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Inactivation of vascular smooth muscle cells photosensitised by liposome-delivered Zn(II)–phthalocyanine

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Abstract

Photodynamic therapy is a promising approach for the prevention of arterial restenosis, which frequently occurs after balloon angioplasty, largely owing to abnormal proliferation of vascular smooth muscle cells (VSMC) and their migration from the media to the intima, where they originate intimal hyperplasia (IH). We investigated the efficacy of Zn(II)–phthalocyanine-photosensitised processes in promoting the inactivation of VSMC. This liposome delivered phthalocyanine is readily taken up by VSMC, largely partitions in the Golgi apparatus, and upon photoactivation causes >95% cell mortality using mild irradiation conditions (e.g. 5 min irradiation at 1 μM ZnPc). Cell death occurs through the parallel development of random necrotic and apoptotic processes.

Keywords: Intimal hyperplasia; Smooth muscle cells; Photodynamic therapy; Photosensitisation; Phthalocyanines

1. Introduction

The use of photosensitised processes to inactivate a variety of cell types has been widely studied [1] and was shown to have interesting perspectives in the medical field. In particular, the photosensitisation of cells by porphyrins and their analogues is opening the way for novel therapeutic modalities, since porphyrinoid compounds are accumulated with some selectivity in several diseased tissues and are efficiently activated by red light wavelengths, which have a deep penetration into most human tissues [2]. Recently, phthalocyanines (Pcs) have been proposed as photoactivatable agents for the prevention of arterial restenosis, which frequently occurs after balloon angioplasty [3,4]: the presently investigated protocols involve the local delivery of the phthalocyanine immediately after angioplasty followed by irradiation with laser light in the 680–700 nm range, which is specifically absorbed by Pcs. This technique is yielding promising results in preliminary clinical trials using motexafin lutetium as a phototherapeutic agent [5], however it clearly needs optimisation before a widespread clinical use is accepted.

Restenosis is often induced by the abnormal proliferation of VSMC and their migration from the media to the intima, where they originate intimal hyperplasia (IH). The incidence, timing, clinical and anatomic correlations of restenosis have been studied in depth, which led to the development of novel technologies, such as brachitherapy, drug-releasing stents, and local delivery of immunosuppressive and increased the immediate success with reduction of restenosis after percutaneous interventions. In spite of such important advances, in-stent restenosis still represents an important clinical problem, whose incidence (5–50%) calls

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for alternative or additional treatments [6,7]. In this connection, PDT could play a very useful role, by promoting early reendothelization, control of IH and matrix production [8,9].

The lipophilic ZnPc selected for our studies is a second-generation photosensitiser that is accumulated in large amounts by a variety of experimental tumours in vivo owing to its preferential transport by serum low-density lipoproteins and, after irradiation, causes regression of the neoplastic lesion by inducing both necrosis and apoptosis [10,11] according to the technique named photodynamic therapy (PDT). In vitro, low ZnPc concentrations (1 μM) photosensitise the killing of various types of transformed cells with high efficiency. Previous data showed that PDT, performed immediately after balloon injury, is also effective in reducing IH in an experimental animal model [12]. In the present paper, we investigate the mechanisms involved in ZnPc-photosensitisation of VSMC in order to obtain potentially useful information for improving the scope and potential of this therapeutic modality.

2. Materials and methods

2.1. Chemicals

The liposomal formulation of ZnPc was supplied by NOVARTIS (Basel, Switzerland) in freeze–dried form and rehydrated by addition of water immediately before use [10,13]. Rhodamine 123 (R123), NBD[C_6]ceramide and LysoSensor^{TM} used for fluorescence microscopy were obtained from Molecular Probes (Leiden, Netherlands). The ApoAlert CPP32 kit for apoptosis test was purchased from Clontech (Palo Alto, CA) and annexin-V-FLUOS for annexin staining from Roche Biochemicals (Milano).

2.2. Cell line

The immortalized rat vascular smooth muscle cell line SV40LT-SMC was used [14]. The cells were cultured in monolayer at 37 °C in a humidified atmosphere with 5% CO_2 and grown in Dulbecco's modified Minimal Essential Eagle Medium (DMEM, Sigma) containing 10% heat-inactivated Fetal Calf Serum (FCS) and supplemented with 1.5 gr/l $NaHCO_3$ and neomycin 0.2 mg/l (Sigma).

2.3. Cell uptake of Zn(II)–phthalocyanine

VSMC cells were seeded in 25 cm^2 tissue culture flasks (Falcon, Lincoln Park, NJ) at a density of 600,000 cells/cm^2; after 48 h, the medium was changed to DMEM + 3% FCS. The cells were incubated for 1 h with ZnPc concentrations in the 1–15 μM range; moreover, the accumulation of 10 μM ZnPc was studied as a function of the incubation time (0.25–8 h). The incubation was performed at 37 °C in a dark humid atmosphere containing 5% CO_2. At the end the medium was removed and the cell monolayer was washed with cold phosphate-buffer saline (PBS) containing Ca^{2+} and Mg^{2+} ions. Then, 2 ml of a 2% aqueous dispersion of sodium dodecylsulfate (SDS) were added to each flask. After magnetic stirring for 1 h, each sample was divided into two portions: 0.2 ml were stored for the determination of the protein content by the bicinchoninic acid assay [15], while the remaining volume was analysed spectrophotometrically in the 640–800 nm interval in order to measure the amount of cell-bound ZnPc. The uptake of ZnPc by cells was expressed as nanomoles of photosensitiser per milligram of protein.

2.4. Fluorescence microscopy studies

The intracellular localization of ZnPc after incubation for 0.5 and 6 h was determined with an Olympus IMT-2 fluorescence microscope equipped with a refrigerated CCD camera (−12 °C; single Peltier cooled; Micromax; Princeton Instruments). A 75-W xenon lamp was used as the excitation source. Fluorescence images obtained with a 60*x* 1.4 NA oil immersion objective (Olympus) were acquired and analysed with the imaging software Metamorph 6.1 (Universal Imaging). The cellular distribution of ZnPc fluorescence was compared with that of R123, NBD[C_6]ceramide and LysoSensor^{TM} used as markers for the mitochondria, Golgi apparatus and lysosomes, respectively. Appropriate excitation and emission cubes (Chroma Technology Corp.) were used for the fluorescent dyes: 616 ± 25/770 nm longpass for ZnPc and 475 ± 15/525 ± 25 nm longpass for R123, NBD[C_6]ceramide and LysoSensor.

2.5. Cell phototoxicity experiments

VSMC cells were seeded in Petri dishes (35 mm diameter) at a density of 300,000 cells/cm^2 and grown for 20 h in DMEM with 10% FCS. The medium was removed, replaced with DMEM + 3% FCS at the desired ZnPc concentration (0.5–10 μM), and the cells were incubated at 37 °C for 0.5 or 6 h. After incubation, the ZnPc-containing medium was removed, the cells were washed twice with 2 ml PBS containing Ca^{2+} and Mg^{2+} and irradiated in the same buffer for various periods of time with 600–700 nm light at a fluence rate of 10 mW/cm^2. The effect on cell survival of ZnPc alone and ZnPc in combination with light was evaluated by the trypan blue exclusion test [16].

2.6. Caspase-3 activity

The activity of caspase-3 was measured in photosensitised cells at various post-treatment times using the ApoAlert CPP32 kit. According to the manufacturer-recommended procedure, 10^6 cells were collected by centrifugation, resuspended in 50 μl of lysis buffer, and held for 10 min on ice. Then, 50 μl of reaction buffer containing dithiothreitol and 5 μl of Asp–Glu–Val–Asp–7-amino–4-trifluoromethyl–coumarin were added to the cell lysate and, after 1 h incubation at 37 °C, the fluorescence emitted at 505 nm ($\lambda_{ex} = 400$ nm) was measured with a Perkin–Elmer LS50

spectrofluorometer. The caspase-3 activity in the treated cells was expressed as *x*-fold increase of the emitted fluorescence, taking the fluorescence from untreated cells as reference.

2.7. Annexin staining

Typically, VSMC cells were seeded onto uncoated 24 mm diameter round glass coverslips and incubated for 20 h in growth medium. After phototreatment (30 min incubation with 1 μM ZnPc, 3 and 5 min irradiation at 10 mW/cm^2), each coverslip was incubated for 15 min at 25 °C with 0.25 ml of a solution containing 140 mM NaCl, 5 mM $CaCl_2$ and 10 mM Hepes/NaOH, pH 7.4, 2 μM propidium iodide, and annexin-V-FLUOS to a final dilution of 1:25 (v/v). Cells were washed twice with PBS before analysis. The fluorescence of annexin-V-FLUOS and propidium iodide was obtained by the Olympus IMT-2 fluorescence microscope, using excitation/emission cubes of 488 ± 15/525 ± 25 nm bandpass and 568 ± 7/585 nm longpass, respectively. Images were stored, and cells in four fields were counted and classified for each specimen.

2.8. Electron microscopy studies

At predetermined times after PDT treatment, the cells were trypsinized, collected by centrifugation, fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and post-fixed with 1% OsO_4. Dehydration was performed in a graded series of ethanol solutions followed by embedding in Epon. The cells were microsectioned, stained with uranyl acetate and lead citrate, and observed in a Hitachi H-600 transmission electron microscope.

3. Results

3.1. Cell uptake studies

The effect of ZnPc concentration on the uptake of the phthalocyanine by VSMC cells after 1 h incubation is shown in Fig. 1A. The amount of cell-bound ZnPc steadily increased with the photosensitiser concentration in the incubation medium, up to at least 10 μM. Analogoulsy, for 10 μM ZnPc, the amount of phthalocyanine accumulated by the cells increased upon prolonging the incubation time up to 8 h.

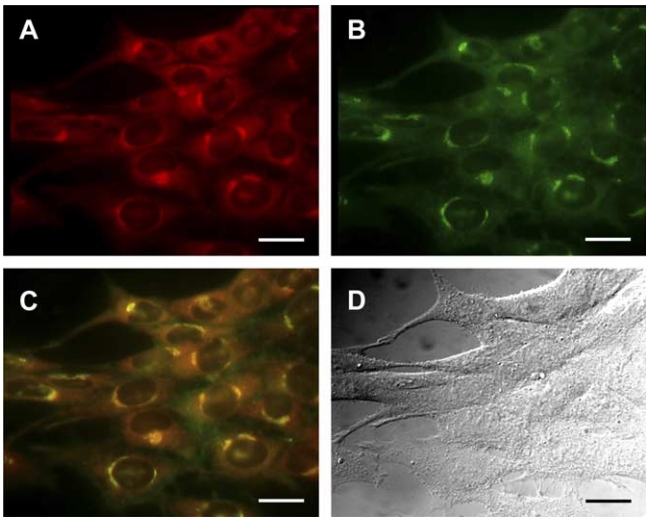

Fig. 2. Intracellular localization of 1 μM ZnPc in VSMC cells after incubation for 6 h. ZnPc fluorescence (A) is mainly co-localized with the green fluorescence of NBD [C_6]ceramide (B) as shown by the yellow fluorescence of the superimposed images (C). Bright field image (D). Bars = 20 μm.

3.2. Fluorescence microscopy studies

VSMC cells incubated for 6 h with 1 μM ZnPc exhibited the phthalocyanine-typical red fluorescence, which was mainly localized in cytoplasmic districts (Fig. 2A). The green fluorescence typical of the Golgi probe NBD[C_6]ceramide (Fig. 2B) was well superimposed with the phthalocyanine fluorescence (Fig. 2C) suggesting a colocalization of the two fluorophores. On the other hand, no overlap was detected between the ZnPc fluorescence and that emitted by the mitochondrial probe R123 or lysosomal probe

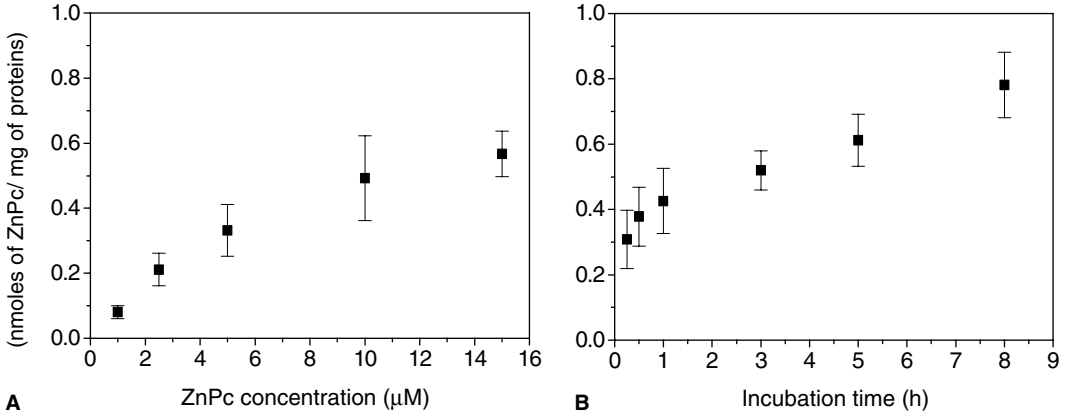

Fig. 1. Effect of ZnPc concentration on the phthalocyanine uptake by VSMC cells. The incubation time was 1 h (A). Effect of the incubation time on the uptake of 10 μM ZnPc by VSMC (B).

LysoSensor (data not shown). An essentially identical distribution pattern was observed after 0.5 h cell incubation with ZnPc.

3.3. Cell phototoxicity experiments

As one can observe in Fig. 3A, ZnPc exhibited a high phototoxicity towards VSMC after 1 h incubation: the cell survival decreased by at least 85% after 1 min irradiation. Reducing the incubation time to 30 min, ZnPc appeared to be less effective: a 20% drop in cell survival was measured after 1 min irradiation. We also evaluated the cell photosensitivity as a function of ZnPc concentration for a constant 30 min incubation (Fig. 3B). The phototoxicity decreased as the ZnPc concentration decreased in the incubation medium. In particular, the cell survival after 1 min irradiation (0.5 μM ZnPc) was similar to that found for the controls.

3.4. Studies of cell apoptosis

With the aim of understanding the mechanisms involved in cell photoinactivation, we determined the expression of caspase-3, a typical marker of apoptotic processes. Fig. 4 shows that the activity of caspase-3 was clearly stimulated by ZnPc-photosensitisation and maximal expression was measured at 2 h after the phototreatment. These data were confirmed by annexin staining. The appearance of annexin V-reactive phosphatidylserine on the membrane surface is one of the earliest events in commitment to apoptosis [17]. Our findings document that photosensitisation with ZnPc induced apoptosis at both 2 h and immediately after the phototreatment (Fig. 5E). In particular, the number of annexin-V-positive cells was slightly higher at 2 h after irradiation than at time 0, and there was no evidence of necrosis in 3 min irradiated cells (Fig. 5E); the necrotic processes became more important at longer post-irradiation times.

3.5. Electron microscopy studies

Cells dark-incubated with ZnPc showed (Fig. 6B) the typical morphology of control cells (Fig. 6A). The morphological changes occurring in VSMC cells after photosensitised 70% killing were typical of apoptotic processes, such as chromatin condensation on the nuclear envelope

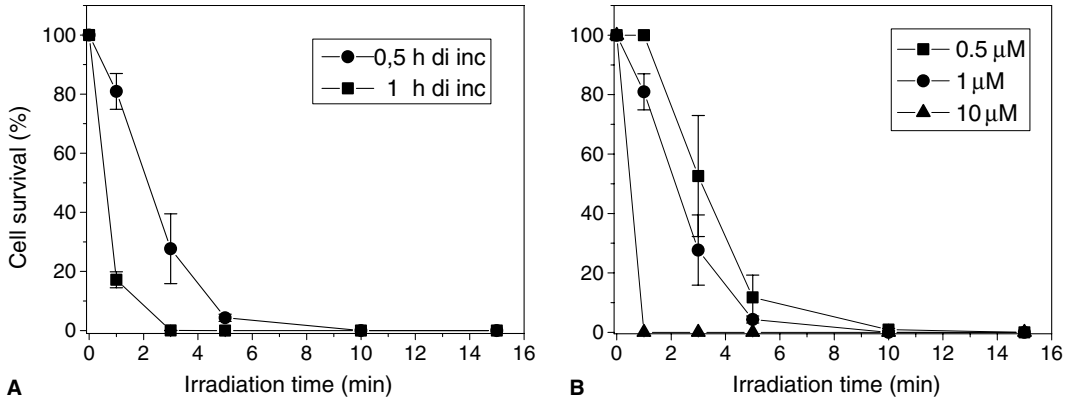

Fig. 3. (A) Effect of irradiation time (600–700 nm, 10 mW/cm^2) on the survival of VSMC cells incubated for 0.5 and 1 h with 1 μM ZnPc. (B) Effect of irradiation time (600–700 nm, 10 mW/cm^2) on the survival of VSMC cells incubated for 0.5 h with different ZnPc concentrations. Each minute of irradiation corresponds with the delivery of 0.6 J/cm^2.

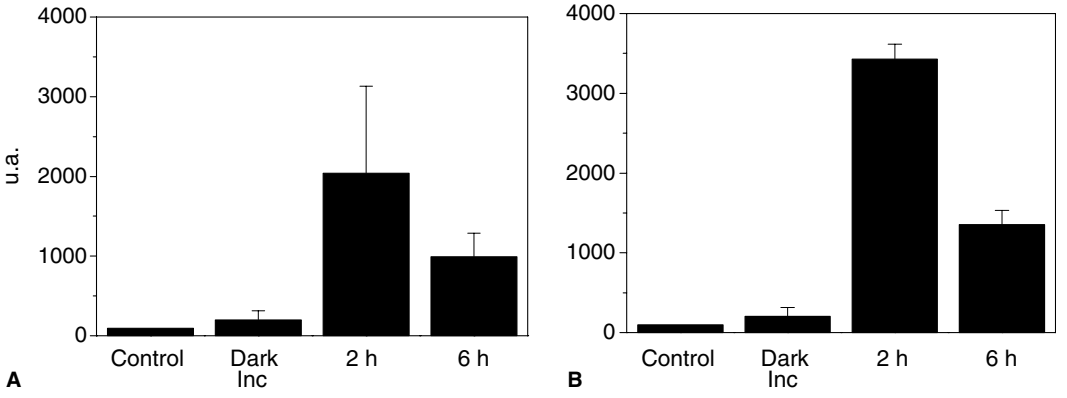

Fig. 4. Activation of caspase-3 in VSMC cells at various time periods (2 and 6 h) after photosensitisation. VSMC cells were incubated for 30 min with 1 μM ZnPc and irradiated for 3 min (A) or 5 min (B) with 600–700 nm light at a fluence-rate of 10 mW/cm^2 to reduce cell survival by 70% and 95%, respectively.

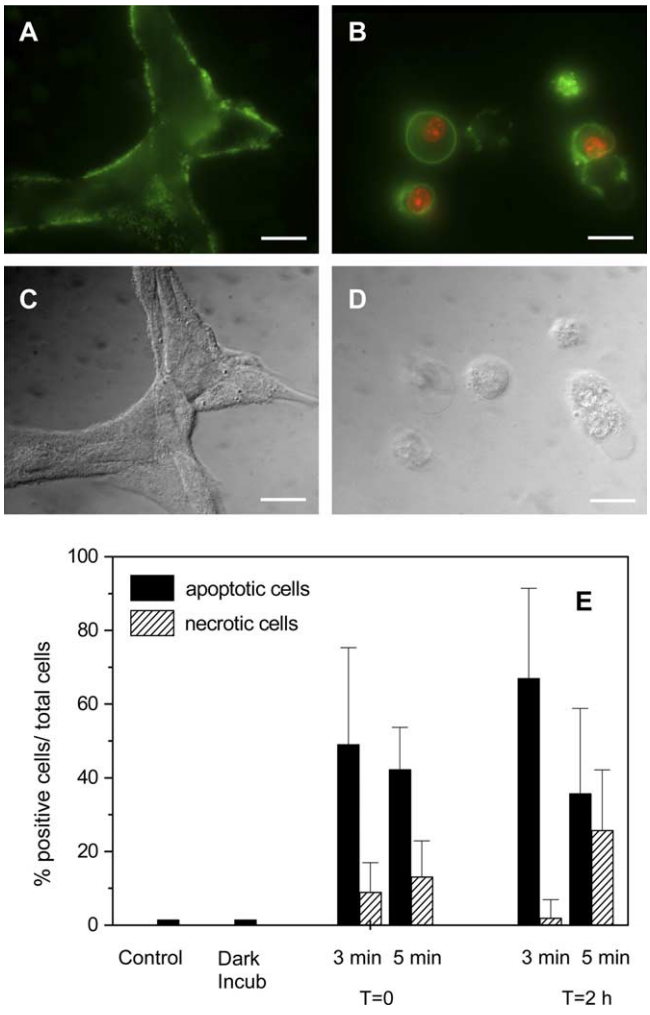

Fig. 5. Fluorescence microscopy images of VSMC cells incubated with 1 μM ZnPc for 0.5 h and irradiated for 3 min (A) or 5 min (B) with 600–700 nm light (10 mW/cm^2); (C), (D): bright field images, (Bars = 20 μM). Green fluorescence indicates apoptotic cells, while the presence of both green and red fluorescence is indicative of necrotic cells; (E) % positive cells/total cells (in each experiments the number of counted cells ranged between 15 and 27); T = time after irradiation.

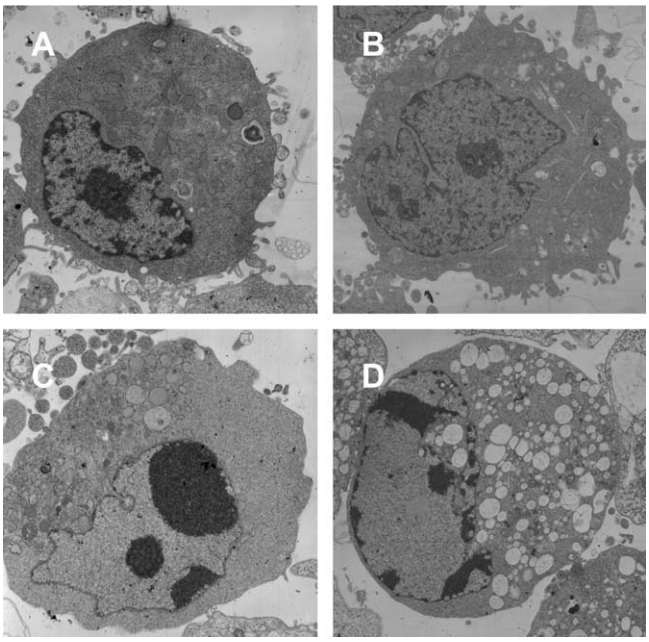

Fig. 6. Morphological alterations induced by ZnPc photosensitisation of VSMC cells. Untreated VSMC cells after trypsinization (A, 6000x). VSMC cells incubated for 30 min with 1 μM ZnPc and not irradiated (B, 5000×); irradiated for 3 min (C, 6000×); or 5 min (D, 6000×). After 3 min irradiation, cells show chromatin condensation at the periphery of the nucleus (C). At 5 min irradiation, cells show chromatin condensation, as well as a pronounced vacuolization in the cytoplasm (D).

(Fig. 6C). Cells that underwent a 95% killing, after 5 min irradiation, predominantly exhibited morphological alterations typical of necrosis, including a pronounced vacuolization of the cytoplasm and alteration of the plasma membrane, while a fraction of the damaged cells showed alterations typical of both necrotic and apoptotic processes (Fig. 6D).

4. Discussion

The susceptibility of VSMC to the action of photodynamic sensitisers has been observed in previous investigations. Thus, Chen et al. [18] showed that a Lutetium-texaphyrin promotes the efficient killing of vascular cells largely throw apoptotic processes, involving release of cytochrome *c*, DNA fragmentation and caspases activation. Similar findings were described by Granville et al [19] using Verteporphyrin as a photosensitiser, who observed a maximum expression of apoptotic events at 1–2 h after the end of photosensitisation.

The present findings clearly confirm the hypothesis that VSMC represent a likely major target during PDT treatment of arteries as a prevention of restenosis. Such cells appear to accumulate ZnPc amounts in the nmole/mg of cell protein range, which are evidently sufficient to induce an extensive cell inactivation even when a mild irradiation protocol is adopted, including a phthalocyanine concentration in the 1–10 μM range and a fluence-rate as low as 10 mW/cm^2 (see Figs. 1 and 3). These data were obtained after incubation times of the order of 30 min, which approximate the time interval elapsing between ZnPc deposition and laser irradiation in arteries subjected to angioplasty in vivo [14].

Apparently, the hyperproliferation of VSMC starts shortly after the injury [20]. Therefore it appears essential to utilize a photosensitiser which is rapidly taken up by such cells so that the application of PDT in order to inhibit cell proliferation can start at relatively short times after angioplasty. Our data suggest that PDT with locally delivered ZnPc fulfills these criteria, since this phthalocyanine appears to be endowed with a large affinity for such cells and a high phototoxic activity.

The efficiency of VSMC photoinactivation could be further enhanced by the observed subcellular distribution of liposome-delivered ZnPc. As shown in Fig. 2, the phthalocyanine is largely localized in the Golgi apparatus.

This organelle plays a critical role in cell metabolism and should be an early site of ZnPc photosensitisation owing to the high reactivity and short lifetime of the photogenerated intermediates, such as singlet oxygen or radical-type species [21]; in general, both proteins and unsaturated lipids which are present in the microenvironment of the photosensitiser are oxidatively modified at comparable rates [1,22]. This overall picture is in agreement with the development of both apoptotic and random necrotic pathways leading to cell death (Figs. 4–6). Thus, apoptotic mechanisms appear to prevail at short times after the end of irradiation, as indicated by both the annexin and caspase-3 tests, which probe different subcellular sites, in agreement with the above-mentioned observations [18,19]. The weight of necrotic processes becomes more pronounced at later times, since such processes are the consequence of a cascade of events which are usually characterised by an overall rather slow development [22]. As shown in Fig. 6D, apoptotic and necrotic processes can also take place in the same cell.

The competition between apoptosis and necrosis as a result of cell photosensitisation is modulated by a delicate balance between a variety of experimental parameters [23]. Our investigations will explore the possibility to further enhance the contribution of either pathway to VSMC death in order to define whether the occurrence of one modality is to be preferred from the point of view of efficacy, as well as of extrapolation of the protocols developed at a cellular level to in vivo situations.

5. Abbreviations

IH	Intimal hyperplasia
PDT	Photodynamic therapy
VSMC	Vascular smooth muscle cells
ZnPc	Zn(II)–phthalocyanine

References

[1] J. Moan, K. Berg, Photochemotherapy of cancer: experimental research, Photochem. Photobiol. 55 (1992) 931–948.

[2] B.C. Wilson, W.P. Jeeves, D.M. Lowe, In vivo and post-mortem measurements of the attenuation spectra of light in mammalian tissues, Photochem. Photobiol. 42 (1985) 153–162.

[3] G. Valassis, I. Pragst, C. Adolfs, D. Paclik, F. Wiens, C. Vogel-Wiens, S. Milz, P. Gonschior, Local photodynamic therapy reduces tissue hyperplasia after stenting in an experimental restenosis model, Basic Res. Cardiol. 97 (2002) 132–136.

[4] P. Gonschior, C. Vogel-Wiens, A.E. Goetz, T.Y. Huehns, F. Breger, F. Gerheuser, M. Fleuchaus, U. Welsch, R. Sroka, M. Dellian, H.A. Lehr, B. Hofling, Endovascular catheter-delivered photodynamic therapy in an experimental response to injury model, Basic Res. Cardiol. 92 (1997) 310–319.

[5] D.J. Kereiakes, A.M. Szyniszewski, D. Wahr, H.C. Herrmann, D.I. Simon, C. Rogers, P. Kramer, W. Shear, A.C. Yeung, K.A. Shunk, T.M. Chou, J. Popma, P. Fitzgerald, T.E. Carroll, D. Forer, D.C. Adelman, Phase I drug and light dose-escalation trial of motexafin lutetium and far red light activation (phototherapy) in subjects with coronary artery disease undergoing percutaneous coronary intervention and stent deployment: procedural and long-term results, Circulation 108 (2003) 1310–1315.

[6] R.O. Cannon, Restenosis after angioplasty, New England J. Med. 346 (2002) 1182–1183.

[7] A.R. Marks, Sirolimus for ther prevention of in-set restenosis in a coronary artery, New England J. Med. 349 (2003) 1307–1309.

[8] J. Heckenkamp, M. Aleksic, M. Gawenda, S. Breuer, J. Brabender, A. Mahdavi, F. Aydin, J.S. Brunkwall, Modulation of human advetitial fibroblasts function by photodynamic therapy of collagen matrix, Eur. J. Vasc. Endovasc. Surg. 28 (2004) 651–659.

[9] F. Adili, T. Scholz, M. Hille, J. Heckenkamp, S. Barth, A. Engert, T. Schmitz-Rixen, Photodynamic therapy mediated induction of accelerated re-endothelization following inury to the arterial wall: implication for the prevention of post-interventional restenosis, Eur. J. Vasc. Endovasc. Surg. 24 (2002) 166–175.

[10] K. Schieweck, H.G. Capraro, U. Isele, P. van Hoogevest, M. Ochsner, T. Maurer, E. Batt, CGP 55847 liposome-delivered zinc(II)–phthalocyanine as a phototherapeutic agent for tumors, in: G. Jori, J. Moan, W.M. Star (Eds.), Photodynamic Therapy of Cancer, Proceedings of the SPIE 2078 Bellingham, Washington, 1994, pp. 107–118.

[11] C. Zhou, D. Shunji, L. Jinsheng, G. Junlin, G. Jori, C. Milanesi, Apoptosis of mouse MS-2 fibrosarcoma cells induced by photodynamic therapy with Zn(II)–phthalocyanine, J. Photochem. Photobiol. B: Biol. 33 (1996) 219–223.

[12] Visonà, A. Angelini, S. Gobbo, A. Bonamone, G. Thiene, A. Pagnan, D. Tonello, E. Bonandini, G. Jori, Local photodynamic therapy with Zn(II)–phthalocyanine in an experimental model of intimal hyperplasia, J. Photochem. Photobiol. B: Biol. 57 (2000) 94–101.

[13] G. Valduga, G. Bianco, G. Csik, E. Reddi, L. Masiero, S. Garbisa, G. Jori, Interaction of hydro- or lipophilic phthalocyanines with cells of different metastatic potential, Biochem. Pharmacol. 51 (1996) 585–590.

[14] C.F. Reilly, Rat vascular smooth muscle cells immortalized with SV40 large T antigen posses defined smooth muscle cells characteristics including growth inhibition by heparin, J. Cell Physiol. 142 (1990) 342–351.

[15] P.K. Smith, R.I. Kron, G.T. Hermanson, A.K. Mallia, F.H. Garten, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[16] S. Rockwell, In vivo–in vitro tumour cell lines: characteristics and models for human cancer, Br. J. Cancer 41 (1980) 118–126.

[17] V.A. Fadok, D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, P.M. Henson, Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, J. Immunol. 148 (1992) 2207–2217.

[18] Z. Chen, K.W. Woodburn, C. Shi, D.C. Adelman, C. Rogers, D.I. Simon, Photodynamic therapy with motexafin lutetium induces redox-sensitive apoptosis of vascular cells, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 759–764.

[19] D.J. Granville, B.A. Cassidy, D.O. Ruehlmann, J.C. Choy, C. Brenner, G. Kroemer, C. van Breemen, P. Margaron, D.W. Hunt, B.M. McManus, Mitocondrial release of apoptosis-inducing factor and cytochrome *c* during smooth muscle cell apoptosis, Am. J. Pathol. 159 (2001) 305–311.

[20] C. Bauters, T. Meurice, M. Hamon, E. McFadden, J.M. Lablanche, M.E. Bertrand, Mechanisms and prevention of restenosis: from experimental models to clinical practice, Cardiovasc. Res. 31 (1996) 835–846.

[21] G. Jori, Photosensitised processes in vivo: proposed phototherapeutic applications, Photochem. Photobiol. 52 (1990) 439–443.

[22] K. Berg, Mechanisms of cell death in photodynamic therapy, in: H. Hönigsmann, G. Jori, A.R. Young (Eds.), The Fundamental Basis of Phototherapy, OEMF, Milano, 1996, pp. 181–207.

[23] N.L. Oleinick, R.L. Morris, I. Belichenko, The role of apoptosis in response to photodynamic therapy: what where why and how, Photochem. Photobiol. Sci. 1 (2002) 1–21.