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Research Article

Hypericin photoactivation triggers down-regulation of matrix metalloproteinase-9 expression in well-differentiated human nasopharyngeal cancer cells

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Abstract. Recently, we have shown that hypericin-mediated photodynamic therapy (PDT) is a promising modality for the treatment of nasopharyngeal cancer (NPC). The present study evaluated the expression of matrix metalloproteinase-9 (MMP-9) following hypericin-PDT in well-differentiated HK1 NPC cells. Down-regulation of MMP-9 by hypericin-PDT was observed at the mRNA level in HK1 cells *in vitro* and *in vivo* and at the protein level *in vitro*. Transcriptional activities of the activator protein-1 (AP-1) and nuclear factor (NF)-κB regulatory elements were inhibited by PDT. We also found that PDT reduced secreted granulocyte-macrophage colony stimulating factor (GM-CSF), which is known to activate transcription of NK-κB and AP-1. However, incubation of untreated HK1 cells with exogenous GM-CSF abrogated the reduction of MMP-9 production in hypericin-PDT-treated cells. It would appear that PDT down-regulates MMP-9 expression via inhibition of GM-CSF production, which in turn modulates AP1/ NF-κB transcriptional activities. Suppression of MMP-9 by hypericin-PDT may have therapeutic implications.

Keywords. Hypericin, photodynamic therapy, nasopharyngeal cancer, matrix metalloproteinase-9, nuclear factor-κB, activator protein-1.

Introduction

Hypericin, a perylenequinone, with known antiviral activity [1, 2] is a natural photosensitizer found in the *Hypericum* species of herbaceous plants. Fluorescence produced by photosensitizers upon activation by light has been utilized to discriminate between normal and malignant cells in bladder cancer [3]. The three photosynthesizers currently used for bladder cancer detection (photodynamic diagnosis) are 5-aminolevulinic acid [4], hexaminolevulinate [5] and hypericin [6]. Hypericin-based fluorescence diagnosis of bladder carcinoma has been found to be superior

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over the other two photosynthesizers in terms of specificity and sensitivity [3].

When a photosensitizer is activated with light of a specific wavelength that matches its absorption spectrum, high-energy oxygen species (*i.e.*, singlet oxygen and oxygen free radicals) are generated, resulting in tissue injury and cell death. This is the principle behind photodynamic therapy (PDT), a promising modality for the treatment of cancer. PDT has been tested in clinical trials for a wide range of malignancies [7, 8]. Photofrin is presently the most commonly used photosynthesizer. However, Photofrin has been reported to have limitations such as low selectivity for tumor tissues and long-lasting cutaneous photosensitivity [8]. Hence, there is a continual search for potent and effective photosynthesizers with fewer side effects. One potential candidate is hypericin, which has been used for the treatment of several types of tumors, such as recurrent mesothelioma [9], and basal cell and squamous cell carcinoma [10]. Recently, our laboratory has reported the efficacy of hypericin-mediated PDT in nasopharyngeal cancer (NPC) *in vitro* and *in vivo* [11–14].

NPC, a tumor with multifactorial etiology, has a high potential for invasion and metastasis [15]. Early primary nasopharyngeal tumors are frequently accompanied by cervical lymph node involvement [16]. It is well established that besides genetic and environmental factors, NPC is also strongly associated with Epstein Barr virus (EBV) infection. The EBV oncoprotein, LMP1 (latent membrane protein 1), has been shown to influence matrix metalloproteinase-9 (MMP-9) expression [17, 18]. MMPs are known to degrade the basement membrane and extracellular matrix [19], and are therefore regarded as crucial elements for tumor progression [20]. There is a growing body of evidence to suggest that MMPs play central roles in cancer cell invasion, migration, neovascularization and tumorigenesis. Type IV collagen is a major basement membrane component that functions as an important barrier against tumor metastasis, and expression of type IV collagenases (MMP-9 and MMP-2) has been closely correlated with metastasis [21]. Hence, LMP-1 may contribute to invasiveness and metastasis via the induction of MMP-9 transcription and enzymatic activity [22]. In the same manner, MMPs would be suitable molecular targets for the development of cancer treatment strategies.

The aim of the present work was to evaluate the expression of MMP-9 following hypericin-PDT in well-differentiated human NPC cells *in vitro* and *in vivo*, and shed light on the possible mechanism for the regulation of MMP-9 following PDT. We show that down-regulation of MMP-9 expression by hypericin-PDT is associated with inhibition of granulocyte-macrophage colony-stimulating factor (GM-CSF) production and, consequently, modulation of downstream events such as those affecting the transcriptional activities of nuclear factor (NF)-κB and activator protein-1 (AP-1).

Materials and methods

Materials. Hypericin (Molecular probes, Eugene, OR, USA) was prepared as a stock concentration of 1 mg/ml hypericin in dimethyl sulfoxide and stored at –20°C in the dark. The chemical was further diluted in fetal bovine serum (FBS)-free culture medium immediately before use. Hypnorm was purchased from Janssen Pharmaceutica (Geel, Belgium) and dormicum from David Bull Laboratories (Scottsdale, AZ, USA).

Cell culture. Well-differentiated HK1 NPC cells were established from an NPC patient diagnosed with recurring well-differentiated squamous cancer at the original site after radiotherapy [23]. This cell line was kindly provided by Professor K. M. Hui, National Cancer Center, Singapore. The HK1 cells were cultured in RPMI 1640 medium with 10% FBS at 37°C as previously described [12]. Normal human lung fibroblasts (NHLF) were purchased from Clonetics-Whittaker (MD, USA) and cultured in Fibroblast Basal Medium (BioWhittaker, MD, USA) supplemented with 2% FBS, human growth factor-B (1 μg/ml), insulin (5 mg/ml) and gentamicin (50 mg/ml) according to the supplier's instructions.

Tumor model. HK1 tumors were generated by injecting 1.5×10^6 cells subcutaneously into the lower right flanks of BALB/c nude mice of 6–8 weeks of age [11]. All procedures for *in vivo* experimentation were approved by the Institutional Ethics Committee.

Cell photosensitization. Cells were incubated with 0.25 μM or 0.5 μM hypericin for 4 h in subdued light conditions prior to illumination. A wide-band illumination with wavelength above 585 nm was carried out using a bank of fluorescence tubes (Philips Type OSRAM L30w11 – 860, 30 W) filtered with a red acetate filter (No. 17 Roscolux, Rosco, CA, USA). The resulting light output within the wavelength of 585–620 nm was 12% of the total delivered light dose. HK1 cells were treated with light dose of 0.5 J/cm^2 as calculated by the formula: light dose (J/cm^2) = fluence rate (W/cm^2) / irradiation time (s). A drug dose and light dose response analysis was initially conducted, and, under the conditions described above, 0.25 μM hypericin produced 50% cell death and 0.5 μM hypericin induced 70% cell death (data not shown). PDT doses inducing higher cytotoxicity instead of sublethal doses were used in this study so as to attain therapeutic efficacy.

***In vivo* PDT treatment protocol.** Hypericin, 2 mg/kg, prepared in 0.1 ml phosphate-buffered saline (PBS) was injected through the tail veins of tumor-bearing mice. After hypericin administration, the animals were anaesthetized by intraperitoneal injection of a 90-μl cocktail of hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml), dormicum (5 mg/ml) and deionized water (1:1:2). The tumors were subsequently illuminated after a drug-light interval of 4 h with a light beam emitted by a 360 W halogen lamp (Osram, Mexico) that was filtered by Red acetate filter (No. 17, Roscolux) to give a wide-band illumination above 585 nm. A cold mirror (No. 42414, Edmund Scientific, NJ, USA) was placed in front of the lamp to remove the infrared components of the spectral output. A 14-inch Fresnel lens (Edmund Scientific) was used to focus the light to a spot of 1-cm diameter on the tumors. The resulting light output within the wavelength of 585–620 nm was 12% of the total delivered light dose. A final light dose of 14 J/cm^2 and fluence rate of 27 mW/cm^2 was used. We have previously shown that under these conditions, hypericin-PDT induced tumor necrosis and significant regression of subcutaneously implanted tumors in the mice [11].

Detection of EBV transcript by *in situ* hybridization. Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS at pH 7.2 and 5% acetic acid for 5 min at room temperature. After rinsing twice in PBS with 0.5% Tween-20 for 5 min, the coverslips

were dehydrated and air dried. Subsequently, 20 µl of a commercially available fluorescein-labeled EBV probe cocktail (BD PharMingen, CA, USA) was applied on the coverslips and incubated for 1 h in a humidified chamber at 45°C. The. stained cells were examined under a confocal laser scanning microscope (LSM 510, Karl Zeiss, Esslingen, Germany) equipped with a Plan-Neofluar 63× oil immerse lens. The 488-nm line of an Argon/Krypton (Ar/Kr) laser was used. Green fluorescence was detected using the channel with a FITC filter. NHLF cells were taken as control cells.

Western blot analysis. Cells were harvested at selected time points after PDT treatment. Whole cell extracts were prepared by lysing the cell pellets in 150 µl lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 µM Tris at pH 7.4) at 95°C for 5 min. After centrifugation, the supernatants were stored at –20°C until use. The cell lysate was aliquoted to contain 50 µg protein and mixed with sample buffer (0.5 M Tris pH 6.8, 20% glycerol, 10% SDS and 0.2% bromophenol blue). The samples were loaded on a 12% polyacrylamide gel after being heated at 95°C for 4 min. Following electrophoresis, the protein was transferred to a nitrocellulose membrane. After incubation with 0.5% non-fat milk in Tris-buffered saline to block nonspecific binding, the membrane was incubated with primary antibody against human MMP-9 (1:1000, Oncogene, MA, USA) or β-actin (1:5000, Sigma, MO, USA) at 4°C overnight. It was reprobed with rabbit anti-mouse IgG conjugated with horseradish peroxidase at 1:1000 for 1 h at room temperature. Detection was carried out by enhanced chemiluminescence followed by densitometric analysis.

Real-time PCR. Total RNA was extracted from 3×10^6 cells with the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA was synthesized from 2 µg DNA-free total RNA with M-MLV reverse transcriptase and oligo(dT)15 primer. A 1:10 final dilution of the reaction was used as the template for PCR analysis. Real-time PCR was performed with the LightCycler-Faststart DNA Master SYBR Green I kit (Roche, ID, USA). The primer sequences for MMP-9 were: ccctggagacctgagaacca (forward) and cccgagtgtaaccatagcgg (reverse) (Gene accession no. J05070). The housekeeping gene was glyceraladehyde-3-phosphate dehydrogenase (G3PDH). PCR was carried out with 0.5 µM primer with 3 mM $MgCl_2$ at annealing temperature of 58°C (for G3PDH) or 60°C (for MMP-9); 45 cycles were performed followed by melting curve analysis to verify the specificity of the amplicon. The $2^{-\Delta\Delta CT}$ method for quantification of mRNA was used in analyzing the data [24].

Immunofluorescence. Cells grown on cover slips were fixed with 4% paraformaldehyde. After preincubation with 0.2% Triton X in PBS for 10 min, the cells were incubated with 2% bovine serum albumin (BSA) to block nonspecific binding. The cells were then incubated with primary mouse anti-human MMP-9 antibody (Oncogene Research Products, MA, USA) diluted to 1:200 in PBS for 2 h at room temperature followed by incubation with secondary FITC-conjugated anti-mouse IgG1 (Oncogene Research Products) at 1:200 for 1 h at room temperature. Cells incubated in PBS without primary antibody were taken as controls. The immunofluorescence was examined using a Confocal laser scanning microscope equipped with a Plan-Neofluar 20× lens. The 48-nm line of an Ar/Kr laser was used.. Green fluorescence was detected using the channel with a FITC filter.

Determination of active MMP-9. The amount of active MMP-9 in the cell culture supernatant was measured by a commercially available human active MMP-9 fluorescence assay kit (R&D Systems, MN, USA). The fluorescence signals were measured with a fluorescence plate reader with excitation wavelength set to 320 nm and emission wavelength set to 405 nm. The amount of active MMP-9 was determined by a standard curve plotted by relative fluorescence unit (RFU) against known concentrations of MMP-9. Total amount of MMP-9 (including the pro-enzyme and active enzyme) and endogenous active enzyme in the samples can be differentiated by adding 4-aminophenylmercuric acetate (APMA), which cleaves the 92-kDa MMP-9 proenzyme to produce a 82-kDa active MMP-9 or a C-terminal truncated form of 65 kDa with activity comparable to the 82-kDa form [25]. APMA has been found to directly react with the sulfhydryl group of the cysteine residue in the pro-peptide domain, resulting in its displacement from the active site [26].

Cytokine analysis. The concentration of GM-CSF in culture medium was measured using a commercially available ELISA kit (R&D Systems). The optical density was measured at 450 nm with a correction wavelength set at 540 nm or 570 nm.

Evaluation of MMP-9 expression with exogenous GM-CSF. Cells (5×10^6) in serum-free medium were plated in 10-cm petri dishes and incubated overnight. After hypericin-PDT, cells were incubated for 24 h in the presence or absence of human recombinant GM-CSF (R&D Systems) at a final concentration of 10 ng/ml. The conditioned media (supernatant) were collected and centrifuged at 300 *g* for 10 min to remove cell debris and analyzed for MMP-9.

Luciferase reporter assay. The luciferase assay is designed to measure the transcriptional activity of AP-1 and NF-κB. Cells were seeded in 60-mm petri dishes 24 h before transfection in FBS and antibiotic-free RPMI1640 culture medium. pAP-1-Luc (Clontech, CA, USA), p-NF-κB-Luc (Clontech) or the control counterpart vector was co-transfected with the β-galactosidase reporter plasmid (Clontech) using Lipofectamine™ 2000 (Invitrogen, CA, USA). At 4 h after transfection, cells were rinsed and supplemented with fresh medium containing 10% FBS. Following incubation for 36 h, the untreated or hypericin-PDT treated cells were harvested 6 h after treatment. The transfection efficiency was normalized by β-galactosidase activity measured with a β-galactosidase enzyme assay system (Promega, WI, USA). The reporter gene activity was determined by a commercially available luciferase assay system (Promega) using a LUMI-ONE luminometer (Trans Orchid Inc, FL, USA).

Statistical analysis. Results are expressed as mean ± SEM. The significance of differences were evaluated by Student's *t*-test and One-way Analysis of Variance (ANOVA) followed by post-hoc Dunnett test.

Results

Detection of EBV in HK1 cells. In view of the association of EBV with MMP-9 expression, the EBV status in HK1 cells was first ascertained. EBV-encoded RNA 1 (EBER1) transcript was detected by a cocktail containing a specific fluorescein-labeled probe. The existence of the EBER1 transcript is indicated by the green fluorescence in HK1 cells (Fig. 1a) as opposed to the absence of fluorescence in the NHLF cells, which served as the negative controls (Fig. 1b), thus providing evidence of the EBV genome in HK1 NPC cells.

MMP-9 expression following exposure to hypericin-PDT. Western blot analysis revealed the presence of MMP-9 proenzyme in HK1 cells (Fig. 2a). A time-dependent reduction of this protein was observed post hypericin-PDT (Fig. 2b; $p=0.001$). This was confirmed by the immunofluorescence study, which showed dampening of green fluorescence (representing MMP-9 expression) in HK1 cells as compared with pretreated cells (Fig. 3).

The total amount (including both pro-enzyme and active forms) of MMP-9 in the cell culture supernatant was reduced by hypericin-PDT in HK1 cells (Fig. 4a; $p=0.014$) compared with pretreated cells. As the latent

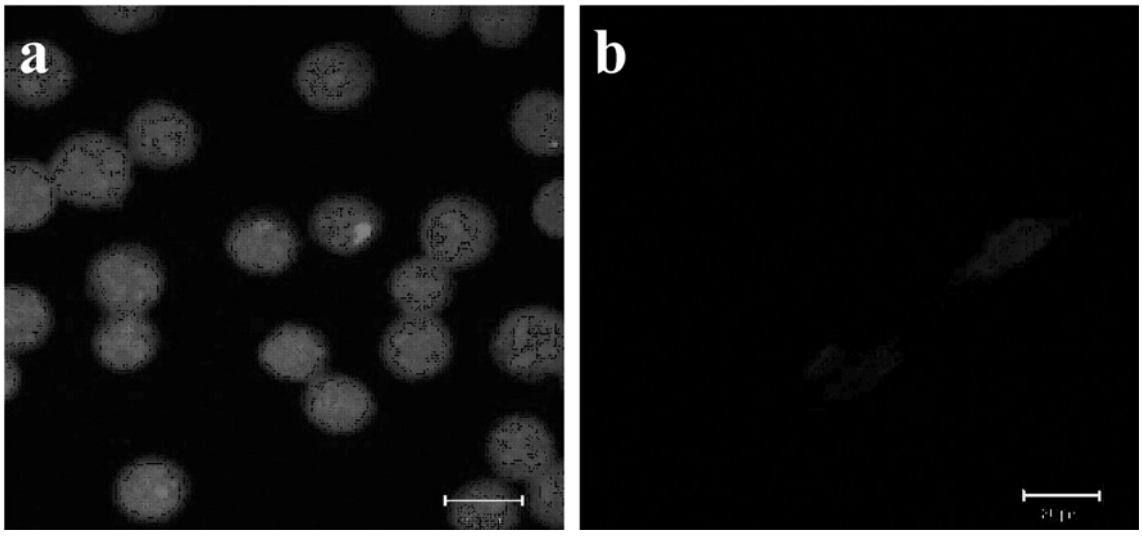

Figure 1. EBV-encoded RNA 1 (EBER1) *in situ* hybridization in nasopharyngeal cancer (NPC) cells. (*a*) Presence of highly abundant and non-translated EBER1 was detected by a fluorescence-labeled EBV probe cocktail via *in situ* hybridization, demonstrating a positive identification of the EBV genome in HK1 cells. (*b*) No hybridization was found in NHLF cells. Bar=20 μM. Result is representative of two independent experiments.

precursor of MMP-9 has to be activated to the active form before it is able to degrade the substrate [27], it is also essential to measure the amount of active MMP-9 in the cell culture supernatant. To detect the amount of MMP-9 proenzyme in the cell culture supernatant, APMA was added to the samples, which cleaves the pro-peptide domain to produce the active enzyme. It can be seen that the active MMP-9 comprised only 3 % of total MMP-9, inferring that the majority of MMP-9 is in the pro-enzyme form (Fig. 4b).

Down-regulation of MMP-9 transcription by hypericin-PDT was also observed in HK1 cells as the mRNA was reduced by 76 % at 1 h post PDT (Fig. 5a; p=0.0047). Although the relative expression of the MMP-9 transcripts at 2, 4 and 6 h appeared to be higher than untreated cells, the results were not statistically different because of the large SEM especially at 4 h post PDT ($p>$ 0.05). Similarly, MMP-9 transcription was also observed to be down-regulated in the HK1 tumor tissues (Fig. 5b). MMP-9 mRNA levels in PDT-treated tumors declined to 0.48-fold as compared to the control at 2 h post PDT (p=0.0094) and further decreased to 0.21-fold at 12 h after treatment (p=0.0094). The MMP-9 mRNA level appeared to rise to about 2.6-fold at 18 h but the result was not statistically significant ($p>$0.05) before declining to 0.29-fold at 24 h post PDT (p=0.029). Verification of the specificity of the MMP-9 RT-PCR product is shown in Figure 6.

Evaluation of NF-κB and AP-1 transcriptional activity after hypericin-PDT. Since the promoter region of the MMP-9 gene contains AP-1 and NF-κB regulatory elements, we determined if the transcriptional activities of these two transcription factors were modified by PDT treatment. Figure 7 shows that NF-κB and AP-1 transcriptional activities in HK1 cells were both inhibited by more than 50 % with hypericin-PDT ($p<$0.01).

Effect of hypericin-PDT on the production of GM-CSF. As GM-CSF is a cytokine, which has been shown to modulate MMP-9 expression, we evaluated its involvement in the regulation of MMP-9 by hypericin-PDT. Secreted GM-CSF produced by HK1 cells was decreased by 25–30 % following PDT (Fig. 8; p=0.0012 and p=0.0082 for 0.25 μM and 0.5 μM hypericin, respectively). It can be seen that there was a decline in GM-CSF production up to 4 h post PDT with 0.5 μM hypericin having a more pronounced effect on the production of this cytokine than a dose of 0.25 μM hypericin in HK1 cells.

Effect of exogenous GM-CSF on MMP-9 expression. The amount of endogenously active MMP-9 (which was determined without the addition of APMA) was shown to be reduced post treatment (Fig. 9; p=0.030). However, down-regulation of MMP-9 was abrogated when PDT-treated cells were incubated with GM-CSF (p=0.023). When untreated HK1 cells were incubated with GM-CSF, the amount of active MMP-9 in culture medium was shown to be enhanced, increasing from 1.302±0.045 ng/mg protein before treatment to 1.453±0.022 ng/mg after hypericin-PDT (p=0.032).

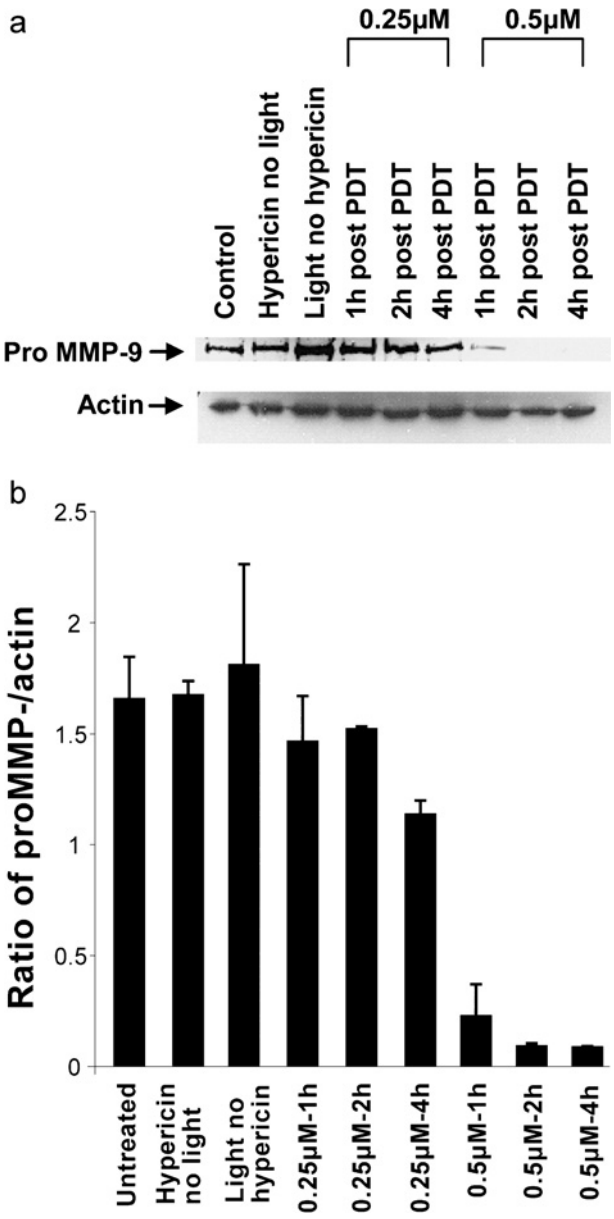

Figure 2. Matrix metalloproteinase-9 (MMP-9) protein expression in photodynamic therapy (PDT)-treated HK1 cells. (*a*) Hypericin-PDT down-regulates MMP-9 expression in HK1 cells. HK1 cells were incubated with 0.25 or 0.5 μM hypericin for 4 h followed by light irradiation (585–620 nm) at a dose of 0.5 J/cm^2. Cell lysates were prepared at 1–4 h post PDT and analyzed for MMP-9 levels by Western blot analysis as detailed in 'Materials and methods'. (*b*) Densitometric analysis of the MMP-9 protein bands was performed on two independent experiments. Values are means ± SEM.

Discussion

In the present study, existence of the EBV genome was confirmed in HK1 NPC cells by EBER1 *in situ* hybridization. HK1 cells were first reported to be EBV negative as the presence of EBNA1 (nuclear antigen 1 of EBV) was not demonstrated in cultured cells [23]. However, the same investigators also noted that the results in touch smears from biopsy tissue were inconclusive. In general, EBNA1 is expressed only at low levels and its epitopes are vulnerable to common fixatives, whereas EBERs are well preserved in conventional fixatives [28]. This could explain the discrepancy in the positive results in HK1 cells demonstrated in the present study as compared with that of Huang et al. [23]. Recent findings have also indicated that EBV could persist for a long time in NPC cells established from tumor biopsies as evidenced by the partial integration of EB viral LMP1 in the chromosomal DNA observed in several types of NPC cell lines including CNE-2 cells and HK1 cells as demonstrated by *in situ* hybridization [29]. Tsai and co-workers [30] have also reported that detection of EBER1 by *in situ* hybridization is more sensitive and specific than PCR methods.

As we have shown that HK1 cells are EBV positive, constitutive expression of the MMP-9 transcript and protein in the HK1 cells is in accord with previous reports that the EBV oncoprotein LMP1 is associated with MMP-9 expression [17]. We have also observed that hypericin photoactivation induced down-regulation of MMP-9 production in HK1 cells (both at the mRNA level and protein levels) and MMP-9 transcription in the NPC/HK1 mouse tumor model. At the mRNA level, the decrease in MMP-9 expression in HK1 cells *in vitro* was especially obvious at 1 h post PDT and generally so in tumor tissues except at 18 h post PDT (which had higher values than the control tissues but was not statistically significant because of the large SEM). Down-regulation of MMP-9 in HK1 cells following PDT, as shown in this study, is opposite to the finding that photofrin-mediated PDT induced the expression and activation of MMP-9 in the mouse C3H/BA mammary tumor model [31]. However, it must also be emphasized that the BA mammary tumor cells in the above study did not secrete any detectable amount of MMP-9 nor induce MMP-9 production upon PDT treatment. The modulatory effect on MMP-9 was rather the result of MMP-9 induction in endothelial cells and an influx of MMP-9-expressing inflammatory host cells.

It should also be noted that the cancer cell line used in our present study express MMP-9 endogenously, and this enabled effective analysis of the inhibitory effect of PDT. There have been two very recent reports that appear to support our finding that PDT reduces MMP-9 production. Au et al. [32] observed that PDT using photofrin, 5-aminolevulinic acid and calphostin C reduced migration of glioma cells and partially attributed the phenomenon to reduction in MMP-9 production. Salih and co-workers [33] have shown that PDT suppressed MMP-9 production in keratinocyte

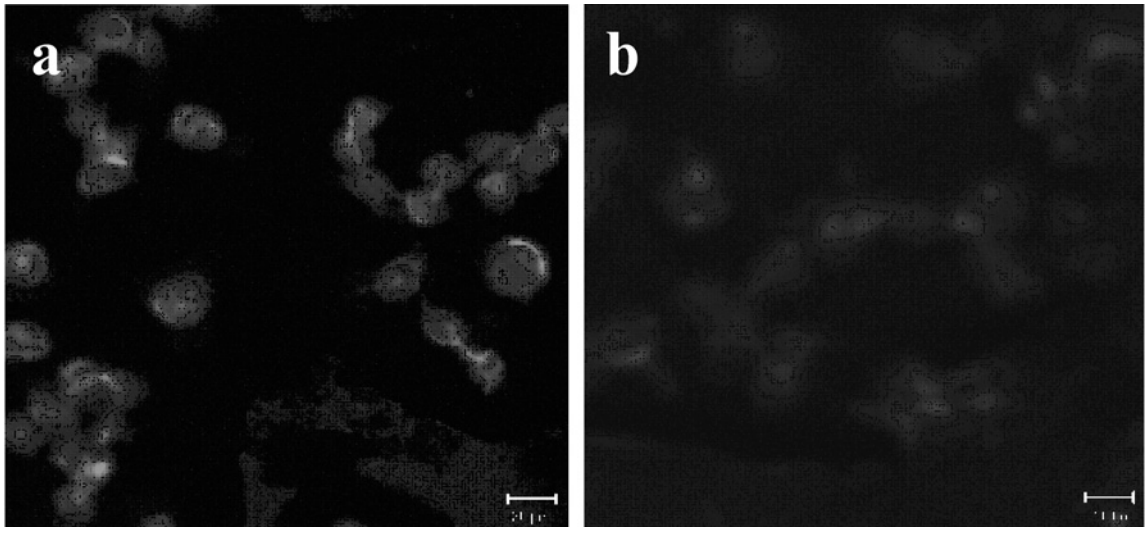

Figure 3. Immunofluorescence analysis of MMP-9 expression in HK 1 cells by confocal microscopy. (*a*) Untreated HK 1 cells. (*b*) Cells treated with 0.25 μM hypericin followed by light illumination at 0.5 J/cm^2. Bar=20 μm.

cell lines derived from human oral squamous cell carcinomas. Moreover, UV-A irradiation, which shares common photochemical properties such as the generation of reactive oxygen species, has also been shown to down-regulate MMP-9 expression in human epidermal keratinocytes via the production of singlet oxygen [34].

MMP gene expression is known primarily to be regulated at the transcriptional level. The promoter regions of the individual MMPs exhibit a high degree of similarity in their *cis* acting elements, which includes an AP-1 site and multiple polyoma enhancer A binding protein-3 (PEA3) sites, with the exception of MMP-2 and MMP-11 [35]. The promoter region of MMP-9 gene includes two AP-1 binding sites and one NF-κB regulatory element. The two AP-1 binding sites (located at –79 bp and –533 bp) are perfect consensus sequence that can bind members of the AP-1 transcription factor family [36]. Dimeric complex Jun/Jun or Jun/Fos, or Fos alone are known to bind with the AP-1 element to activate the transcriptional machinery [37]. The proximal AP-1 site is believed to play an essential role in the transcription activation of MMP promoters [38, 39].

Recent studies have shown that hypericin-PDT could modulate mitogen-activated protein kinases (MAPK) signaling pathways, including ERK, JNK/SAPK and p38 MAPK cascades [40], which are found to regulate AP-1 transcription factors. Our current study has demonstrated that hypericin-PDT inhibited AP-1 activation. This is contrary to previous reports that AP-1 DNA binding activity was increased by photofrin-PDT in murine keratinocyte [41] and in HeLa cells [42]. However, in the latter study, it was reported that enhancement of the AP-1 binding ability by phorbol esters and pyrrolidine dithiocarbanate (an antioxidant), which are known inducers of AP-1 DNA binding, was inhibited by PDT.

Deletion and mutational analysis have also shown that the induction of MMP-9 requires cooperation with other *cis* acting elements, such as NF-κB [43]. NF-κB is known to play a crucial role in the regulation of genes that participate in cell adhesion, immune and inflammatory responses, cell survival, as well as invasion and metastasis [44, 45]. As reviewed by Piette and colleagues [46], there have been several reports that PDT mediates NF-κB activation in different types of cells including mouse L1210 leukemia cells, human colon cancer cells and endothelial cells. In contradiction, our present study has shown that hypericin-PDT inhibited the transcriptional activity of NF-κB. NF- κB, as an oxidative-stress responsive transcription factor, was observed to be suppressed by singlet oxygen in certain type of cells such as keratinocytes [47]. Photofrin photosensitization also did not induce increased NF-κB binding in HeLa cells [48]. It is therefore possible that regulation of NF-κB by PDT is cell-type dependent.

Cytokines have been associated with the regulation of MMPs expression under physiological and pathological conditions [49]. In this study, we observed that HK1 cells endogenously expressed GM-CSF, a cytokine that has been implicated in the tumorigenesis of NPC and associated with cancer invasion and metastasis [50, 51]. In this study, we have also shown that incubating HK1 cells with exogenous GM-CSF induced elevation of MMP-9 activity. This is consistent with the finding

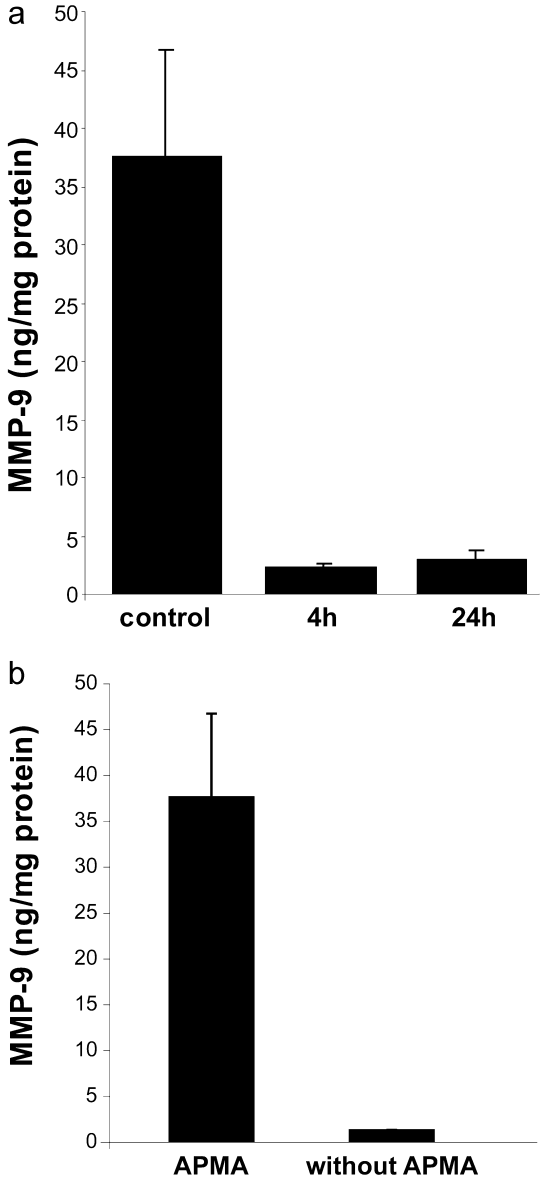

Figure 4. MMP-9 expression in supernatants of PDT-treated HK1 cells. (*a*) Total MMP-9 produced in supernatants of HK1 cells. Supernatants from untreated and treated cells were collected at 4 or 24 h post PDT. Total amount of MMP-9 in the supernatants was determined using a human active MMP-9 fluorescence assay kit. Protein was quantified by using Coomassie blue with colorimetric assay. Values are means ± SEM of triplicate samples from two separate experiments. (*b*) Expression of active form of MMP-9 in the supernatants of HK1 cells. Cells (5×10^6) in serum-free medium were seeded on petri dishes and incubated for 24 h. Cells were centrifuged and supernatants collected. Total amount of MMP-9 in the supernatants with and without addition of 4-aminophenylmercuric acetate (APMA) were determined using a human active MMP-9 assay kit.

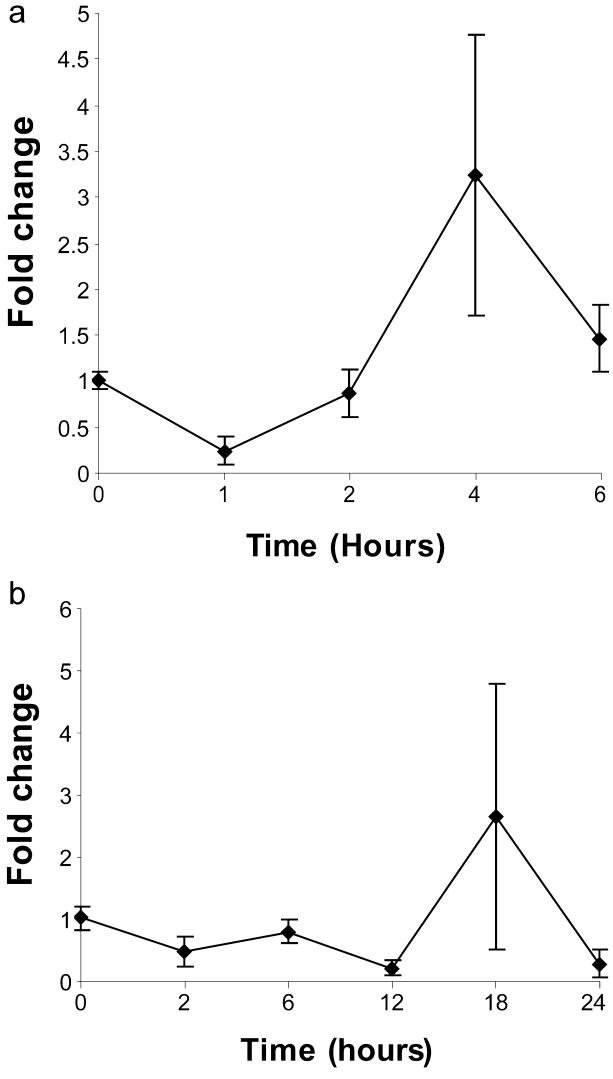

Figure 5. MMP-9 mRNA expression in PDT-treated HK1 cells *in vitro* and *in vivo*. (*a*) MMP-9 mRNA expression in HK1 cells *in vitro* following treatment with 0.25 μM hypericin and light illumination at 0.5 J/cm^2. (*b*) MMP-9 mRNA expression in HK1 tumor tissues. HK1 tumor bearing mice received a 2 mg/kg i.v. injection of hypericin before treatment with light at a dose of 14 J/cm^2. Fold change of MMP-9 at designated time points was calculated by: $\Delta\Delta C_T = (C_{T,\ MMP\text{-}9} - C_{T,\ G3PDH})_{\ Time\ x} - (C_{T,\ MMP\text{-}9} - C_{T,\ G3PDH})_{\ Time\ 0}$. Values are means ± SEM of triplicate samples from two to three separate experiments.

that GM-CSF up-regulates MMP-2 and MT1-MMP in human head and neck squamous cell carcinoma cells [52]. GM-CSF has also been observed to increase MMP-2 and MMP-9 activity in human lung cancer cells [50]. As GM-CSF has been shown to activate NK-κB and AP-1 [53], it is possible that the regulation of MMP-9 by GM-CSF is via the binding activity of these transcription factors in the promoter region.

In our study, inhibition of MMP-9 expression was observed to be accompanied by a reduction in GM-CSF production. Inhibition of MMP-9 expression by hypericin-PDT was abrogated by exogenous treatment with GM-CSF in HK1 cells, suggesting that GM-CSF could modulate the production of MMP-9 in HK1 cells in response to hypericin-PDT. Therefore,

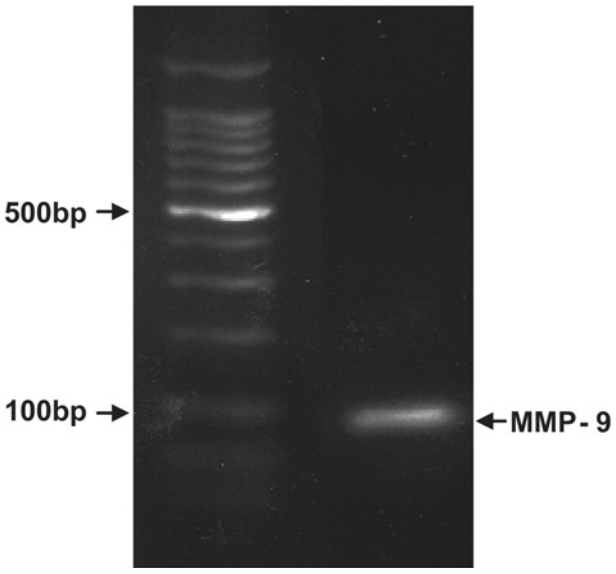

Figure 6. Determination of MMP-9 gene fragment by electrophoresis. The amplified products of MMP-9 were removed from LightCycler reaction capillaries and analyzed by agarose gel electrophoresis. 10 µl of the PCR reaction mixture was electrophoresed on 2 % agarose gel in the presence of ethidium bromide in Tris-buffered saline. The product size was determined by comparison to a 100-bp ladder run on the gel.

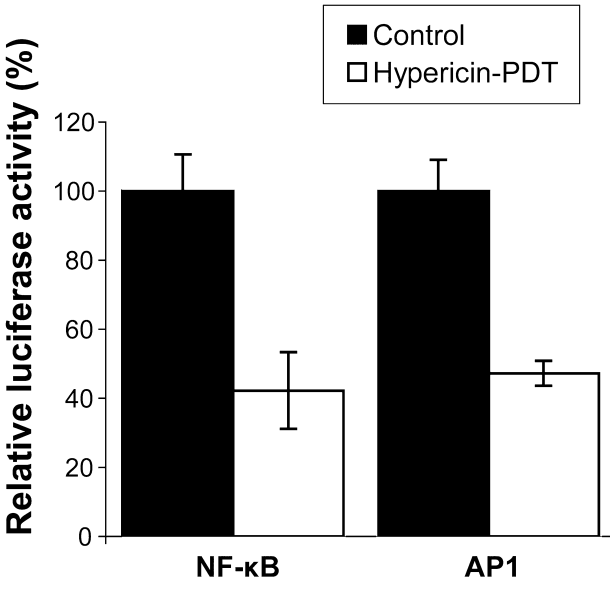

Figure 7. Binding activity of NF-κB and AP-1 in HK1 cells post hypericin-PDT. HK1 cells were transfected with pAP-1-Luc or p-NF-κB-Luc using Lipofectamine™ 2000. At 36 h after transfection, cells were treated with 0.25 µM hypericin followed by light irradiation at dose of 0.5 J/cm^2. Cells were harvested 6 h after the treatment for luciferase assay. Values are means ± SEM of triplicate samples from three separate experiments.

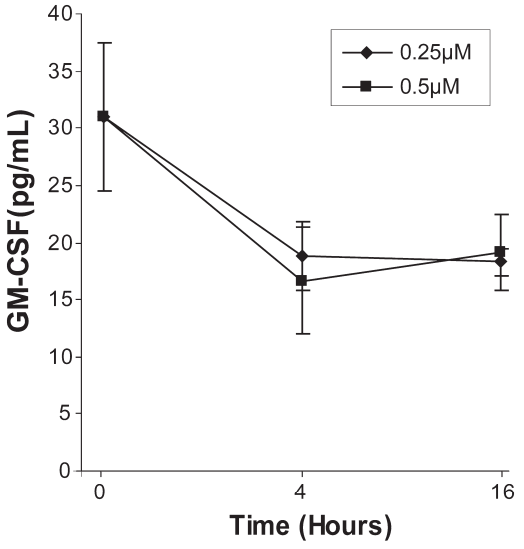

Figure 8. GM-CSF production in hypericin-PDT-treated HK1 cells. Cells (1×10^6) were incubated with 0.25 or 0.5 µM hypericin for 4 h before exposure to light irradiation at a dose of 0.5 J/cm^2. At 4 and 16 h post treatment, culture media were collected for determination of GM-CSF using an ELISA kit. Values are means ± SEM of triplicate samples from two separate experiments.

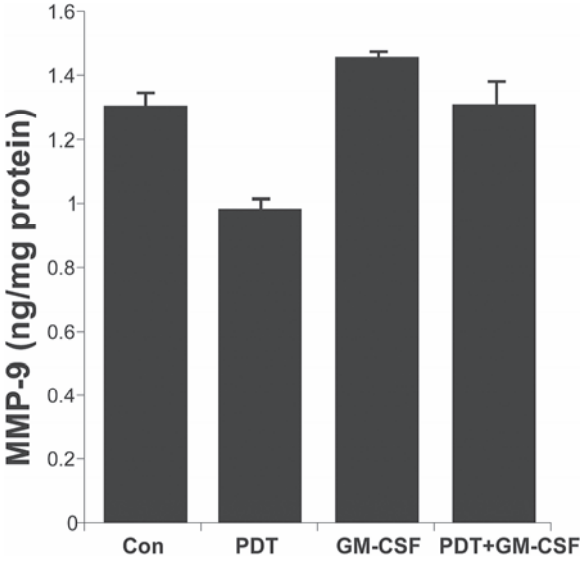

Figure 9. Effect of exogenous GM-CSF on MMP-9 secretion in HK1 cells. Cells (5×10^6) in serum-free medium were incubated for 24 h before hypericin-PDT. PDT-treated cells were further incubated for 24 hr in the presence or absence of human recombinant GM-CSF, added at final concentration of 10 ng/ml. The cell conditioned media (supernatant) were collected to analyze active MMP-9 by a human active MMP-9 fluorescence assay kit. Protein was quantified using Coomassie blue with colorimetric assay. Values are means ± SEM of triplicate samples from two separate experiments.

we hypothesize that hypericin-PDT down-regulates MMP-9 via inhibition of GM-CSF, which results in decreased binding activity of downstream NF-κB and AP-1 transcription factors (Fig. 10).

The observation that hypericin-PDT suppresses MMP-9 expression is of special clinical interest because radiotherapy (which is the main treatment for NPC) has been shown to enhance MMP-9 and

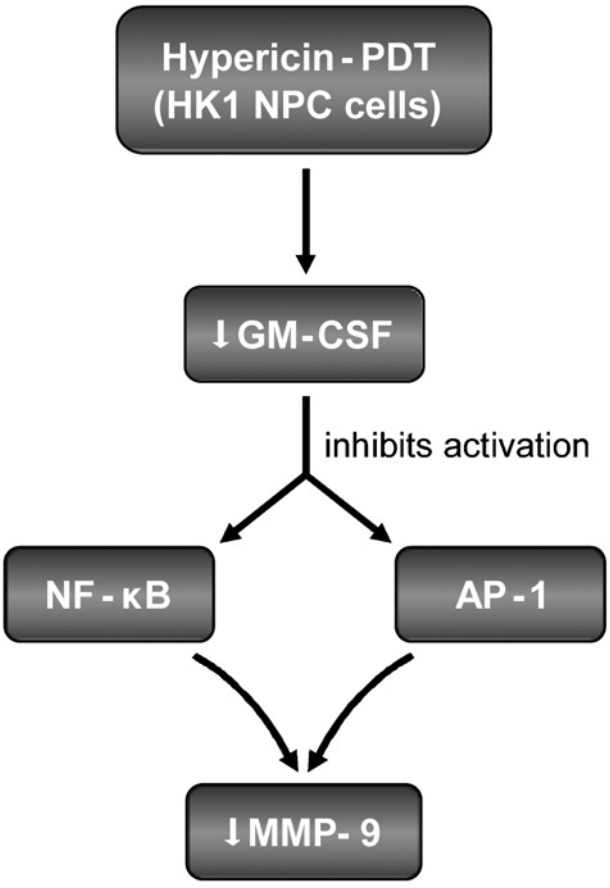

Figure 10. Overview of the mechanism by which hypericin-PDT regulates MMP-9 expression.

MMP-2 expression and activity in human glioblastoma *in vitro* and *in vivo* [54, 55], and may therefore promote metastasis and invasiveness of tumor cells. Ionizing irradiation has been shown to induce 72-kDa collagenase in rat astrocytes [56], and radiation-induced collagenase production was also observed in diploid human fibroblasts [57]. Preoperative radiotherapy was also reported to induce increased type IV collagenase activity in patients with rectal cancer [58]. In conclusion, down-regulation of MMP-9 would be an added advantage of PDT over radiotherapy with regard to decreasing the metastatic potential of tumor cells surviving the treatment.

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