以活性氧分子形成的腫瘤溶解液來增加癌症疫苗的效果

計畫類別：個別型計畫
計畫編號：
執行期間：92年08月01日至93年07月31日
執行單位：國立成功大學醫學系皮膚科

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報告類型：精簡報告
處理方式：本計畫涉及專利或其他智慧財產權，1年後可公開查詢

中華民國93年11月2日
Summary. Photodynamic therapy (PDT), which uses the activation of tumor localizing photosensitizing agents by visible light, is an effective therapy for local malignant tumors, achieving palliation of advanced disease and cure of early disease. Our research team has showed that PDT-generated tumor cell lysates are effective antitumor vaccines that are tumor specific and are superior at eliciting cytolytic activity and providing protection in compared with those prepared by UVB or IR. However, the mechanism of why PDT-generated cell lysates are superior to other cell lysates remains unknown. In this study, we examined whether PDT-generated tumor cell lysates could enhance DCs functions, especially on cell migration. PDT-generated tumor cell lysates were compared with freeze/thaw (F/T)-generated tumor cell lysates and LPS stimulation in their ability to enhance murine dendritic cell (DC) maturation, cell adhesiveness and migration by flow cytometry and time-lapse cinematography. We found PDT-generated tumor cell lysates can strongly elicit DCs maturation but to a lesser extend in cell migration, probably related to different activation of mitogen-activated protein kinases. The understanding of how tumor cell lysates regulate DC phenotypes might potentially improve the design of cancer immunotherapy.

Key words: Photodynamic therapy (PDT), immunotherapy, vaccine, dendritic cell, migration
Photodynamic therapy (PDT)-generated tumor cell lysates strongly stimulate dendritic cell maturation but to a lesser extend in cell migration

Introduction

PDT uses a photoreactive drug or photosensitizer in combination with a specific wavelength of light to kill tumor tissue (1). Clinical trials have shown a palliation of advanced disease and long-term control of early disease (1). PDT is approved for multiple indications in the United States, Canada, Japan, and 17 countries of the European Union. Numerous preclinical studies have demonstrated that PDT enhances the host antitumor immune response (1, 2), but the mechanisms behind this enhancement are unknown. Among the potential contributing factors are alterations in the tumor microenvironment via stimulation of proinflammatory cytokines and direct effects of PDT on the tumor that increase immunogenicity. Previous studies have shown that PDT stimulates the expression of several inflammatory mediators, including tumor necrosis factor-α, IL-6 (3, 4), and IL-1 (1, 3, 5). However, little is known about the direct effects of PDT on the inherent immunogenicity of the tumor cells. Gollnick et al. (6) showed that photodynamic therapy- (PDT) generated tumor cell lysates are effective antitumor vaccines that are functional in the absence of adjuvant, and direct comparison of PDT-generated vaccines with those prepared by UVB or IR indicated that PDT-generated vaccines were superior at eliciting cytolytic activity and providing protection. PDT vaccines are tumor specific and appear to induce a cytotoxic T-cell response. Although both UV and PDT-generated tumor cell lysates are able to induce phenotypic dendritic cells (DCs) maturation, only PDT generated lysates are able to activate DCs to express IL-12 which is critical to the development of a cellular immune response. However, the mechanism of why PDT and UV-generated cell lysates are superior to other cell lysates is unknown.

In this study, we examined whether PDT generated tumor cell lysates could enhance DCs functions especially on cell migration. We found PDT generated tumor cell lysates could enhance DCs maturation but less effective in cell migration.

Material and Methods

Reagents. Recombinant mouse cytokine GM-CSF was purchased from R&D Systems (Wiesbaden, Germany). Other chemicals were purchased from Sigma Chemical Co.

Photosensitizer. Indocyanine green (Diagnogreen Injection™, 2.5mg/ml) was a gift from Daiichi Pharmaceutical Company, Tokyo, Japan.
**Cell cultures.** Murine colon cancer cell line (CT26) was grown in 90% RPMI with 2mM L-glutamine to contain 1.5 g/l sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate and 10% FBS and antibiotics (6). Cells were kept in a humidified atmosphere of 5% CO2 in air at 37°C.

**Generation of Lysates.**

**PDT-generated lysates.** Exponentially growing CT26 cells were exposed to 250 μM Indocyanine Green (ICG)(7) in complete medium for 24 h, followed by exposure to serum-free medium (Ham’s F12; Life Technologies, Inc.) at 1 x 10^7 cells/ml and irradiated with an infrared lamp (PAR38, Philips, Holland) from above with a dose (30 J/cm^2 at 40mW/cm^2) equivalent to the LD99 determined by tetrazolium salt MTT assay. The lamp emits light between 700 and 2000 nm, peaked around 1000nm. PDT treated cells were incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 h. Supernatants were collected and spun at 800 x g to clear cell debris and any remaining live cells.

**Freeze/thaw (F/T)-generated lysate.** F/T lysates are generated by subjecting cells (1 x 10^7 cell/ml) to three F/T cycles, followed by centrifugation at 800 x g to remove the cell debris and any remaining live cells. The resulting supernatant was collected and frozen at -70°C until use. A minimum of two independent lysate preparations by each method was used. All of the above supernatants are collected and passed through a 0.2-μm filter. The protein concentration of the lysate is determined by a commercial assay (Bio-Rad, Munich, Germany).

**Flow Cytometetry Analysis.** Antibodies used to phenotype the cells were anti-CD11c-PE, anti-CD86-FITC, anti-MHCII-PE (PharMingen, San Diego, CA). For staining, 10^6 cells were suspended in 100 µl of PBS and were incubated with 10 µl of the antibodies for 30 min on ice. Flowcytometry was performed in the FACS Core Facility available at the University Hospital.

**Generation of Tumor Cell Lysate-pulsed Mature DCs.** Bone marrow-derived DCs from BALB/c mouse are generated as described elsewhere (8). Briefly, bone marrow cells were cultured in complete RPMI 1640 medium containing 20ng/ml recombinant mouse GM-CSF at 37°C in a humidified atmosphere with 5% CO2. The cultures were fed every second day with medium containing fresh GM-CSF. On day 6 of culture, nonadherent cells were removed and washed once in complete medium. Flow cytometry analysis of these cells showed that 40–50% were positive
for CD11c and CD86, and were defined as immature DCs. Immature DCs were primed with tumor cell lysates by incubating $10^6$ DCs with tumor cell lysates generated from $3 \times 10^6$ CT26 cells for 20h in complete medium at 37°C in a humidified atmosphere with 5% CO2. As a positive control DCs were incubated with lipopolysaccharide (LPS) (1 μg/ml; Sigma Chemical Co.); negative control DCs were incubated with medium alone. After incubation DCs were analyzed for CD11c and CD86, and also MHC class II and CD86 expression by flow cytometry as described (6). Each experiment was performed a minimum of two times with separate DC preparations.

**Adhesion and migration studies.** After priming with different tumor lysate, $9 \times 10^4$ primed BMDC were plated on fibronectin (FN, 20 μg/ml)-coated 35mm culture dish. DCs migration was monitored under phase contrast time-lapse cinematography (Leica DM IRE2, Germany) for 3h. Images were acquired every 10 minutes and were analyzed with Metagraph software. Cells adhered to fibronectin were counted under 6 randomly selected high power field under microscope. The percentage of migratory cells was calculated by divided the number of migrated cells by the number of attached cells to the substrate. The speed is defined as the traversed path during the entire experiment divided by the imaging time.

**Study of the MAPK pathways with Western blot analysis.** DCs are cultured as described above. On day 6, the cells are incubated with lysates of CT26 or medium (control) at a final concentration of 120μg of protein/ml for 3 h. Some DCs samples were pretreated with Erk inhibitors PD98059 or a p38 specific inhibitor, SB202190. The DCs were then lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% w/v bromphenol blue) and boiled for 5 min. Equivalent amounts of protein are loaded onto polyacrylamide gel, subjected to electrophoresis, transferred to a PVDF membrane and incubated with phospho-specific p44/42 Erk, JNK, or p38 MAPK antibodies. The immune complexes are detected by ECL system. After probing with the phospho-specific antibodies, the membrane is washed and reprobed with the corresponding control p44/42 Erk, JNK, or p38 MAPK to check the loading and calculate the ratio to non-activated kinase.

**Statistical Analysis.** One-way analysis of variance was performed to determine whether there were significant differences between the different test conditions. Bonferroni correction was applied to allow for the effect of multiple testing. Regression analysis was performed to determine whether a dose-dependent
relationship existed between tumor cell lysate or LPS concentrations. Comparisons with a difference of $P<0.05$ were considered significant.

**Results**

**PDT Lysates Are More Potent in Stimulation of DCs Maturation.**  DCs differentiation was enhanced from around 30% on day 6 culture (Fig. 1A, upper half) to around 50% (Fig. 1B, upper half) on day 7 in control. Incubation with F/T-generated lysates significant increased the maturation of DCs when compared with control (medium) (Fig. 1C-E), $p < 0.01$, Bonferroni $t$ method. Incubation with PDT-generated lysate strongly enhanced DCs maturation with higher number of cells with CD11c+ and CD86+ (Fig. 1F, $p < 0.001$, Bonferroni $t$ method) and also CD86+ and MHC class II$^{hi}$ cells (Fig. 2E and F). The trend was similar in Fig. 2 as showed by the expression of MHC class II$^{hi}$ and CD86 though not significant difference. The results implied that PDT-generated lysates might be a stronger immunogen that stimulated DCs differentiation and maturation than F/T-generated lysates. Challenged DCs with a higher concentration of PDT lysates could not provide stronger stimulation (data not shown), possible related to a saturated antigen processing ability of DC.

**Tumor Cells Lysate-pulsed DCs Are More Adhesive to Fibronectin.**  To examine whether differences in adhesive and migratory behavior between DCs being pulsed with different tumor cell lysates, we investigated their capacity to bind to the extracellular matrix protein fibronectin. Binding to fibronectin and cell spreading of DCs was much stronger in DCs being pulsed with different lysates (Fig. 3B) than of control (Fig. 3A). There was no significant difference between DCs being pulsed with different lysates (Fig. 3C). Adhesion of mature DCs to fibronectin was mediated through $\alpha 5 \beta 1$ integrin (ref Vires), implies that either PDT lysates or F/T lysates or stimulated with LPS can induce expression of this intergrin.

**Migratory Properties of Tumor Cells Lysate-pulsed DCs to Fibronectin.**  Although cell membranes of immature DCs were highly dynamic, and expanding, contracting, and changing shape continuously, none of DCs in control (immature DCs) migrated when followed in time-lapse cinematography (Fig. 4A, E and F). In contrast, DCs being pulsed with PDT or F/T lysates and also those incubated with LPS were highly motile on fibronectin (Fig. 4B-F), using their dendrites to continuously attach and detach from the substrat. As a consequence, the speed of tumor cells lysate-pulsed DCs was higher than of immature DCs (Fig. 4F), 0.71 $\mu$ m/min in F/T lysates-pulsed DCs, 1.41 $\mu$ m/min in LPS stimulated DCs, and 0.60 $\mu$
m/min in PDT lysates-pulsed DCs, versus 0.08 μm/min in immature DCs (all p values < 0.001). Interestingly, although 90% attached DCs being pulsed with F/T-generated lysates migrated on fibronectin, the speed of these cells was less than of LPS stimulated cells (p< 0.001) and was not differed from the speed of PDT-generated lysates pulsed DCs.

**Discussion**

DC plays a critical role in the activation of naive T lymphocytes and in the generation of primary T cell responses. Upon activation they initiate a differentiation process that results in decreased Ag-processing capacities, enhanced expression of MHC and costimulatory molecules, and migration into secondary lymphoid organs, where they trigger naive T cells. It is well documented that generation of tumor immunity by DC migration from peripheral organs to lymph nodes plays a key role in initiating immune responses, whether migratory DCs bring antigen in tow to lymph nodes or position themselves to capture antigen that drains into the lymph node. In general, mature DCs are more effective in migration. De Vries reported that effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state (10). The present study showed PDT-generated tumor cell lysates significantly increased the number of mature DCs with expression of MHC class II^hi^ and CD86. The results are in agreed with studies of Gollnick et al (6). They have demonstrated that PDT-generated lysates are able to induce phenotypic DC maturation and were potent vaccine that augments the host specific antitumor immune response. However, the migratory capacity of these cells have not been fully explored.

Indocyanine green (ICG), a tricarbocyanine dye with a strong absorption band between 600 and 900 nm, has been used in medicine since 1956 (11) and exhibits some characteristics of an ideal photosensitizer, in particular absorption in the near infrared part of the visible spectrum (805 nm in human plasma) allowing deeper tissue penetration. Therefore, the photosensitizing properties of ICG have recently been investigated in vitro (12). Photoactivation of ICG by irradiation with a diode laser in HaCaT cells resulted in cytoplasmic vesiculation, dilation of the rough endoplasmic reticulum, the Golgi complex and the perinuclear cisternae, and the beginning of chromatin condensation, whereas the cell surface and mitochondria remained completely unchanged (13). On the other hand, the plasma membranes and Golgi complex are the main target sites of Photofrin after a brief (3 h) and prolonged (24 h) incubation, respectively in A431 cells (14). The difference in subcellular localization of ICG and Photofrin might result in different cell death phenotypes and elicit different immune responses (15). Sauter et al. reported that
after exposed DCs to necrotic tumor cells, but not primary tissue cells or apoptotic cells induces the maturation of immunostimulatory dendritic cells (15). Ip and Lau showed DCs that had taken up apoptotic cells of early phase acquired a non-fully mature DC phenotype, whereas DCs that had taken up ACs of late phase acquired a mature DC phenotype with enhanced T cell stimulatory capacity (16). Whether ICG mediated or Photofrin mediated PDT-generated lysates provided a better host immunity against tumor remain unclear. The better understanding of the underline mechanisms will lead to a better design of PDT based anticancer immunotherapy.

CCR7 expression on DC prominently controls its migration into afferent lymphatic vessels and the positioning of DCs within the lymph node. Expression of CCR7 is not sufficient for function, as its function is positively regulated by a variety of other extracellular triggers (17). The MAP kinase pathway has been well studied in DCs and exhibits a complex regulatory role in which the activation of different MAP kinase members leads to biologically distinct outcomes that are dependent upon stage of differentiation at the time of activation as well as the duration of signaling. Whereas promoting activation of p38 MAPK or inhibiting ERK1/2 activation improves the maturation of immature DCs, the development of monocytes into immature DCs is impeded by p38 MAPK activation and requires ERK1/2 activation (18). Taking together, activation of p38 MAPK and ERK1/2 might play an important role in DC maturation and migration. The studies concerning how these signaling proteins regulate DC maturation and migration are now undergoing.

In summary, we found PDT-generated tumor cell lysates can strongly elicit DCs maturation but to a lesser extend in cell migration. The understanding of how tumor cell lysates regulate DC phenotypes might potentially improve the design of cancer immunotherapy.
References:


PDT-generated tumor cell lysates activate DCs. Immature DCs were isolated from BALB/c bone marrow cultures and before (A) and after (B-E) incubated overnight with tumor cell lysates (1:3) generated with F/T (C); LPS stimulation (D); PDT (E) and control (medium)(B). Results are reported as percentage of CD11c⁺, CD86⁺ cells in the total population.
FIG. 2. PDT-generated tumor cell lysates increase the number of mature DCs. Immature DCs were isolated from BALB/c bone marrow cultures and before (A) and after (B-E) incubated overnight with tumor cell lysates generated with F/T (C); LPS stimulation (D); PDT (E) and control (medium)(B). Results are reported as percentage of MHC class II$^{\text{hi}}$, CD86$^+$ cells in the total population.
FIG. 3. Cell spreading of DCs after being pulsed with tumor cells lysate. Spreading of DCs was much stronger in DCs being pulsed with different lysates (B) than of control (A). There was no significant difference in the number of cell adhesion to fibronectin between DCs being pulsed with different lysates (C).
FIG. 4. Migration of DCs to fibronectin. B, C and D, different lines, migration paths of DCs: A, immature DCs; B,C,D, mature DCs. E, percentage of migrated DCs ± SD of both control and DCs being challenged with different cell lysates (p < 0.001, one way ANOVA). F, speed of migrated DCs ± SD (p < 0.001, one way ANOVA); speed of migrated DCs in PDT are not differed from those of F/T; data points, the speed of individual cells; horizontal bars, the mean speed. Data are representatives of two experiments.