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Hexadecylphosphocholine causes rapid cell death in canine mammary tumour cells

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Abstract

Hexadecylphosphocholine (HePC, Miltefosine) is an antitumour phospholipid and known inducer of apoptosis in human breast cancer cells. The mechanism underlying the induction of cell death by HePC, however, is not clear yet. In this study, we have investigated the cytotoxic effects of HePC on canine mammary tumour cells (CMTs) in vitro. Upon addition of HePC, CMTs rapidly exhibited several features that resembled apoptotic cell death. Cells showed externalisation of phosphatidylserine, a hallmark of apoptosis, within 5 min after addition of HePC at concentrations as low as 10 μM. Furthermore, rapid swelling of mitochondria was observed. Rounding and detachment of cells followed within 30 min. However, fragmentation of nuclear DNA could not be observed. Overall, HePC was shown to induce a type of cell death in CMTs that in some aspects resembles apoptosis, though the process proceeds much more rapidly than reported for other tumour cell lines.

Keywords: Hexadecylphosphocholine; Apoptosis; Anticancer

1. Introduction

Tumours of the mammary glands are the most frequently occurring type of tumours in both women and female dogs. Similarities in biological behaviour, histology and epidemiology have been shown to occur between these two species (Hellmen, 1992; MacEwen, 1990; Van Leeuwen et al., 1996).

Tumourigenesis in the dog appears to be dependent on the hormonal state of the animal: while dogs that have been spayed before 2 years of age hardly even develop mammary tumours, the incidence of mammary tumours in dogs spayed after this age or non-spayed dogs is extremely high (Gourley, 2000; Perez Alenza et al., 2000; Schneider et al., 1969). Both oestrogen and progesterone have been shown to play an important role during development of canine mammary tumours (Rutteman et al., 1988). While receptors for both of these hormones are expressed at relatively high levels in normal mammary tissue, levels drop in malignant tumours. Metastases of mammary tumours often even fail to express oestrogen receptors (Sheikh et al., 1994).

The absence of oestrogen receptors in tumour cells is correlated with an increased resistance against some of the more frequently used chemotherapeutic agents, like e.g. tamoxifen (Osborne, 1999). Therefore, new cytotoxic agents have been developed that act through different targets like e.g. DNA replication and lipid signalling. The latter pathway is targeted by synthetic antitumour phospholipids (APLs), like e.g. hexadecylphosphocholine (HePC; Miltefosine) and 1-*O*-octadecyl-2-*O*-methyl-*rac*-3-glycero-3-phosphocholine (ET-18-OMe; Edelfosine).

HePC and ET-18-OMe are analogues of lysophospholipids and act as antitumour agents through inhibition of invasiveness (Ruiter et al., 2001, Storme et al., 1985) and induction of apoptosis (Ruiter et al., 2001). For both

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compounds it has been proposed that they enter the cell by means of receptor-independent endocytosis rather than diffusion across the plasma membrane (Bazill and Dexter, 1990; Geilen et al., 1994; Van der Luit et al., 2003). Alternatively, evidence exists that APLs can directly be absorbed into the outer leaflet of the plasma membrane and subsequently be turned to the inner leaflet (Arthur and Bittman, 1998; Fleer et al., 1987; Fleer et al., 1993; Leroy et al., 2003).

Though its exact mode of action is still largely unknown, HePC has been shown to inhibit CTP:phosphocholine cytidylyltransferase (CCT, EC 2.7.7.15) (Wieder et al., 1993) and protein kinase C (PKC) activities (Uberall et al., 1991), whereas it activates phospholipase D (PLD) (Wieder et al., 1996). ET-18-OMe has been shown to inhibit phosphatidylinositol-specific phospholipase C (PI-PLC) (Powis et al., 1992) and CCT activities (Boggs et al., 1995). Based on their relative low toxicity towards normal tissue in comparison with their high toxicity in tumour cells, HePC and ET-18-OMe have been used in several clinical trials, amongst others during treatment of skin metastases of breast cancer (Leonard et al., 2001).

In the present study, the cytotoxic effects of HePC on canine mammary tumour cells (CMTs) have been examined in more detail. Cell morphology, nuclear DNA fragmentation, mitochondrial integrity and externalisation of plasma membrane phosphatidylserine (PS) were investigated.

2. Materials and methods

2.1. Cell line and culture

The canine mammary tumour cell line CMT-U335 originates from a spontaneous primary canine mammary osteosarcoma, as has been described previously by Hellmen (Hellmen et al., 2000). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich; Zwijndrecht, The Netherlands) containing 5% fetal bovine serum (Invitrogen; Breda, The Netherlands) in a humidified atmosphere of air containing 5% CO_2 at 37 °C. Chinese hamster ovary (CHO)-K1 cells and Madin-Darby canine kidney (MDCK) cells were cultured in DMEM containing 10% fetal bovine serum and 100-units/ml penicillin and 100 μg/ml streptomycin.

2.2. Cytotoxicity determination

To determine the cytotoxic effects of HePC on CMT-U335 cells, cell viability was measured using a tetrazolium salt (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; XTT)-based assay (Cell profileration kit II) according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). This assay is based on the conversion by metabolically active cells, but not by dead cells, of XTT to a water soluble formazan dye that can be detected by spectrophotometry. Briefly, cells were seeded in a 96-wells microtiter plate at a concentration of 1×10^3 cells/well and left to adhere in 100 μl culture medium/well for 24 h, reaching subconfluent density.

Subsequently, the cells were washed once with serum-free DMEM (unless stated otherwise) and incubated in serum-free DMEM containing various concentrations of HePC. At 30 min post addition of HePC, 50 μl of XTT labeling mixture was added to each well and incubation was continued for 2 h. Subsequently, spectrophotometrical absorbance was measured in a Benchmark microplate reader (Bio-Rad, Veenendaal, The Netherlands) at 450 nm against a reference wavelength of 655 nm.

In parallel, changes in cell morphology were documented using a Leica DM/IRBE inverted microscope (Leica Microsystems BV, Rijswijk, The Netherlands).

2.3. Statistical analysis

The data was fitted to a sigmoidal dose response curve using GraphPad Prism.

2.4. Detection of plasma membrane phosphatidylserine externalisation and NBD-sphingomyelin breakdown

CMT-U335 cells were inoculated into Delta T4 dishes (BiopTechs, Butler, PA) in DMEM medium (Sigma-Aldrich) containing 5% fetal bovine serum (Invitrogen). After 24 h, subconfluent adherent cells were washed twice with Hanks' balanced salt solution (HBSS) and incubated for 30 min in 900 μl Annexin V-FLUOS incubation buffer (Annexin V-FLUOS staining kit; Roche Applied Science, Almere, The Netherlands) containing 2 μg/ml Annexin V-FLUOS to stain PS in the outer leaflet of the plasma membrane, 0.25 μg/ml propidium iodide to determine plasma membrane integrity and 2 μg/ml Hoechst 33342 to stain nuclear DNA (Sigma-Aldrich) at 37 °C and 5% CO_2. After 30 min, the dish was mounted on a Bioptechs Delta T4 dish incubator (Bioptechs) set at 37 °C under a Radiance 2100 MP multiphoton microscope (Biorad, Veenendaal, The Netherlands). Samples were examined and at different time points, 100 μl of Annexin V-FLUOS incubation buffer was added containing 100 μM hexadecylphosphocholine (HePC; Sigma-Aldrich) to a final concentration of 10 μM HePC. Subsequently, images were taken with interval periods of 1 min. Propidium iodide fluorescence was imaged using a He/Ne laser (λ_{ex} 543 nm) and a 575–625 nm filter; Annexin V-FLUOS fluorescence was imaged using an Ar laser (λ_{ex} 488 nm) and a 500–530 nm filter; Hoechst 33342 fluorescence was detected using a Tsunami multiphoton laser (λ_{ex} 780 nm) and a 410–490 nm filter. Laser intensities were kept as low as possible to prevent possible irradiation damage to the cells.

Conversion of exogenous NBD-sphingomyelin to NBD-ceramide was determined by pre-incubation of CMT-U335

cells for 10 at 37 °C in 90 mm dishes with 5 μM NBD-C_6-sphingomyelin (Molecular Probes, Leiden, The Netherlands) in HBSS and subsequently stimulated with 10 μM HePC or vehicle (ethanol) for 30 min. Lipids were extracted, analysed and quantified essential as described (Tepper et al., 2000).

2.5. Determination of mitochondrial integrity

CMT-U335 cells were inoculated onto Delta T4 dishes as described above. After washing adherent cells with HBSS, cells were incubated in HBSS containing either 5 μM 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF-AM; as a marker to detect possible plasma membrane leakage), 200 nM Mitotracker Green FM to stain mitochondria or 2 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C and 5% CO_2. After incubation, cells were washed twice in HBSS, 1 ml of HBSS was added and cells were imaged using a Radiance 2100 MP multiphoton microscope (Biorad). BCECF, Mitotracker fluorescence was imaged using an Ar laser (λ_{ex} 488 nm) and a 500–530 nm filter.

2.6. DNA fragmentation analysis

A modification of the DNA fragmentation analysis as described by Pushkareva et al. (1999) was used. Briefly, cells were grown on 90 mm dishes to subconfluency and treated with 10 μM HePC. Control cells were treated with 1 μM staurosporine (Roche Applied Science), which is known to induce apoptosis and DNA fragmentation. At various intervals, cells were detached by scraping into the media and collected by centrifugation at 5000×*g* for 1 min. Cells were washed by resuspending them in phosphate buffered saline (PBS) (pH 7.4), subsequent pelleted by centrifugation at 5000×*g* for 1 min and resuspended in 20 μl lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.5% Na laurylsarcosinate) containing 1.25 mg/ml proteinase K (Roche) and incubated for 1 h at 50 °C. Subsequently, 1 μl (10 mg/ml) of DNAse-free RNAse A (Roche) was added and incubation was continued for 1 h at 50 °C. Reactions were terminated by addition of 10 μl loading buffer (15% Ficoll 400, 10 mM EDTA, 0.1% Orange G, 0.1% xylene cyanol) and incubation at 70 °C for 10 min. Whole samples were loaded onto 1.25% agarose gels and electrophoresed for 1.5 h at 120 V in Tris–Boric acid–EDTA buffer (TBE; 100 mM Tris, 83 mM boric acid, 1 mM EDTA pH 8.3). Gels were stained post electrophoresis with ethidium bromide (0.5 μg/ml) in TBE.

2.7. Ultrastructural analysis

CMT-U335 cells were inoculated onto 100-mm dishes and grown as described above. After 24 h, cells were washed twice with HBSS and subsequently incubated at room temperature in serum-free DMEM medium containing 10 μM HePC. Samples of treated cells were taken every minute between 0 and 15 min after addition of the HePC-containing medium by briefly washing the adherent cells with HBSS and subsequent fixation in Karnovsky's fixative.

Cells were scraped from the dishes after fixation, briefly spinned down in microcentrifuge tubes and post-fixed for 2 h with 2% osmium tetroxide buffered with 0.1 M sodium cacodylate (pH 7.2). The pelleted cells were then washed in distilled water, block stained for 1 h with 2% uranyl acetate, dehydrated in a graded acetone series and infiltrated with Durcupan epoxy resin. After polymerisation of the resin at 60 °C, 50–70 nm ultrathin sections were cut on a Reichert Ultracut S ultramicrotome and collected on 200-mesh copper grids. The sections were then stained with lead citrate and subsequently viewed and photographed using a Philips CM10 transmission electron microscope.

3. Results

3.1. HePC induces detachment of canine mammary tumour cells

Synthetic APLs like HePC have been shown to induce apoptosis in a number of different cell lines and several different mechanisms have been proposed to be involved in this process (Ruiter et al., 2001; Storme et al., 1985). The onset of apoptosis was reported to occur between 3 and 6 h after addition of the antitumour phospholipid (APL) (Ruiter et al., 1999; Van der Luit et al., 2002) and was found to be maximal at 6–36 h (Ruiter et al., 1999; Van der Luit et al., 2002; Vrablic et al., 2001), strongly dependent on the cell type used.

In order to determine whether canine mammary tumour cells are sensitive towards HePC-induced apoptosis, CMT-U335 cells were incubated in serum-free media (SFM) containing different concentrations of HePC. As shown, HePC clearly was cytotoxic to CMT-U335 cells and caused detachment of the cells in a dose-dependent manner within 30 min. As shown in Fig. 1A, 10 μM HePC had an almost maximal effect, whereas 5 μM HePC caused almost no cell detachment within an hour after addition (data not shown). CMT-U335 cell viability was determined upon addition of increasing concentrations of HePC by means of a standard XTT-assay. Data obtained thus were fitted to a sigmoidal dose–response curve (Fig. 1B) with a 50% effective dose of 8.8 μM. Loss of viability at increasing concentrations of HePC corresponded to cell detachment as expected. Furthermore, addition of a broad spectrum caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (zVAD-fmk; Promega; 20 μM) did not prevent detachment or cell death induced by 10 μM HePC (data not shown). The rapid HePC-induced cell death was relative

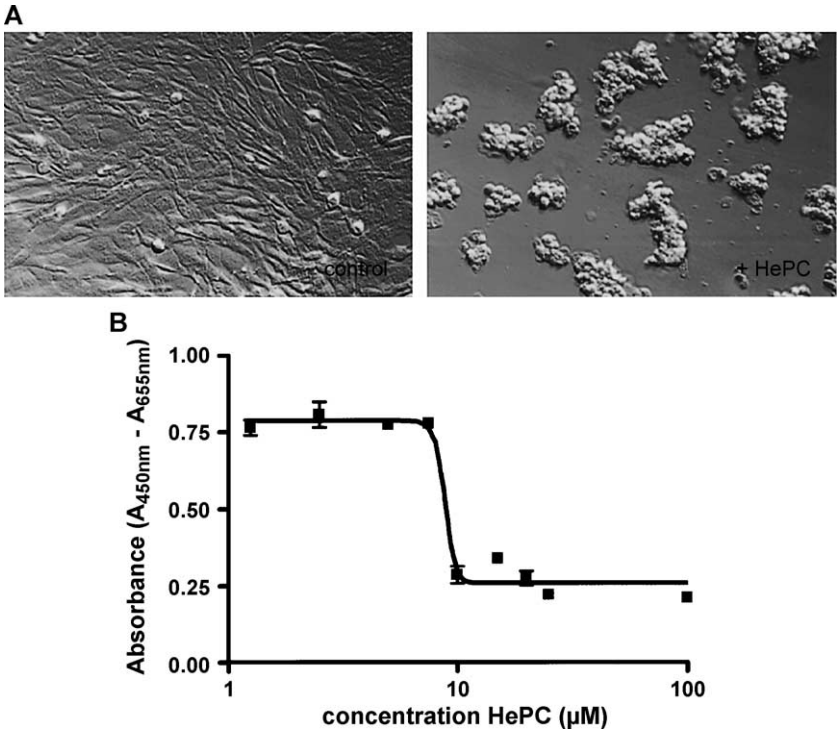

Fig. 1. (A) HePC induces rapid detachment of canine mammary tumour cells. HePC was added at a final concentration of 10 μM in DMEM to subconfluent monolayers. Subsequently, cells were monitored using a Leica DM/IRBE inverted microscope. Left panel: unstimulated CMT-U335 cells; right panel: CMT-U335 cells, stimulated for 30 min with HePC. (B) Dose–response curve for HePC in canine mammary tumour cells. HePC was added to CMT-U335 cells at increasing concentrations. Subsequently, cell viability was measured using an XTT-based assay in a microplate reader at 450 nm against a reference wavelength of 655 nm. Bars represent mean values of four individual samples ±S.D. Data were fitted to a sigmoidal dose response curve using GraphPad Prism; goodness of fit was R^2=0.96.

specific for the CMT cells, as HePC even at 25 μM did not cause detachment of hamster ovary CHO-K1 or canine kidney MDCK cells within 1 h under similar conditions (data not shown).

Because HePC is a phospholipid analogue, it can be expected that HePC will form micelles above its critical micelle concentration in aqueous solutions. Upon contact with cells, it will immediately be integrated into the cell membrane. Hence, the cytotoxic effects of HePC will be dependent on its concentration in the cell culture media in combination with the number of cells/cell density used. Therefore, from here on we used HePC at a final concentration of 10 μM and subconfluent cell density, a condition at which we observed clear effects.

3.2. CMT-U335 cells show several hallmarks of apoptosis after incubation with HePC

To identify whether morphological changes, detachment and eventual loss of viability of CMT-U335 cells upon addition of HePC were caused by induction of apoptosis or necrosis, we examined several markers for apoptosis.

First, translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane was examined by staining the cells with Annexin V-FLUOS and propidium iodide. As shown in Fig. 2, upon stimulation with HePC, the plasma membrane of CMT-U335 cells stained positively with Annexin V-FLUOS, indicating that translocation of PS had occurred. Staining could be observed as early as 3 min

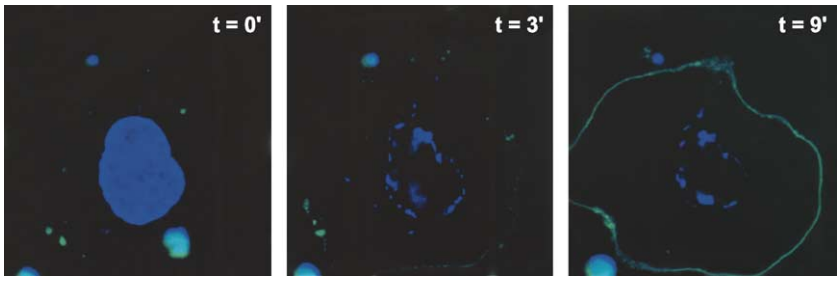

Fig. 2. HePC-stimulated CMT cells show rapid phosphatidylserine externalisation. CMTs were stained using Hoechst 33342 (blue) for nuclei, PI (red) for possible plasma membrane leakage and Annexin V-FLUOS (green) for PS. Images were taken at 0, 3 and 9 min post addition of 10 μM HePC, as indicated.

post addition (Fig. 2). No staining of nuclear DNA by propidium iodide could be observed at these time points, suggesting that the integrity of the plasma membrane was still retained.

To confirm that HePC did not cause Annexin V-FLUOS staining by disrupting plasma membrane integrity, in a second experiment cells were loaded with the cytosolic marker BCECF-AM for 30 min prior to addition of HePC. No leakage of BCECF from the cells could be observed after HePC stimulation, confirming that the plasma membrane remained intact (data not shown). Exposure of PS on the external leaflet might be caused by an activation of a scramblase. Therefore we tested the possible effect of HePC on scramblase activity by determining the formation of NBD-ceramide from exogenous NBD-sphingomyelin upon translocation of this lipid to the inner leaflet, where sphingomyelinases are located (Tepper et al., 2000). However we could not detect a rapid increase in NBD-ceramide formation upon addition of HePC (10 μM; data not shown).

Loss of mitochondrial membrane potential is a second feature that generally occurs during apoptosis. To determine the effect of HePC on mitochondrial integrity, cells were preincubated with Mitotracker Green in order to stain mitochondria. Five minutes post addition of HePC mitochondria started swelling and lost their characteristic rod-like structure (Fig. 3A), which was confirmed by electron microscopy (Fig. 3B,C).

To investigate whether all intracellular membrane structures were equally affected by HePC, cells were fixed at different time points post addition of HePC and ultrastructural analysis was performed. Four min post addition of HePC, membrane vesicles were shed from the plasma membrane and the rough endoplasmic reticulum (rER) stacks started expanding (Fig. 4A). This continued until 14 min post addition, at which time point the rough endoplasmic reticulum stacks had collapsed into vesicular structures (Fig. 4B).

No visible rupture of the nuclear membrane or plasma membrane could be seen, consistent with the earlier observation that the fluorescent marker BCECF did not leak out of the cells and propidium iodide did not stain the nuclei at this time point. However, at 14 min after addition of HePC nuclear DNA condensation could clearly be observed (Fig. 4C,D). Ultrastructurally these nuclei showed many similarities to the nuclei of other types of apoptotic cells.

DNA fragmentation is a hallmark of late apoptosis, caused by a caspase-activated DNAse (CAD) (Enari et al., 1998). However, no DNA fragmentation could be observed in HePC-treated CMT cells, whereas a characteristic “DNA ladder” pattern was seen in control CMT cells treated with

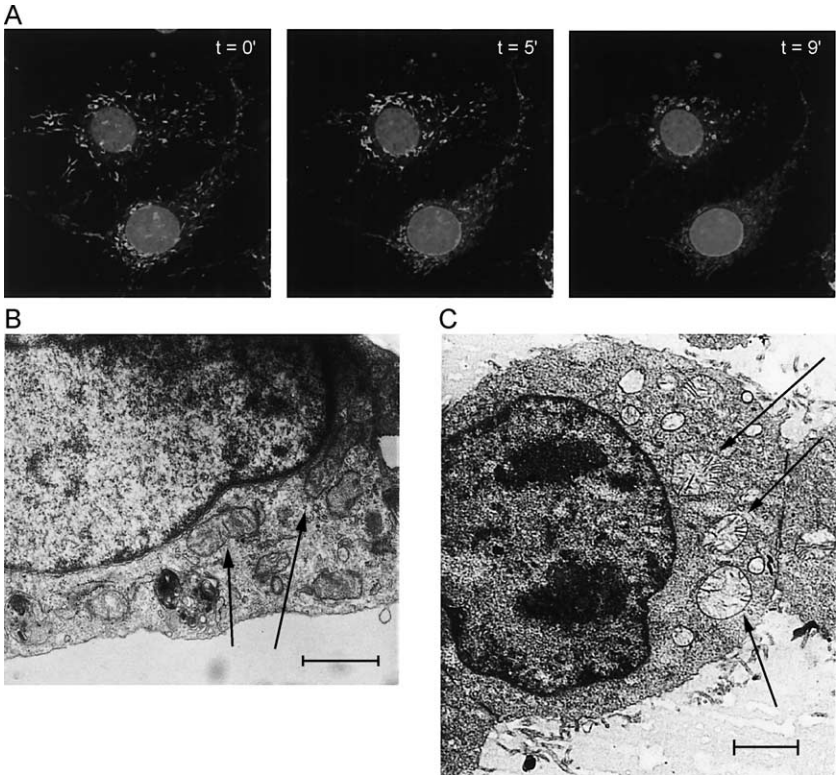

Fig. 3. Mitochondrial integrity is disrupted by HePC. (A) CMTs were stained using Mitotracker Green (green) for mitochondria and Hoechst 33342 (blue) for nuclei. Images were taken at 0, 5 and 9 min post addition of 10 μM HePC. Mitochondrial swelling was also imaged by electron microscopy. (B) CMTs at 5 min post addition of HePC. Magnification 11,500×: bar represents 1 μm; (C) CMTs at 14 min post addition of HePC. Magnification 3900×: bar represents 2 μm. Arrows indicate mitochondria.

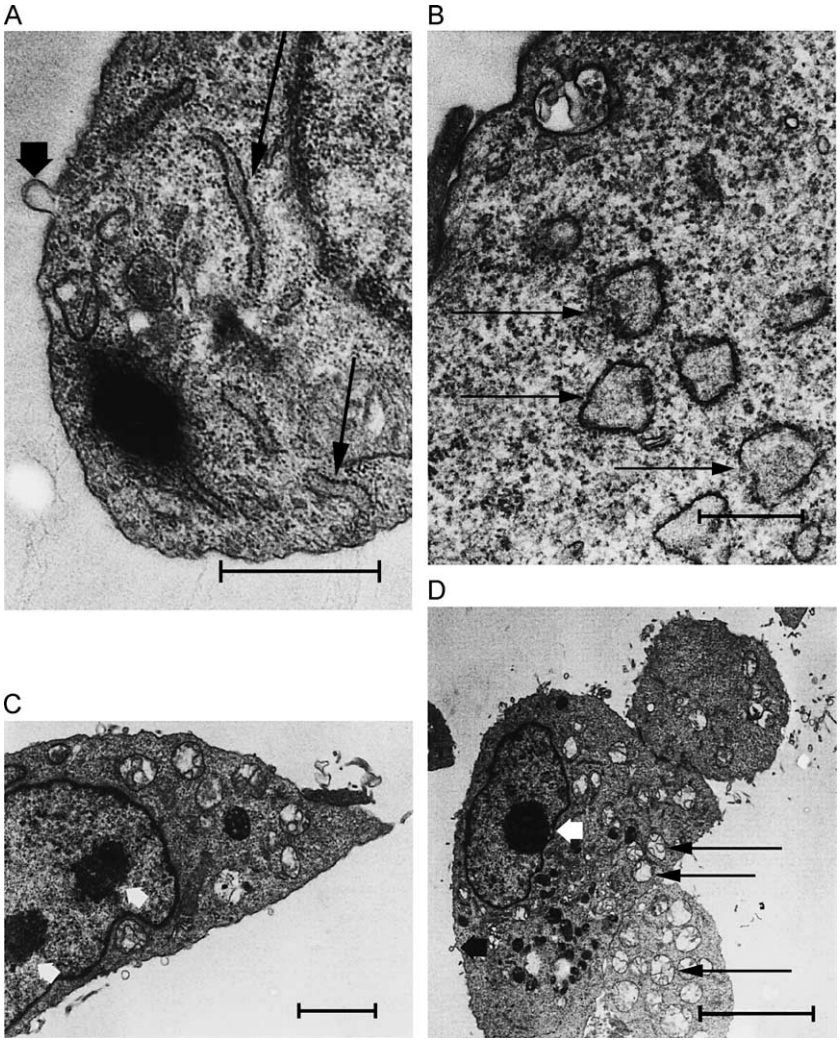

Fig. 4. HePC causes general membrane stress in CMTs. CMTs were stimulated with 10 μM HePC and at different time points samples were taken and imaged using electron microscopy. (A) Vesicles are shed from the plasma membrane (block arrow) and rough endoplasmic reticulum lumen volume increases (dotted arrows) at 4 min post addition of HePC. Magnification 15,500×: bar represents 1 μm; (B) Vesiculation of the rough endoplasmic reticulum (straight arrows; magnification 28500 x: bar represents 500 nm) and (C) nuclear DNA condensation occur (white block arrows) at 14 min post addition of HePC (magnification 5200×: bar represents 2 μm); (D) Vesicle shedding continues at 14 min post addition (black block arrow), nuclear DNA condensates (white block arrow) and mitochondrial integrity is lost (straight arrows) (magnification 2950×: bar represents 5 μm).

staurosporine (Fig. 5). This indicates that HePC rapidly induces cell death in CMT cells that shares some, but not all, characteristics of apoptosis.

4. Discussion

HePC at micromolar concentrations induces cell death in canine mammary tumour cells, a process which in some aspects resembles apoptosis.

CMT-U335 cells rapidly detach from the culture dish after addition of HePC, possibly due to modulation of cell attachment molecules. It has been described that ET-18-OMe, a related alkylphospholipid, modulates the adherens junction-E cadherin complex in human mammary carcinoma cell lines MCF-7/6 and MCF-7/AZ (Steelant et al., 2001). ET-18-OMe restored the E-cadherin mediated cell adhesion of the adhesion-deficient MCF-7/6 cells, whereas it disrupted the same type of cell adhesion in the adhesion-proficient MCF-7/AZ cells (Steelant et al., 2001). In addition to this, Leroy et al. (2003) described that epithelial tight junctions of T84 human colon carcinoma cells are reversibly opened by several different APLs, demonstrating that APLs influence several cell–cell adhesion complexes. In both of these studies HePC and ET-18-OMe were used at concentrations varying between 20 and 60 μM. Comparably, the CMT-U335 cells used in our study showed a similar sensitivity towards HePC, resulting in complete detachment of the cells within 30 min after addition of 10 μM HePC.

HePC is thought to enter the cell either by means of receptor-independent endocytosis (Bazill and Dexter, 1990; Geilen et al., 1994; Van der Luit et al., 2002) or by direct absorption into the outer leaflet of the plasma membrane and subsequent translocation to the inner leaflet (Arthur and

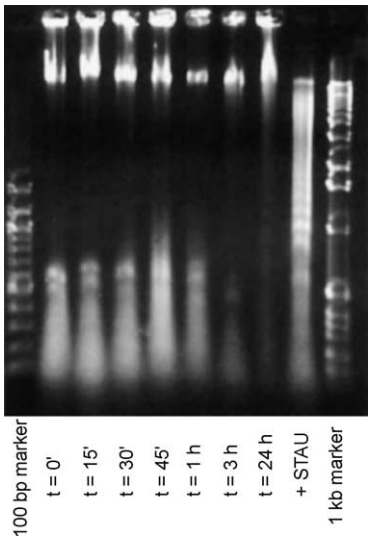

Fig. 5. HePC-induced cell death is not accompanied by DNA fragmentation. CMT cells were treated with 10 μM HePC and harvested at different time points. Total DNA was isolated and separated on a 1.2% agarose gel. Lane 1: 100 bp molecular marker; lanes 2–8: DNA isolated from HePC-treated CMT cells at 0, 15 min, 30 min, 45 min, 1 h, 3 h and 24 h, respectively; lane 9: DNA isolated from CMT cells treated with 1 μM staurosporine for 24 h; lane 10: 1 kb molecular marker.

Bittman, 1998; Fleer et al., 1987; Fleer et al., 1993; Leroy et al., 2003). Strikingly, several min after addition of HePC to CMT-U335 cells, translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane could be demonstrated by Annexin V-FLUOS staining. The PS externalization is presumably not mediated by a general activation of a scramblase since the PS exposure was not accompanied by the formation of NBD-ceramide from exogenous NBD-sphingomyelin as expected in case of scramblase activation (Tepper et al., 2000).

Shortly after PS exposure was observed, vesicles were shed from the plasma membrane. This occurred without disruption of the plasma membrane integrity, as no leakage of the fluorescent marker BCECF from the cells could be detected. This microvesiculation has been described to occur in several different cell types treated with lysoPC analogues (Culvenor et al., 1982; Kobayashi et al., 1984): also in these studies, no significant release (<5%) of lactate dehydrogenase (LDH) into the supernatant could be seen, indicating that also in these studies plasma membrane integrity was not disrupted during APL-induced vesiculation.

In contrast to the plasma and nuclear membranes, mitochondrial membrane integrity was rapidly lost upon HePC treatment. Loss of mitochondrial integrity is a common feature during induction of apoptosis and is accompanied by release of cytochrome *c* into the cytosol of the cell (Green and Reed, 1998). APLs have been shown to induce disruption of mitochondrial membrane potential and production of reactive oxygen species, thereby eventually causing the cells to go into apoptosis (Cabaner et al., 1999; Vrablic et al., 2001). Indeed, mitochondria started swelling as early as 5 min after addition of HePC and had lost their characteristic rod-like shape and cristae completely after 8 min, as can be seen frequently in different cell types during apoptosis, like e.g. dexamethasone-induced thymocyte apoptosis (Petit et al., 1995; Petit et al., 1997), though mitochondrial swelling occurs much slower in these thymocytes as compared to the CMT-U335 cells used in our study.

At 4 min post addition, rough endoplasmic reticulum stacks dilated and eventually collapsed within 10 min, while at this point in time only few clearly distinguishable mitochondria could still be observed. Overall, HePC caused massive collapse of intracellular membrane structures, eventually leading to cell death. This was preceded by translocation of PS to the outer leaflet of the plasma membrane, condensation of nuclear DNA and destabilisation of mitochondria, but without rupture of the plasma membrane, resembling an extremely rapid type of apoptotic cell death rather than necrosis.

In contrast to most types of apoptosis, the HePC-induced cell death in CMT-U335 cells appeared to be independent of caspase activation, as addition of a broad spectrum caspase inhibitor could not prevent detachment and cell death. It has been reported previously that another synthetic APL, erucylphosphocholine (ErPC), induced caspase-dependent apoptosis in glioma cells, but that addition of caspase inhibitors could not prevent cell death in these cells (Kugler et al., 2002). During the caspase-independent type of cell death, no fragmentation of nuclear DNA could be observed, corresponding to our findings on HePC-induced cell death in CMT-U335 cells. The fact that synthetic lipid analogues can induce both caspase-dependent and-independent types of cell death has also been demonstrated for synthetic ceramides (Metkar et al., 2000; Zhao et al., 2004). Also during apoptosis induced by short-term ceramide treatment, no DNA fragmentation could be seen (Zhao et al., 2004).

In vivo, HePC is used as an antitumour agent and is thought to kill tumour cells not only by direct action on the tumour cell, but also by activation of tumour cytotoxic macrophages (Eue, 2001).

Apoptotic cells are quickly eliminated in vivo by activated macrophages that recognise the apoptotic cell by exposure of phosphatidylserine (PS) on the outer leaflet of its plasma membrane (Krahling et al., 1999). As CMT cells rapidly expose PS upon treatment with HePC, it seems feasible that in this way they can be recognised by activated macrophages and subsequently killed. Thus, this mechanism could contribute to the in vivo antitumour activity of HePC.

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