytes interacting with stained lymphocytes was determined. Data presented in Fig. 5 indicate, that percentage of monocytes PKH26-positive (due to association or phagocytosis of red lymphocytes) is much higher in samples where they were mixed with lymphocytes forced to apoptotic cell death by UVA irradiation with photosensitizers. This strongly suggests, that monocytes interact with apoptotic cells, possibly recognizing them for removal in process of phagocytosis.

DISCUSSION

Various cellular and molecular mechanisms including modulation of cytokines release and apoptosis have been
Fig. 3. Changes of lymphocytes mitochondrial membrane potential at 24 h following treatment with TMA (0.22 nmol/mL and 0.44 nmol/mL), CPZ (0.28 nmol/mL and 0.56 nmol/mL) or 8-MOP (0.47 nmol/mL and 0.93 nmol/mL) irradiated or not with UVA (10 kJ/m²). Also cells treated twice with UVA are shown. The values are the mean value of the seven independent experiments ± SD. Asterisk indicates statistically significant differences vs. cells treated with the same compound in the dark (the t-Student test, p < 0.05).

Fig. 4. Percentages of apoptotic cells among the CD3+ (A), CD19+ (B) and CD56+ (C) subpopulations of lymphocytes. Cells were double stained with CD3, CD19 and CD56 and later with AnV to determine apoptosis of cells. The results are the mean value of the three independent experiments ± SD. Asterisk indicates statistically significant differences vs. cells treated with the same compound in the dark (the t-Student test, p < 0.05).
suggested to be involved in the therapeutic action of ECP (7, 15, 16, 28, 29).

Our results confirm that the apoptosis-inducing effect of photochemotherapy can be reproduced in primary lymphocytes and that this effect is dose and time dependent. Comparing different photosensitizers we found that on molar basis TMA is the most effective drug in induction of apoptosis in lymphocytes compared to 8-MOP and CPZ, that is a similar observation to that found on Jurkat cells (15). This difference might be explained in part by different extinction coefficients in the UVA range, partition in cells membrane and photochemistry of the photosensitizers used (30).

Apoptosis is a complex event subjected to a variety of regulatory mechanisms and other signals including mitochondrial depolarization and subsequent cytochrome c release, CD95 (FAS), caspases, and p53 activation have been described to contribute to the experimental and clinical effects of photochemotherapy (10, 17-19, 29).

We present data which show that regardless of the photosensitizer used in phototreatment, the percentages of apoptotic lymphocytes in the same subpopulation does not differ significantly. However, only subpopulations of CD3+ and CD56+ cells show higher susceptibility to the apoptosis induced by irradiation with UVA in the presence of the photosensitizers, whereas CD19+ cells seem to be more resistant to this type of apoptosis induction. Perhaps B cells are less vulnerable to the damage caused by the phototreatment, but this phenomenon would require further studies for the precise explanation. The mechanism underlying the apoptosis induction by UVA treatment of cells with the photosensitizers is unknown by now. The toxic effect of the UV light for cells is well known, however the UV light with a shorter wavelength (means UVB and UVC) is the most potent inducer of cell death. In this study the UVA light was used with the intensity that should not induce the apoptosis by itself. Therefore the apoptosis induction observed here is dependent on the presence of photosensitizers. Their mechanism of action is not certain yet though. TMA, CPZ and 8-MOP may bind to different targets in cells, including DNA, proteins or lipids (31). It is possible than that while bound to that targets and irradiated with UVA, they may induce intracellular stress that results in apoptotic cell death. Several reports indicated that JNK kinases, which are sensitive to changes in a redox potential in cells, are involved in apoptosis induction under the cellular stress conditions (32, 33). What is more, all JNKs are important in a UV-activated mitochondrial-dependent death signaling pathway (34). It is possible that the photosensitizers together with UVA irradiation can induce cell death that is dependent on JNK kinases, however further experiments would be required to prove that hypothesis. Recent studies shown that in antitumor photodynamic therapy that also uses a photosensitization effect, an excess of nitric oxide (NO) may exert an antiapoptotic effect in tumor cells by inhibition of JNK and p38α activation and downregulation of expression of proapoptotic proteins Bax and Bid (35). Interestingly in human lymphocytes, some of the reactive nitrogen species, similarly with cigarette smoke, may activate an NF-κB, which is an important transcription factor that promotes cell survival (36, 37). Similarly, it was shown that the administration of neuraminidase into rats with Morris tumor decreases death of lymphocytes from peripheral blood (38). Also peripheral blood cells showed greater susceptibility to activation when treated with low-energy laser irradiation (39). It would be interesting to check if these phenomena could interfere with apoptosis induced by phototreatment of lymphocytes with UVA in the presence of TMA, CPZ or 8-MOP.

Many studies showed that apoptotic cells could induce an increased production of anti-inflammatory cytokines in monocytes after their activation by LPS (19, 20, 40). Cracium et al. (8) and Di Renzo et al. (9) reported that after treatment with 8-MOP monocytes responded to T cells with increased production of IL-10. Additionally, Bzowska et al. (19) proved, that physical interaction of monocytes with apoptotic cells was necessary for the production of IL-10. The photosensitisers used in this study similarly induce apoptosis in lymphocytes (this report) and in Jurkat cells (16). On the other hand there were reports indicating that TMA did not act similarly as CPZ and 8-MOP in the treatment of contact hypersensitivity. Iperen et al. (12) demonstrated that TMA did not induce specific immune suppression of contact hypersensitivity induced by 2,4-dinitrofluorobenzene (DNFB) in contrast to CPZ and 8-MOP (13). The question arose than that there may be any differences in recognition of cells in which apoptosis was induced by different photosensitizers. Our results indicate that recognition of apoptotic lymphocytes by monocytes is similar for both TMA and 8-MOP induced apoptosis. This interaction was lower in cells that were irradiated with UVA in presence of CPZ, however closer analysis of lymphocytes from that samples revealed, that the percentage of AnV+/PI+ positive cells was much higher, which may indicate their very late stage of apoptosis passing to necrosis. Results from the TUNEL assay seem to support this hypothesis. This would explain lower level of interaction between monocytes and lymphocytes in this samples. The interaction of monocytes and apoptotic lymphocytes may suggest that this process and the production of IL-10 resulting from it, are not responsible for the observed differences in the suppression of CHS by TMA, CPZ or 8-MOP. Interestingly Rivas and Ullrich (41) showed that IL-10 did not induce suppression of CHS, although it could block type IV hypersensitivity (DTH). This can be due to fact that while TH1 lymphocytes take part in
DTH, in CHS a main role is played by lymphocytes CD8+, which do not depend on IL-10 (42, 43). It is possible that the mechanism of suppression of CHS have a completely different mechanism and both CPZ and 8-MOP induce immune suppression on IL-10 independent way. Taken together, our data indicate that all three photosensitizers may induce apoptosis in human peripheral blood lymphocytes, and the effect is dose and time dependent. We show that this effect correlates with a decrease of mitochondrial potential in lymphocytes and also with recognition of apoptotic lymphocytes by freshly isolated monocytes. Additionally, induction of apoptosis is higher in CD3+ and CD56+ subpopulations, although the type of the photosensitizer used for the phototreatment seems not to play a role in the level of cell death.

**Abbreviations:** CHS: contact hypersensitivity; CPZ: chlorpromazine; ECP: extracorporeal photoimmunotherapy; PBL: peripheral blood leucocytes; PUVA: psoralen + UVA; 8-MOP: 8-methoxypsoralen; TMA: 4,6,4’-trimethylangelicin; TUNEL: TdT-mediated dUTP Nick-End Labeling.

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