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Expression of Fas antigen and apoptosis caused by 5,10,15,20-tetra(4-methoxyphenyl)porphyrin (TMP) on carcinoma cells: implication for photodynamic therapy

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Abstract

The photodynamic effects of 5,10,15,20-tetra(4-methoxyphenyl)porphyrin (TMP) on a Hep-2 cell line were investigated. TMP toxicity in the dark and in relation to illumination with visible light was examined. Hep-2 cells were treated with different TMP concentrations (1, 5 and 10 μM). The uptake of TMP by Hep-2 cells increased with TMP concentration and an increase of the initial uptake rate was observed with increasing TMP concentrations. However, after 24 h of incubation, a similar value of intracellular TMP concentration was reached at all three concentrations of TMP added. Cell toxicity induced by TMP was analyzed in the dark at different concentrations of the photosensitizer and at several incubation periods. The cell mortality obtained after exposure of the cell cultures to visible light was exclusively due to the photosensitization effect of TMP produced by light irradiation. Staining with the hematoxylin–eosin method demonstrated that treatment with TMP, followed by exposure to visible light, notably increased the apoptotic figures. Fas antigen was only expressed in these conditions. The results contribute to the understanding of the photodynamic therapy (PDT) mechanism produced by TMP on Hep-2 carcinoma cell line.

Keywords: Apoptosis; Fas; Porphyrins; Photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) is based on the administration of a photosensitizer that becomes concentrated in tumor cells, and upon subsequent irradiation with visible light in the presence of oxygen specifically destroys the cells (Dougherty, 1987; Penning and Dubbelman, 1994). The treatment is effective for cancerous tissues and malignant hemopoietic cells (Chen et al., 1998). Among phototherapeutic agents applied to the treatment of tumors, porphyrins and their analogs, in combination with visible light, have attracted much

Abbreviations: μM, micromolar; mM, millimolar; TMP, 5,10,15,20-tetra(4-methoxyphenyl)porphyrin.

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attention (Milgrom and O'Neill, 1993; Grosseweiner, 1994).

It is generally accepted that singlet oxygen ($^1O_2$), produced after exposure of the sensitizer to light, is the main species responsible for cell inactivation. Nevertheless, the photodynamic process of the sensitizers on neoplastic tissues is still not well understood (Jori et al., 1996). It is believed that PDT-induced cell death may be divided into two main types, namely apoptosis and necrosis (Agarwal et al., 1991). Apoptosis is characterized by cell shrinkage, chromatin condensation, the expression of surface antigens like Fas and formation of apoptotic bodies. Fas is a type I membrane protein with a molecular mass of 45 kD that mediates apoptosis. Its cytoplasmic domain (70 aminoacids), named the 'death-domain', is necessary and enough to translate apoptotic signals. Inappropriate expression of this receptor leads to disorders and/or autoimmune diseases. In many experimental systems Fas is utilized as an apoptosis marker (Chinnaiyan et al., 1995; Furukawa et al., 1996).

An important element of a successful PDT drug may be the ability to induce a rapid apoptotic response (Luo et al., 1996). The search for alternative photosensitizers seeks to find one that exhibits good selectivity, not so much in their affinity for, malignant cells but rather in the ultimate photokilling. The mode of cell death should also be investigated once their potency is established (Chen et al., 1998).

In this paper, we describe the photodynamic effects of 5,10,15,20-tetra(4-methoxyphenyl)-porphyrin (TMP) on a Hep-2 cell line. The goal of this research was to establish the toxicity of TMP in the dark, and in relation to the illumination with visible light. We also determined which kind of death TMP produces (necrosis or apoptosis) and whether Fas expression is an indicator of an apoptotic event.

2. Material and methods

2.1. Cell culture

A human larynx-carcinoma cell line (Hep-2), purchased from ABAC (Buenos Aires, Argentina) and kept in liquid nitrogen, was used. The cells were grown as a monolayer employing Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 μg/ml gentamycin. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and the medium was changed daily. The cell line was routinely checked for the absence of mycoplasma contamination.

2.2. Chemicals

4-Methoxybenzaldehyde, pyrrole, trifluoroacetic acid and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone from Aldrich were used without further purification. All solvents were high performance liquid chromatography (HPLC) quality. Dipalmitoyl phosphatidylethanolamine from Sigma was used in liposome preparation. Silica gel 230–400 mesh for column chromatography from Aldrich was used.

2.3. Photosensitizer

5,10,15,20 - Tetra(4 - methoxyphenyl)porphyrin was synthesized using the Lindsey et al. (1987) method. A solution of 4-methoxybenzaldehyde (0.152 ml, 1.5 mmol) and pyrrole (0.10 ml, 1.5 mmol) in 100 ml of dichloromethane was purged with argon for 15 min. Trifluoroacetic acid (35 μl, 0.45 mmol) was added. The solution was stirred for 60 min at room temperature. After that, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (0.34 g, 1.5 mmol) was added and the mixture was stirred for an additional 60 min, open to the atmosphere. The solvent was removed under reduced pressure and flash column chromatography (silica gel, dichloromethane) yielded 98 mg (35%) of pure TMP. MS [m/z] 734.3 (M^+) (734.2895 calculated for $C_{48}H_{38}N_4O_4$).

2.4. Liposome preparation

The incorporation of TMP into the phospholipid bilayer of the dipalmitoyl phosphatidylethanolamine was done by a modification of the ethanol injection procedure of Kremer et

al. (1977). Typically, 2 ml of a solution bearing 9.60 mM of phospholipid, 1.91 mM of cholesterol and 0.27 mM of TMP in ethanol–tetrahydrofuran binary mixture (1:1, v/v) was injected into 10 ml of phosphate-buffered saline solution (PBS) at 80°C. The injection was performed at a speed of 50 μl/min with magnetic stirring.

2.5. Irradiation

The visible light source used was a slide projector equipped with a 150 W lamp. The light was filtered through a 3 cm water layer to absorb heat. The light intensity at the treatment site was 18 mW/cm^2 (Radiometer Laser Mate-Q, Coherent).

2.6. TMP uptake and quantification

The uptake of TMP by Hep-2 cells was determined by fluorescence spectroscopy. About 1×10^6 cells were inoculated in 25 cm^2 culture flasks and incubated to obtain nearly confluent cell layers. An appropriate volume of the TMP incorporated into liposomes was added to the culture flask containing 5 ml of medium. Cells were treated with different TMP concentration for several incubation times. The medium containing the photosensitizer was then discharged and the cells were washed three times with PBS and suspended in 1 ml of PBS. The number of cells in each suspension was estimated by trypan blue (TB) exclusion test using a Neubauer chamber counter. Next, 1.0 ml of 4% sodium dodecyl sulphate (SDS, Merck) was added to the cellular suspension, which was incubated further for 15 min (in the dark and at room temperature) and centrifuged at 9000 rpm for 30 min. The concentration of the sensitizer in the supernatant was measured by spectrofluorimetry ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 658$ nm). The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension. The concentration of TMP in this sample was estimated by comparison with a calibration curve obtained with standard solutions of TMP in 2% SDS ([TMP] ~ 0.5–10 μM). Each experiment was repeated three times and four culture flasks were used for each incubation time.

2.7. Cell photosensitization studies

Cells were seeded, incubated and treated with TMP in 25 cm^2 culture flasks for the selected time in the dark, as described above. Afterwards, the medium containing the photosensitizer was removed. Cells were washed three times with medium and kept in 5 ml of it. The dishes were exposed for different time intervals to visible light.

2.8. Cell viability

After each irradiation time, the viability of the cells was estimated by microscopy using TB. The same procedure without irradiation was carried out for determining dark toxicity. Four culture flasks were used for each incubation time. Every experiment was compared with a culture control without TMP.

2.9. Apoptosis determination

To differentiate among necrosis and apoptosis, the hematoxylin–eosin method was utilized. Results were corroborated with a specific kit (TdT Frag EL™ DNA Fragmentation Detection Kit, Oncogene Research Products).

2.10. Fas-Ag determination

The Fas-Ag expression was determined in control cells grown as monolayers. The cells were incubated with TMP for 24 h in the dark. Other cultures were treated with TMP for 24 h, illuminated with 18 mW/cm^2 and maintained during 5 h at 37°C in the dark. The expression of Fas was analyzed by Western Blot. Cell monolayers were washed with PBS buffer, released by using a rubber policeman, collected in Eppendorf tubes and centrifuged at room temperature at 10 000 rpm for 2 min. The pellet was collected and the supernatant discarded. An extraction buffer (150 mM NaCl, 1% NP-40, 0.5% deoxicolate, 0.1% SDS, 50 mM Tris, pH 8) was added, and after a 25 min incubation at 0°C, the cell suspension was centrifuged at 150 000 $\times g$, the supernatant collected and the protein content was determined.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), in slab 10% gels. Proteins were transferred to a nitrocellulose membrane previously stabilized in TBS buffer (5 mM NaCl, 10% albumin, 1 M Tris, pH 7.6) added with 3%-decreamed skim milk. The membrane was incubated 45 min with the mouse anti-Fas antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250, followed by incubation with anti-mouse-IgG (1:1000) labeled with peroxidase. Fas expression was visualized by a final incubation with 4-Cl-α-naphtol and hydrogen peroxide. The reaction was stopped by washing with water.

3. Results and discussion

Since TMP is a hydrophobic photosensitizer, it was added to the medium in a liposomal solution of dipalmitoyl phosphatidylethanolamine and cholesterol. This technique optimizes the release of lipophilic sensitizer to low density lipoproteins (LDL). Hep-2 cells were treated with TMP (1 μM). Fig. 1 shows the behavior of TMP, in relation to its uptake by Hep-2 cells, after different periods of incubation with the sensitizer. The uptake increased with TMP concentrations up to the highest concentration studied (10 μM). In all cases, TMP incorporation was initially very rapid with short incubation times (< 5 h), and tended to reach a saturation value after long incubations (≥ 24 h). An increase of the initial uptake rate was observed with increasing TMP concentrations. However, after 24 h of incubation, a similar value for total uptake was reached. It is interesting to note that by comparison with a calibration curve obtained with standard solutions of TMP in 2% SDS ([TMP] ~ 0.5–10 μM), a value of TMP uptake ~ 0.018 μmol/10^6 cells was calculated after 24 h of incubation.

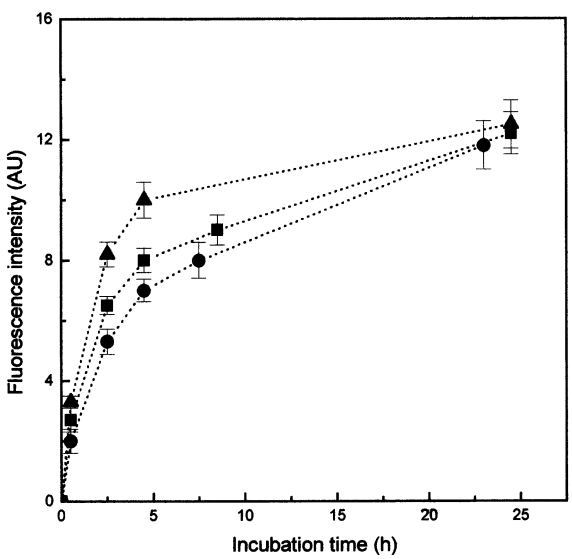

Fig. 1. Uptake of TMP into Hep-2 cells as a function of incubation time for different TMP concentrations, (●) 1 μM; (■) 5 μM; (▲) 10 μM of TMP. The amount of the drug taken up by the cells was measured by fluorescence spectroscopy ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 658$ nm). Values represent mean ± S.D. of three separate experiments.

Cell toxicity induced by TMP was examined in the dark at different concentrations of the photosensitizer and after several incubation periods. The cells were treated with 1 μM TMP for 24 h at 37°C. Cell survival was determinate by microscopy in the presence of TB at 0, 4, 8, 12 and 24 h. In all irradiation experiments, a control kept in darkness containing TMP was analyzed at each time. No toxicity, in terms of cell survival, was detected at any evaluated time. The same occurred when cell cultures not treated with TMP were irradiated under similar conditions. Therefore, the cell mortality obtained after the exposure of the cell cultures to visible light was exclusively due to the photosensitization effect of TMP produced by light irradiation. (Fig. 2).

Fig. 3 shows the results obtained with control cultures (a), incubated with TMP 1 μM for 24 h, maintained in the dark (b), illuminated for 30 min with 18 mW cm^2 (c), and stained with hematoxylin–eosin. It can be seen that the treatment with TMP followed by exposure to visible light notably increased the apoptotic figures. This result was corroborated by utilizing a specific kit (data not shown).

Fas antigen expression was investigated under three conditions (control, TMP treated cultures in

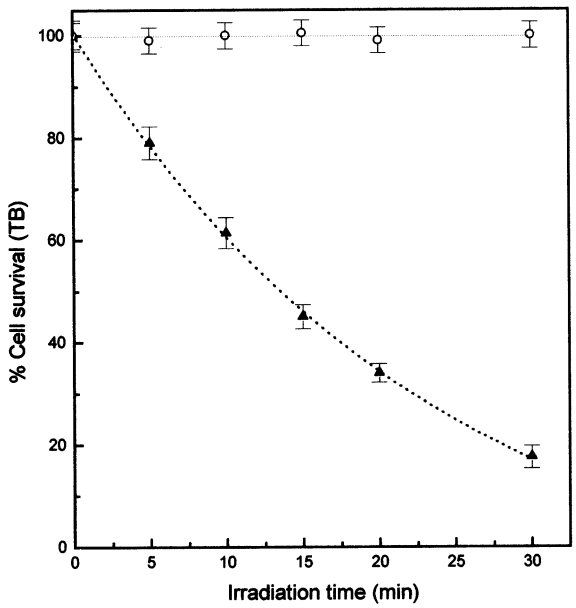

Fig. 2. Inactivation of the cells incubated with 1 μM of TMP exposed to different irradiation times with visible light. Incubation times with TMP, (▲) 24 h. Irradiated controls (○). Values represent mean ± S.D. of three separate experiments.

darkness, and illuminated). In Fig. 4 it can be seen that Fas was only expressed in the last group, when cultures were treated with the porphyrin following illumination.

Noodt et al. (1999) studied the induction of apoptosis by exposing V79 Chinese hamster fibroblasts to PDT with various porphyrins and light. They found different kinetics for the induction of DNA strand breaks characteristic of apoptotic cells. PDT-induced damage to membranes resulted in an increasing number of apoptotic cells for about 12 h after PDT.

In the present study, the uptake kinetics of TMP on Hep-2 cells was initially very fast, depending on TMP concentration. Saturation of TMP intracellular concentration was seen at ~24 h of incubation. No toxicity was observed for either cells containing TMP under dark conditions or by irradiating the cells without the photosensitizer. The survival of the irradiated cells incubated with TMP was dependent on the light dose. The kind of death produced is apoptosis, probably through the expression of Fas. These results

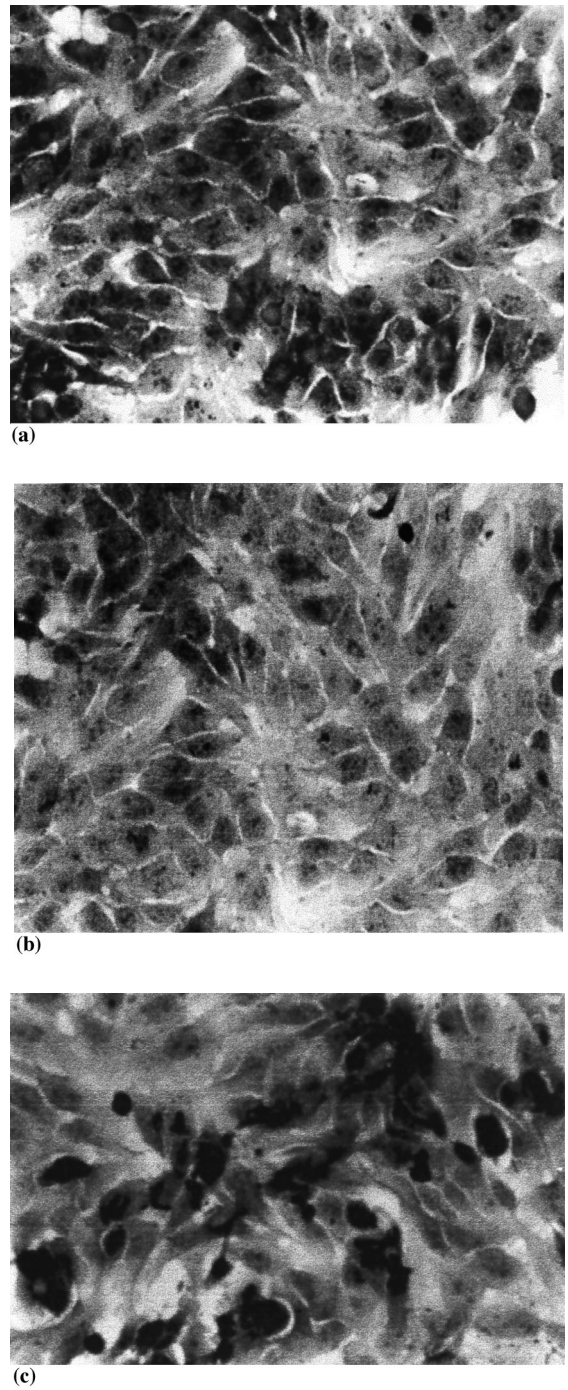

Fig. 3. Apoptotic figures as seen by the hematoxylin–eosin method in, (a) control cultures; (b) cultures incubated with TMP 1 μM for 24 h and maintained in the darkness; (c) cultures incubated with TMP 1 μM for 24 h and illuminated for 30 min with 18 mW/cm^2.

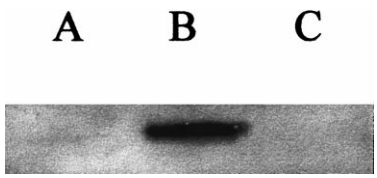

Fig. 4. Fas antigen expression in, (A) control cultures; (B) cultures incubated with TMP 1 μM for 24 h and illuminated for 30 min with 18 mW/cm^2; (C) cultures incubated with TMP 1 μM for 24 h and maintained in the darkness.

contribute to the knowledge of the PDT mechanism produced by TMP on Hep-2 carcinoma cell line.

5,10,15,20-Tetra(4-methoxyphenyl)porphyrin might be a candidate for studying its effects on laboratory animals, since it is a non-toxic molecule in the dark that causes cell death upon illumination within a short time (30 min). Photodynamic cell death is by apoptosis and not necrosis, which could trigger other reactions and inflammation. Further studies are being planned to study the effects of TMP on experimental animals.

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References

Agarwal, M., Clay, M., Harvey, E., Evans, H., Antonez, A., Oleinick, N., 1991. Photodynamic therapy induces rapid cell death by apoptosis in L5178 lymploma cells. Cancer Res. 51, 5993–5996.

Chen, J.Y., Mak, N.K., Wen, J.M., Leung, W.N., Chen, S.C., Fung, M.C., Cheung, N.H., 1998. A comparison of the photodynamic effects of temoporfin (mTHPC) and MC540 on leukemia cells: efficacy and apoptosis. Photochem. Photobiol. 68, 545–554.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., Dixit, V., 1995. FADD a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81, 501–512.

Dougherty, T.J., 1987. Photosensitization: therapy and detection of malignant tumors. Photochem. Photobiol. 45, 879–889.

Furukawa, Y., Iwase, S., Terui, Y., Kikuchi, J., Takao, S., Nakamura, M., Kitagawa, S., Kitagawa, M., 1996. Transcriptional activation of the *cdc*2 gene is associated with Fas/induced apoptosis of human hematopoietic cells. J. Biol. Chem. 271, 45, 28469–28477.

Grosseweiner, L.I., 1994. The science of phototherapy. In: Photodynamic Therapy. CRC Press, London, pp. 139–155.

Jori, G., Schindl, L., Schindl, A., Polo, L., 1996. Novel approaches towards a detailed control of the mechanism and efficiency of photosensitized process in vivo. J. Photochem. Photobiol. A: Chem. 102, 101–107.

Kremer, J.M.H., van der Esker, M.W.J., Pathmanoharan, C., Wiersema, P.H., 1977. Vesicles of variable diameter prepared by a modified injection method. Biochemistry 16, 3932–3935.

Lindsey, S.J., Schreiman, I.C., Hsu, H.C., Kearney, P.C., Marguerettaz, A.M., 1987. Rothemund and Adler–Longo reactions revisited: synthesis of tetraphenylporphyrins under equilibrium conditions. J. Org. Chem. 52, 827–836.

Luo, Y., Chang, C., Kessel, D., 1996. Rapid initiation of apoptosis bt photodynamic therapy. Photochem. Photobiol. 63, 528–534.

Milgrom, L.R., O'Neill, F., 1993. The chemistry of natural products. In: Thomson, R.H. (Ed.), Porphyrins. Blackie, London, pp. 329–376.

Noodt, B., Berg, K., Stokke, T., Peng, Q., Nesland, J., 1999. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. Br. J. Cancer 79, 72–81.

Penning, L.C., Dubbelman, T.M., 1994. Fundamentals of photodynamic therapy: cellular and biochemical aspects. Anti-Cancer Drugs 5, 139–146.