Effects of PEG-liposomal oxaliplatin on apoptosis, and expression of Cyclin A and Cyclin D1 in colorectal cancer cells

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Abstract. Oxaliplatin is one of the agents used against colorectal cancer. Using PEG-liposome encapsulated oxaliplatin may enhance the accumulation of drugs in tumor cells, inducing apoptosis. However, the mechanism of action of PEG-liposome encapsulated oxaliplatin remains unclear. SW480 human colorectal cancer cells were treated with empty PEG-liposomes, free oxaliplatin or PEG-liposomal oxaliplatin. Cell cycle and apoptosis were assessed using fluorescence confocal microscopy and terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end-labeling (TUNEL). Western blotting was used to analyze the expression of pro-apoptotic, anti-apoptotic and cyclin proteins. We found that PEG-liposomal oxaliplatin induced a stronger apoptotic response than empty PEG-liposomes or free oxaliplatin. Moreover, expression of Cyclin D1 increased, whereas expression of Cyclin A decreased after treatment with PEG-liposomal oxaliplatin. Furthermore, the cell cycle was arrested in the G1 phase. The results presented here indicate that PEG-liposome entrapment of oxaliplatin enhances the anticancer potency of the chemotherapeutic agent. The effect of PEG-liposomal oxaliplatin on apoptosis of SW480 human colorectal cancer cells may be through regulation of expression of Cyclin A or Cyclin D1, as well as pro-apoptotic and anti-apoptotic proteins.

Introduction

The antitumor effect of oxaliplatin (L-OHP) is achieved through inhibition of DNA synthesis and duplication. Clinically, oxaliplatin, in combination with other antitumor drugs, has demonstrated significant activity against advanced colorectal cancer. However, all cytotoxic drugs pose significant toxic activity to the human body, leading to conditions such as neurotoxicity, gastrointestinal reaction and cardiotoxicity (1). Moreover, the non-selectivity of cytotoxic drugs between normal tissue and the pathological site poses a challenge for the treatment strategy of tumors.

Targeting of drugs specifically to the colon is advantageous for the treatment, but a chemotherapy drug may not reach its target site in effective concentrations (2,3). Thus, effective treatment may demand an increased dose, which may lead to side effects. To overcome these limitations, new delivery systems with alternative drug release mechanisms have been suggested. Liposomes were shown as good carriers of antineoplastic chemotherapeutics by changing the distribution characteristics of drugs and facilitating extended drug release (3-5). A few reports have shown that liposomes were one of the first nanomolecular drug delivery systems to show increased delivery of small molecular weight anticancer drugs to solid tumors by altering the bio-distribution of associated drugs (6,7). It was repeatedly demonstrated that liposomes could improve the therapeutic index of a variety of drugs. In a recent study, DSPE-PEG modification of the surface of the liposomes may have prevented interactions with the biological in vivo environment. Thus, the circulation lifetime of the liposomes was extended (8-10). This, in turn, resulted in extensive extravasation of the liposomes due to the tumor selective enhanced permeability and retention effect, ultimately leading to enhanced accumulation of the liposomes in the tumor interstitium (11). According to these theories, the liposome containing drugs will show markedly enhanced antitumor activity and induce tumor cells apoptosis.

Recent studies have shown that the inhibitor of apoptosis protein (IAP) and Bcl families of proteins are closely associated with the anti-apoptotic effect. X-linked inhibitor of apoptosis protein (XIAP) is a member of the IAP family and inhibits apoptosis by directly inhibiting the activity of caspase-3, caspase-7 and caspase-9 (12). Bcl-2, Bcl-XL, Bax and Bad are members of the Bcl-2 family. An increase in the expression of...
Bcl-2 and Bcl-XL genes and a decrease in the expression of Bax and Bad genes lead to a reduction in the permeability of the mitochondrial membrane, inhibition of both mitochondrial depolarization and release of cytochrome c, resulting in further inhibition of caspase-9 (13,14). Caspase-9 can activate caspase-7 and caspase-3, the latter being the final executor of apoptosis (15,16). Various proteins are involved in cell division/cell cycle control. Cyclins comprise a family of regulatory proteins that are classified as either G1-, S- or M-phase, depending upon when their levels reach maximum abundance and when they are thought to be functional (17,18). Cyclin D1 is the main protein in the G1-phase, whereas Cyclin A is classified as an S-phase and mitotic cyclin. Interference with cell cycle-related proteins and induction of apoptosis is an important treatment strategy for cancer (19,20). Therefore, cell cycle regulation and apoptosis play a key role in cancer therapy.

In this study, we prepared PEG-modified oxaliplatin liposomes to treat human colorectal cancer SW480 cells, in order to investigate the therapeutic activity of PEG-liposomal oxaliplatin and its effect on the expression of cyclins.

**Materials and methods**

**Reagents.** Oxaliplatin was purchased from Sigma Co. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethyleneglycol)-2000] (DSPE-PEG2000) was obtained from Avanti Polar Lipids, Inc. DIOC18(3), 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was obtained from Vigorous Biotech Co., Ltd. Rabbit polyclonal anti-β-actin, goat anti-rabbit IgG, and peroxidase conjugated secondary antibodies were obtained from Bioscience Co., USA. Rabbit polyclonal antibodies for Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-XIAP, anti-caspase-9, anti-caspase-7, anti-activated-caspase-3 (P17), anti-Bcl-XL, anti-Bad, anti-Cyclin A and anti-Cyclin D antibodies were obtained from Bioworld Technology, Inc. The TUNEL kit was purchased from Promega. Life Science Academy of Chongqing Medical University provided the human colorectal cancer SW480 cells.

**Preparation of PEG-liposomes, cellular uptake PEG-liposomes and MTT assay.** PEG-liposomes were prepared, then cell uptake and cell viability were measured using the MTT assay, as described previously (21,22).

**Colony formation assay for long-term cell survival.** Human colorectal cancer SW480 cells were trypsinized and cultured in 6-well plates (105/well). After the cells were grown for 14 days, cells were treated with free oxaliplatin (28 µg/ml), PEG-liposomal oxaliplatin (containing 28 µg/ml oxaliplatin) or empty PEG-liposomes (2.6 µmol/ml phospholipids) for 24 h, with no treatment as a control. Then cells were washed twice in PBS, fixed with 70% ethanol and stained with crystal violet (0.5% in ethanol). The plates were rinsed with water, air-dried, photographed and evaluated for colony estimation.

**Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end-labeling (TUNEL) assay.** SW480 cells (5x10⁴/slide) were incubated on glass slides for 24 h, treated with empty PEG-liposome, free oxaliplatin and PEG-liposomal oxaliplatin for 12 h, respectively. After removal of culture solution, cells were fixed with freshly prepared 4% paraformaldehyde (dissolved in pH 7.4 PBS) for 30 min at room temperature and washed with PBS. The slides were blocked with 0.3% H₂O₂, MeOH solution for 30 min at room temperature to block endogenous peroxidase activity. Then cells were incubated with permeation mixture (0.1% Triton X-100 dissolved in 0.1% sodium citrate solution) for 2 min in an ice bath. The cells were subsequently incubated with 50 µl TUNEL reaction mixture for 1 h prior to addition of 50 µl transforming agent-POD for 30 min. Stained cells were visualized by 3,3'-diaminobenzidine (DAB) coloration and analyzed under a light microscope. Cells treated with deoxyribonuclease I served as positive control for DNA fragmentation.

**Flow cytometric analysis of cell cycle.** The empty PEG-liposome, free oxaliplatin and PEG-liposomal oxaliplatin-treated cells were collected and fixed with 70% ice-cold ethanol overnight at 4°C. Cells were centrifuged, resuspended in 400 µl 1X binding buffer (>1x10⁶/ml) and incubated with Annexin V-FITC (5 µl) in the dark for 15 min. Cells were subsequently treated with PI (10 µl) and incubated in the dark for 5 min prior to detection and analyzed using a BD FACS Calibur (BD Biosciences).

**Western blot analysis.** After 12 h of treatment, cells were collected, disrupted using cell lysis solution, and protein was extracted as previously reported (23,24). The protein concentration was determined with the Bradford assay. Fifty micrograms of protein were separated by 12 or 15% SDS-PAGE, and the proteins were transferred to a PVDF membrane. The membrane was incubated overnight at 4°C using Bcl-2, Bax, Bcl-XL, Bad, XIAP, caspase-9, caspase-7, activated-caspase-3 (P17), Cyclin A, Cyclin D and β-actin antibodies. The membrane was subsequently incubated at room temperature for 1 h with a HRP-labeled secondary antibody (goat anti-rabbit IgG) and developed using an ECL chemiluminescent reagent for visualization on a BIO-Rad imaging system. β-actin was used as an internal standard to normalize protein expression. Band intensities of target proteins are expressed as the percentage of the β-actin band intensity, which was set at 100%.

**Data analysis.** All the data are presented as the mean ± SD. Treatment group comparisons were performed using Student’s t-test analysis, with P<0.05 being considered statistically significant. The statistical data were analyzed using SPSS17.0 software.

**Results**

PEG-liposomal oxaliplatin induced growth inhibition. The sensitizing effects of the treatment of SW480 cells with PEG-liposomal oxaliplatin were further confirmed using the colony forming assay (Fig. 1). The colony formation of cultures exposed to empty PEG-liposomes was indistinguishable from the untreated controls, while the free oxaliplatin (28 µg/ml) and PEG-liposomal oxaliplatin (containing 28 µg/ml oxaliplatin) treatments showed a decrease in colony formation. However, after treatment of cells with PEG-liposomal oxaliplatin there was a drastic reduction in the colony formation owing to the severe synergistic effects on the survival of SW480 cells.
Analysis of apoptosis. During apoptosis, genomic DNA is disrupted, which results in the presence of DNA fragments. Terminal deoxynucleotidyl transferase-catalyzed polymerization of tagged deoxynucleotides at the free 3'-terminal in a non-template-dependent manner allows for labeling the gaps at the broken ends of the DNA. DNA fragments can be detected using an HRP conjugated anti-Fab fragment. The karyons assumed a yellowish-brown color after the DAB reaction. The results of the TUNEL assay confirmed the flow cytometry data with regard to the change in the levels of apoptosis (Fig. 2).

Expression levels of pro-apoptotic and anti-apoptotic proteins. Apoptotic death was further confirmed by western blot analysis. The IAP and Bcl families of protein are associated with apoptosis. XIAP, a member of the IAP family, is the most potent inhibitor of caspase activity. After treatment with PEG-liposomal oxaliplatin, there was not only increased apoptosis, but also the expression of XIAP was markedly decreased (Fig. 3A). Expression of relative protein was lower (0.16±0.06) as compared with the other treatments (P<0.01). However, expression of caspase-9, caspase-7 and activated-caspase-3 were increased after treatment with PEG-liposomal oxaliplatin (Fig. 3B). Expression of caspase-9, caspase-7 and activated-caspase-3 was 13.61±4.19, 7.63±2.75 and 14.05±3.16, respectively. We also detected protein expression levels of Bcl family members. After treatment of cells with PEG-liposomal oxaliplatin, expression of anti-apoptotic Bcl-2 and Bcl-XL decreased to 0.32±0.07 and 0.31±0.05, respectively, whereas
pro-apoptotic Bax and Bad proteins increased to 6.10±1.02 and 7.30±0.94, respectively (Fig. 3C). These results indicate that apoptosis was strongly induced by PEG-liposomal oxaliplatin. 

**Expression levels of Cyclin A, Cyclin D and cell cycle arrest.** PEG-liposomal oxaliplatin treatment dramatically reduced the levels of Cyclin A (0.22±0.03), compared with other treatments,
We also found that the Cyclin D1 level was elevated in these cells (3.2±0.42), compared with other treatments (P<0.01) (Fig. 4). This analysis indicates that altered expression of Cyclin A and Cyclin D1 was associated with changes in the cell cycle. Progression of the cell cycle was examined using flow cytometry. As expected, there were a high proportion of G1-phase cells after treatment with PEG-liposomal oxaliplatin. We found that the cell cycle was distinctly arrested in the G1-phase, with the percentage of cells greater in those treated with PEG-liposomal oxaliplatin (60.9±3.24%), than in control cells (54.5±1.04%) (P<0.05). In contrast, the number of cells in S-phase was less after treatment with PEG-liposomal oxaliplatin (28.7±0.29%), as compared to control cells (P<0.01) (Fig. 5).

Discussion

We carried out the present studies to provide further evidence to support the use of PEG-liposome containing drugs in therapy trials and in cancer prevention. Cytotoxic drugs have no target selectivity between normal tissues and pathological sites (25). The dose escalation necessary to overcome even a small increase in cellular resistance can cause severe cytotoxicity to dose-limiting normal tissue. The ideal therapy would deliver the drugs directly to the pathological sites. Thus, strategies containing agents that act through distinct molecular mechanisms, rather than using single agents, represent the most useful alternatives for achieving higher curability with the least toxicity during cancer chemotherapy.

Recently, there has been an increasing interest in evaluating synergistic cancer cell cytotoxicity from chemotherapeutic agents with highly promising results. PEG-modified liposomes have been used as carriers of anticancer drugs to enhance the affinity and uptake of anticancer drugs in cancer cells (26). It has been found that PEG-liposomes are not readily taken up by the macrophages in the reticuloendothelial system (RES) and hence stays in the circulation for a relatively long period of time. Therapeutic studies and pharmacokinetic analysis with
tumor bearing mice revealed that PEG-liposomes have considerable potential as drug carriers for cancer therapy (27,29).

Our in vitro study revealed the uptake of liposomes by cancer cells, and resulted in accumulation of liposomes within the cells, similar to that reported by Gabizon and Goren et al (27,30). As reported previously, we observed PEG-liposome intracellular distribution (21). This suggests that cells may undergo endocytosis of more than a single liposome, which may be related to the electric potential of the liposomes and cells. This study revealed a time-dependent decrease in SW480 cell viability after treatment with PEG-liposomal oxaliplatin. Previous literature was less expansive in explaining the above phenomenon.

Using a flow cytometer and TUNEL technology, it was shown that apoptosis significantly increased after treatment with PEG-liposomal oxaliplatin. We also analyzed the expression levels of pro-apoptotic and anti-apoptotic proteins in the PEG-liposomal oxaliplatin-treated cells. The anti-apoptotic proteins XIAP, Bcl-2 and Bcl-XL decreased in expression. In contrast, the expression of the pro-apoptotic proteins caspase-9, caspase-7, activated-caspase-3, Bax and Bad increased (Fig. 3). The oxaliplatin alone or empty PEG-liposome treatment alone did not appreciably alter the cellular levels of these cell survival regulators. However, these changes were significant in the PEG-liposomal oxaliplatin group. During apoptosis, activated caspase-9 further activates caspase-7 and caspase-3 and augments the apoptotic pathway (31,32). Increased apoptosis induced by oxaliplatin is another feature of cellular response to PEG-liposomal oxaliplatin treatment, which manifests as the synergistic growth inhibitory effect on SW480 cells. Taken together, these results indicate that PEG-liposomal oxaliplatin induces apoptosis.

Further investigation elucidated the potential mechanism of PEG-liposomal oxaliplatin-induced apoptosis. The anti-proliferative effects of PEG-liposomal oxaliplatin have been attributed to changes in the cell cycle and expression levels of cyclins. However, determining the mechanisms regulating the passaging of these cells is important to the understanding of diverse cellular processes including cellular proliferation, DNA repair-mediated cell cycle arrest, terminal differentiation and apoptosis (33,34).

In this study, we observed an increase in the G1-phase cell population in PEG-liposomal oxaliplatin treated cells (P<0.05), whereas the S-phase population was reduced (P<0.01). We hypothesize that DNA synthesis was blocked, and thus, cells proliferation was inhibited. Further analyses indicated that the effects on SW480 cell proliferation were dependent on the PEG-liposomal oxaliplatin-induced apoptosis.

The PEG-liposomal oxaliplatin effect on apoptosis may be through the regulation of expression of Cyclin A or Cyclin D1, as well as pro-apoptotic and anti-apoptotic proteins.

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