Analysis of Apoptotic Effects Induced by Photodynamic Therapy in a Human Biliary Cancer Cell Line

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Abstract. *Background: Photodynamic therapy (PDT) is a relatively new approach for the treatment of biliary tract carcinoma, and its effects have not been investigated in detail to date. This study investigated the mechanisms of human biliary cancer cell death by PDT by focusing on apoptosis induction in vitro and in vivo. Materials and Methods: In vitro, NOZ cells were incubated with porfimer sodium (Photofrin®) for up to 24 hours before exposure to laser light. Cell viability was assessed using a methyltetrazolium assay after PDT. DNA fragmentation, cell cycle analysis and caspase-3 activity assay were performed to evaluate apoptotic cells induced by PDT. In vivo, DNA fragmentation was detected by TUNEL assay. Results: DNA ladder formation and activation of caspase-3 were observed within 24 hours. The proportion of cells with DNA fragmentation on flow cytometric analysis was increased significantly to 22.2% at 24 hours after PDT. In the in vivo model, TUNEL-positive cells began to increase in the implanted tumour from 6 hours after PDT, and peaked 12 hours later. Conclusion: PDT with Photofrin in this human biliary cancer cell line has antitumor effects and induces apoptotic cell death after PDT.*

Photodynamic therapy (PDT) has been described as a technically feasible method for the treatment of non-resectable hilar cholangiocarcinoma (1-5). In two randomised controlled studies, PDT provided longer term survival than bile duct stenting alone (6, 7). A possible explanation for this improved survival is the suspected anti-tumour immunological effects induced by PDT (8).

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PDT is a two-step procedure: first a photosensitiser is administered, and then the region where the photosensitiser accumulates is exposed to light of a specific wavelength, which activates the photosensitiser (9-12). PDT is based on the fact that photosensitisers are absorbed by all cells, but are selectively retained by malignant tissue (13). PDT kills tumour cells *via* apoptosis or necrosis (or both), both *in vivo* and *in vitro*. The particular mode of cell death in response to PDT depends on experimental conditions, such as the dose of PDT and the subcellular localisation of the photosensitiser (14). Furthermore, PDT-mediated apoptosis may have different mechanisms depending on the type of cells being treated, the type of photosensitiser being used, the light delivery protocols employed, and the time lag between the photosensitiser and light treatment (9, 15).

For biliary tract carcinoma, the apoptotic effect of PDT has not been investigated to date *in vitro*. This study analysed the apoptotic effect of PDT on a human gall bladder cancer cell line, NOZ, using Photofrin®, (Quadra Logic Technologies, Vancouver, BC, Canada), a widely used photosensitiser.

Materials and Methods

In vitro study of photosensitiser properties.

Cell culture. NOZ cells, human biliary cancer cell line (JCRB1033: Japanese Collection of Research Bioresources, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM: Nissui Centical Co. Ltd., Tokyo, Japan) with 10% foetal bovine serum, glutamine (0.6 mg/ml), penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Photodynamic treatment. NOZ cells were exposed to the specified levels of Photofrin® (Lederle Japan Co. Ltd., Tokyo, Japan) for 24 hours and were irradiated with an Nd:YAG pumped dye laser (wavelength: 630 nm, energy density range: 4.0 to 16.0 J/cm^2) (Quanta-Ray DCR-3 and PDL-2, Spectra Physics, Mountain View, CA, USA).

Assay of cell viability. The effects of PDT on cell viability in NOZ cells were investigated with methyltetrazolium (MTT; 3[4,5-dimethyl-thiazoyl-2-yl]2,5-diphenyl-tetrazolium bromide; Sigma, St.

Louis, MO, USA). Cells were irradiated in 96-well microplates for 24 hours, and 10 μl of MTT solution (5 mg MTT per 1 ml phosphate-buffered saline [PBS]) were added to each well, followed by incubation for 4 hours. Finally, 100 μl acid-isopropanol were added to each well to solubilise the MTT-formazan. After complete solubilisation of the dye by vortexing the plate, absorbance was read on an Immuno reader (Immuno reader NJ-2000; Nihon Inter Med, Tokyo, Japan) at 570 nm.

Staining for apoptosis. Cells were stained with Hoechst 33342 (Sigma) dye 24 hours after PDT in order to detect chromatin condensation and fragmentation, as a marker for apoptosis under 50% lethal dose (LD_{50}) conditions. Numbers of apoptotic nuclei per field of 100 cells were determined in triplicate.

DNA fragmentation assay. Immediately after PDT, cells were incubated at 37˚C for 24 hours. Cells were collected by centrifugation and DNA was isolated from 1×10^6 cells for each group. After harvesting, cell samples were washed with PBS and centrifuged. Cell pellets were then treated with lysis buffer (0.2% Triton X-100 in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4). To separate fragmented DNA from intact chromatin, samples were centrifuged at 20,000 ×g for 10 minutes, and supernatants were treated with 5 M NaCl in order to remove histones from the DNA. Genomic and apoptotic DNA was precipitated in isopropanol overnight at –20˚C. After centrifugation, DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA), and then loaded onto a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml). Electrophoresis was performed in Tris-acetate/EDTA electrophoresis buffer until the marker dye had migrated 4-5 cm. Gels were examined and photographed under UV light.

Measurement of cellular DNA content. Twenty-four hours after PDT, 10^6 cells from each sample were washed in PBS and the pellet was fixed in ice-cold 70% ethanol at –20˚C. The cell suspension was then centrifuged, washed twice with 1 ml of PBS and resuspended in 1 ml of a PBS solution containing ribonuclease (1 mg/ml) and propidium iodide (PI) (100 mg/ml). The cellular orange fluorescence of PI was detected in a linear scale using a flow cytometer (FACSCanto™ II; BD Bioscience, Mountain View, CA, USA) equipped with an excitation laser line at 488-633 nm. At least 20,000 events were collected for each sample.

Caspase-3 activity assay. Twenty-four hours after treatment, cells were washed twice with PBS, and lysed in a cell lysis buffer (1% Triton-X 100, 1 mM dithiothreitol, 50 mM KCl, 5 mM EGTA, 20 μM cytochalasin B, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml antipain, 3 μg/ml chymostatin, 10 mM HEPES, pH 7.5). Cell lysates were then diluted with caspase assay buffer and Ac-DEVD-pNA substrate (CaspACE Assay System, Promega, WI, U.S.A.) and were incubated at 37˚C for 4 hours. Optical density was measured at 405 nm (Immuno Reader NJ-2000, Nihon Inter Med, Tokyo, Japan).

Animal experiments.

Tumour xenograft. A total of 10^7 NOZ cells were transplanted subcutaneously into the dorsum of 4-week-old nude mice (n=24, BALB/cANcrj *nu/nu*, Charles River Inc., Japan). Tumours that grew to approximately 8×8 mm by approximately 21 days after transplantation (n=24) were used as experimental models.

PDT protocol. Photofrin® (Quadra Logic Technologies, Vancouver, BC, Canada) and red light emitted from the pulsed Nd:YAG dye laser (Quanta-Ray DCR-3 and PDL-2; Spectra Physics, Mountain View, CA, USA) as the photosensitiser and light source, respectively, were used for PDT. The laser was tuned to a wavelength of 630 nm, which was verified with a spectrometric multi-channel analyser (SMA Systems, Tokyo Instruments, Tokyo, Japan), at a frequency of 10 Hz. A power meter (30 A-P; Ophir Optics, Jerusalem, Israel) was used to measure light intensity. The experimental animals received intraperitoneal injection of Photofrin (7 mg/kg body weight) 24 hours before laser irradiation, which was performed with pulses of energy density 10 mJ/cm^2 each for 10 minutes under general anaesthesia using pentobarbital sodium (40 mg/kg body weight) injected intraperitoneally. Each tumour received a total energy fluence of 60 J/cm^2. In the tumour-bearing mouse group (n=24), PDT was administered to the transplanted tumour grown in the dorsum, and four mice in each group were sacrificed at 0, 3, 6, 12 and 24 hours after PDT. Neither the photosensitiser nor laser irradiation was used on control animals (n=4).

TUNEL assay. After excision, tumours were fixed in 3.7% neutral buffered formalin for 24 hours. This was followed by processing for routine paraffin-embedded 4-μm sections for each specimen. Prior to the labeling reaction, samples were deparaffinised by xylene and ethanol. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed using an Apoptosis Detection Kit (Wako, Osaka, Japan) in accordance with the manufacturer's instructions. Slides were analysed under a light microscope. TUNEL-positive nuclei (intensely brown stained) were counted in 3 randomly selected microscopic fields (×400, related to a field size of 0.08 mm^2) per slide with necrotic areas, and were expressed as a percentage of the total nuclei counted. At least 1,000 nuclei were counted on each slide.

Statistical analysis. All assays were set up in triplicate, and the results were expressed as mean±SD. Statistical significance was determined by one-way factorial ANOVA and multiple comparison tests using the statistical package STAT VIEW (Abacus Concepts Inc., Berkeley, CA, USA, 1998). *P*-values of less than 0.05 were considered to be statistically significant.

Results

Viability of NOZ cells after PDT. Viability was determined by MTT assay at 24 hours after PDT. Antitumour activity by PDT against NOZ cells was clearly seen with 10 μg/ml Photofrin. Cell death was induced in a light dose-dependent manner by PDT. In order to induce LD_{50} PDT conditions, a Photofrin concentration of about 10 μg/ml and a laser power 12 J/cm^2 was required for NOZ (Figure 1). Induction of apoptosis in NOZ cells after PDT was investigated by staining the cells with Hoechst 33342 at 24 hours after treatment under LD50 conditions. Apoptotic cells were observed in 19.03% (Figure 2).

DNA fragmentation, cell cycle analysis and caspase-3 activity assay. The DNA ladder formation that is characteristic of apoptosis was seen at 24 hours after

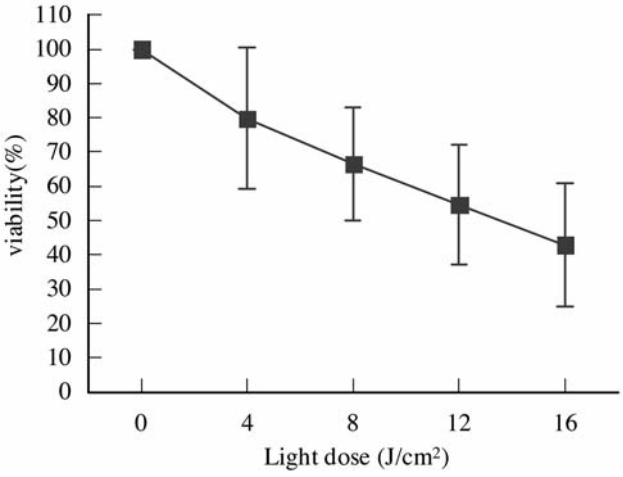

Figure 1. *Cell death was induced in a light dose-dependent manner by PDT. Percentage of surviving NOZ cells after treatment with PDT is shown. Viability was determined by MTT assay at 24 hours after PDT. Values are expressed as the mean±SD and represent the average of three independent experiments.*

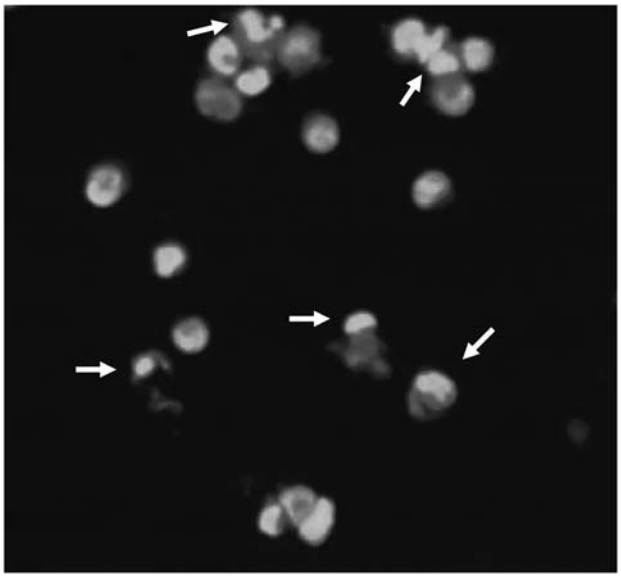

Figure 2. *Fluorescence microscopy of cells stained with Hoechst33342 at 24 hours after PDT (LD_{50} conditions). Arrows show apoptotic cells (original magnification ×100).*

treatment under LD_{50} conditions in NOZ cells. No evidence of DNA ladder formation was seen in untreated controls, or with Photofrin or laser irradiation alone (Figure 3). Frequency distribution histograms of DNA content of PI-stained cells (Figure 4). All treatments showed a population of cells in sub-G_1 phase, corresponding to apoptotic cells. The percentage of sub-G_1 phase cells for PDT was found to be 22.2%, as compared to 3.0% for controls, 2.2% for Photofrin alone and 2.7% for laser alone. Induction of caspase-3 activity was assayed using the specific tetrapeptide substrate Ac-DEVD-pNA. Activation of caspase-3 was also seen at 24 h after PDT under LD50 conditions (Figure 5). There were significant differences between the control and PDT group ($p<0.05$).

Induction of apoptosis in animal model. For induction of apoptosis in the xenograft model, TUNEL-positive cells were distinguished by brown-stained nuclei (Figure 6). They began to increase gradually in the implanted tumour from 6 hours after PDT and peaked 12 hours later (Figure 7). There were significant differences between the control and experimental groups at 6, 12 and 24 hours after PDT ($p<0.01$ at 6, 12 and 24 hours).

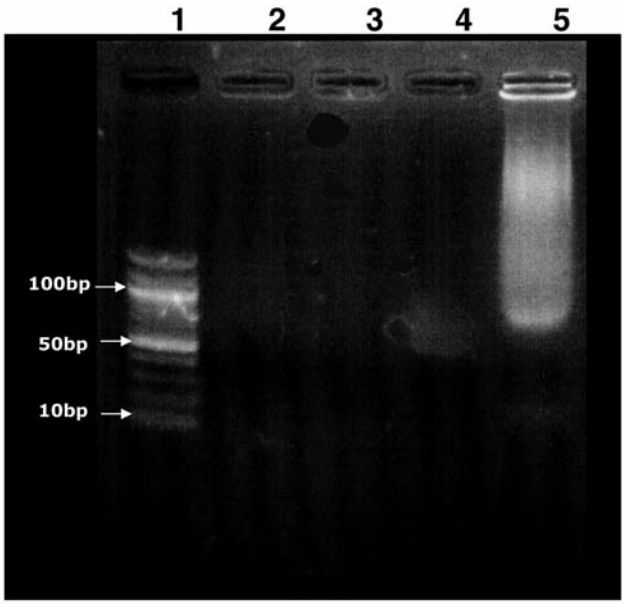

Figure 3. *DNA ladder formation at 24 hours after PDT (LD50 conditions). Column 1: molecular weight markers (100 bp); column 2: control (no treatment); column 3: laser (12 J/cm²) alone; column 4: Photofrin (10 μg/ml) alone; column 5: PDT (10 μg/ml, 12 J/cm²).*

Discussion

PDT has been described as a useful procedure for local ablation for the treatment of non-resectable cholangiocarcinoma (6, 7), for which there are few effective treatment options, even though it is a refractory disease, and some cases are inoperative when first detected. Therefore, PDT appears to have the following merits in the treatment of bile duct cancer: i) in patients with inoperable hilar cholangiocarcinoma, PDT following endoscopic bile duct dilation can achieve long-term stent patency and improve survival (2); ii) when cancer cells remain in the resection

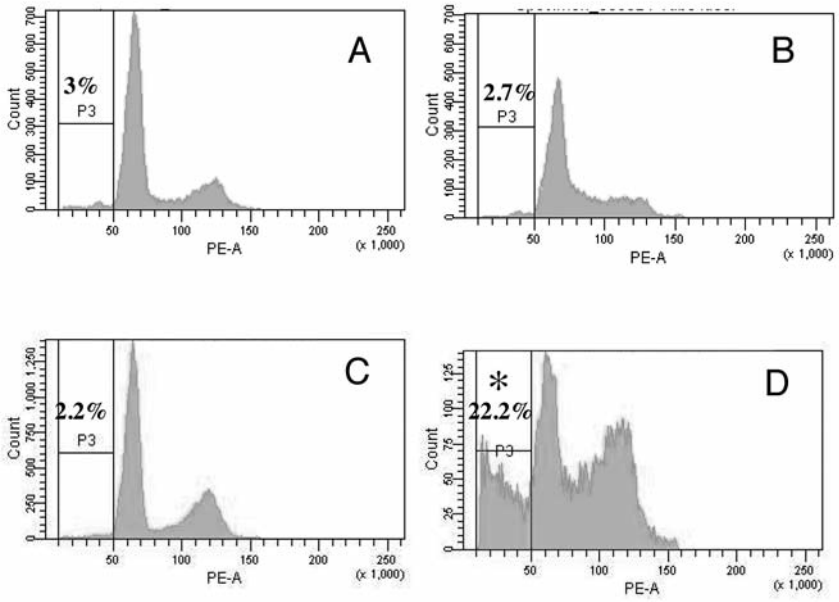

Figure 4. *Frequency distribution histograms of DNA content of PI-stained cells. (A) control (no treatment); (B) laser (12 J/cm^2) alone; (C) Photofrin (10 μg/ml) only; (D) PDT (10 μg/ml, 12 J/cm^2). *p<0.01 Compared to control. In each histogram the vertical lines enclose the sub-G_1 phase cells, corresponding to apoptotic cells and their percentage is also given.*

stump postoperatively, PDT can be performed as a postoperative adjunct therapy (16); and iii) in cases where cholangiocarcinoma has broadly advanced into the bile duct and resection is difficult, preoperative PDT may allow resection (17). Practically, PDT has been proposed as an innovative approach for neoadjuvant or palliative treatment of non-resectable cholangiocarcinoma (6, 16-18) and recent results from clinical studies have shown an increased survival time (3, 6, 16, 17, 19) and quality of life (3-6, 19, 20) in patients with non-resectable cholangiocarcinoma treated with PDT. However, most of the studies have been performed in the setting of palliation.

Based on these results, a possible explanation for the improved survival is the suspected antitumour effect induced by PDT. Earlier studies have reported that PDT induces apoptosis in cancer cells (9,14,15). Nonetheless, there are few reports on induction of apoptosis by PDT in biliary cancer. Compared with necrosis induction, apoptosis induction is advantageous because it leads to a more rapid cancer cell death, and inflammatory symptoms are less likely to occur. Therefore, the present study examined the effectiveness of PDT and the induction of apoptosis in a biliary cancer cell line.

It was found that PDT induced cell death in NOZ cells in a light dose-dependent manner at nontoxic Photofrin concentrations (10 μg/ml). The experiments revealed evidence of condensed choromatin or apoptotic bodies among NOZ cells on Hoechst 33342 staining, and DNA ladder formation was evident on agarose gel electrophoresis at 24 hours after PDT. Furthermore, this study clarified that PDT activated caspase-3 in NOZ cells. PDT with Photofrin apparently led to induction of mitochondrial cytochrome *c* release and initiation of the

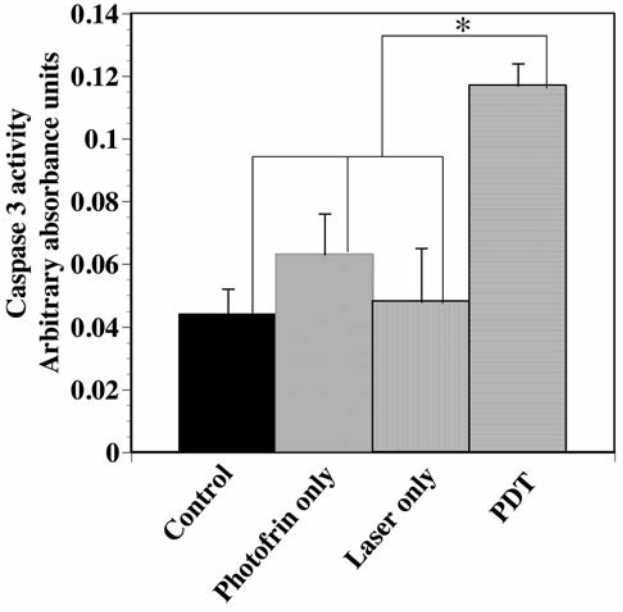

Figure 5. *Caspase-3 activity of cell extract at 24 hours after PDT assayed using the specific tetrapeptide substrate, Ac-DEVD-pNA. Values are expressed as the mean optical density (arbitrary absorbance units) ±SD and the averages of three separate experiments. *p<0.05 Compared to control.*

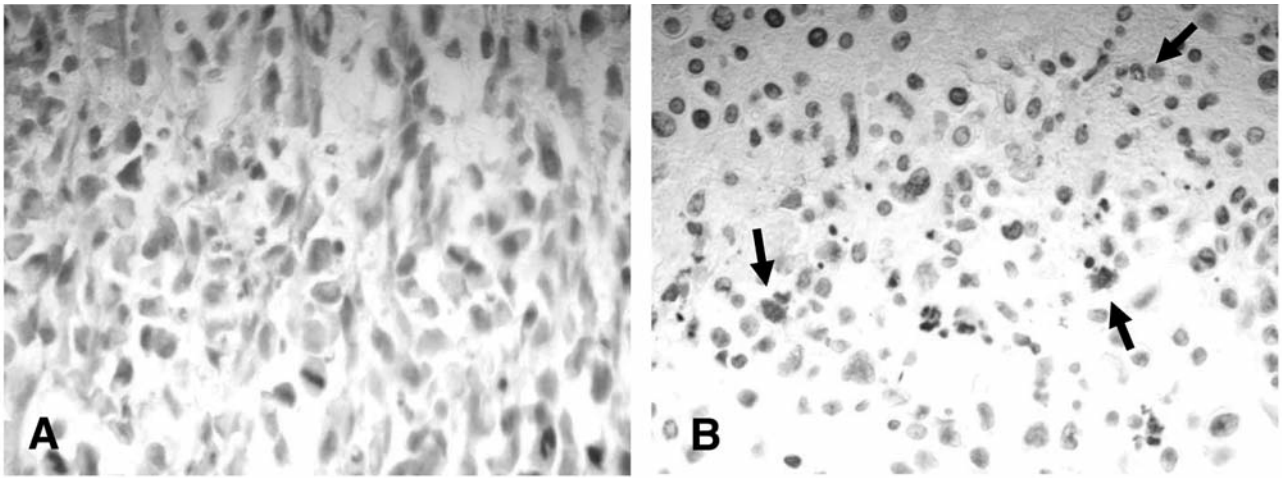

Figure 6. *Induction of apoptosis by PDT. TUNEL-positive cells increased at 6 hours after PDT. (A) control (no treatment); (B) 6 hours after PDT. Arrows show TUNEL-positive cells.*

apoptotic cascade. Following cytochrome *c* release into the cytosol, activation of caspases 9 and 3 has been described for several cell types treated with PDT, and caspase-3 is known to damage DNA (21-24).

The rate of apoptosis induced varies in each cancer cell type. For example, by using 5-aminolevulinic acid as a photosensitiser and V79 cells as a target tissue, 80% cell death by apoptosis is seen; in contrast, only necrosis is seen when the WiDr cell line is used as target tissue (25). In the present study, 19.03% apoptotic cells were observed with Hoechst 33342 staining and the percentage of sub-G_1 phase cells was 22.2% on measurement of cellular DNA contents at 24 hours after treatment under LD50 conditions. However, early apoptosis, at 3, 6 and 12 hours after PDT, was not seen *in vitro*.

In contrast, in the *in vivo* xenograft model, TUNEL-positive cells were significantly elevated, even at 6 hours after laser irradiation, and they peaked at 12 hours. This suggests that a marked induction of apoptosis occurred from 6 to 12 hours after irradiation. The mechanism for tumour responses to PDT *in vivo* has been studied in the literature. Direct tumour cell death, vascular damage and inflammatory responses were all shown to contribute to tumour destruction. A large proportion of Photofrin-mediated PDT damage to tumours *in vivo* was shown to be related to vascular damage (26, 27). Hypoxia has been shown to lead to apoptosis through a variety of mechanisms, including changes in p53 protein levels and caspase activation associated with cytochrome *c* release from mitochondria (28-30). The present study suggested that an increase in early apoptosis at 6 hours after PDT *in vivo* may have been due to the triggering of apoptotic pathways by vascular damage, and that apoptotic cells appearing at 24 hours *in vitro* result in direct tumour cell death.

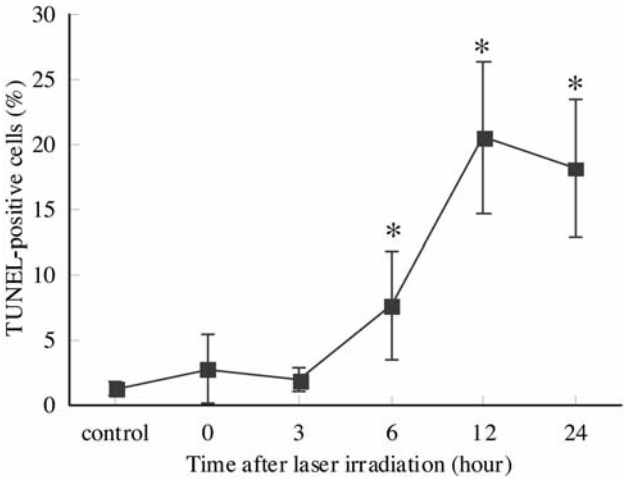

Figure 7. *Histological apoptosis induced by PDT. TUNEL-positive cells increased markedly at 6 hours after PDT and peaked at 72 hours after PDT. Positive cells were observed continuously at 72 hours after PDT. Bars show SD; *p<0.01 compared to control.*

In conclusion, the present study confirmed that PDT can induce apoptotic cell death more rapidly *via* direct and indirect mechanisms, as seen in the clinical settings of PDT treatment. Thus, PDT is a reasonable treatment in patients with hilar cholangiocarcinoma as an adjuvant and neoadjuvant therapy.

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