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HPV-associated cervical cancer cells targeted by triblock copolymer gold nanoparticle curcumin combination

J. Chen-Sandoval[1], C.C. Perry[2], J. Yun[1], P.J. Chan[1]

[1] Gynecology and Obstetrics Department, Loma Linda University School of Medicine, Loma Linda, CA
[2] Department of Basic Sciences, Center for Health Disparities and Molecular Medicine, Loma Linda University School of Medicine, Loma Linda, CA (USA)

Summary

Objective: Curcumin (diferuloylmethane) has promising anti-cervical cancer properties but requires a stabilizing complex such as the Pluronic triblock copolymer gold nanoparticle (GNP). The objectives were to study cytotoxicity of curcumin and to determine the effect of copolymer GNPs curcumin complex on cancer cell necrosis. *Materials and Methods:* The HeLa cells were maintained in Eagle Minimal Essential Medium, fetal bovine serum, and antibiotics, and passaged until 60 % confluency was reached. The cells were exposed to either: (1) control medium, (2) 50 μM curcumin, (3) 100 μM curcumin, (4) 50 μM curcumin with copolymer GNPs complex, or (5) 100 μM curcumin with copolymer GNPs complex. The treated cells were incubated at 37°C with 5% CO_2 in air for 24 hours, and analyzed for viability, apoptosis or necrosis using the dual stains fluorescence procedure. *Results:* A dose-dependent increase in the HeLa necrosis was observed with increasing curcumin concentrations. Cytotoxic effect was decreased by five- to ten-fold when the curcumin was complexed with copolymer GNPs. There were more apoptotic HeLa cells at the higher concentration of curcumin but combination with copolymer GNPs resulted in decreased apoptosis. Cell viability was higher in curcumin with copolymer GNPs (74.4 ± 4.8 versus 2.3 ± 2.2 % live, mean ± SEM, with and without copolymer GNPs, respectively). *Conclusion:* Curcumin increased HeLa cancer cell necrosis but its cytotoxicity was decreased by copolymer GNPs. The results suggested that this specific copolymer GNP did not enhance the curcumin bioavailability in cultured cells possibly due to formation of copolymer GNP aggregates.

Key words: Human papillomavirus; HeLa; Curcumin; Triblock copolymer; Gold nanoparticles.

Introduction

Human papillomaviruses (HPV) are classified Group 1 human carcinogens (International Agency for Research on Cancer, IARC) extensively studied for their role in cervical cancer [1]. HPV are double-stranded DNA viruses with a well-defined affinity for epithelial cells. The high-risk HPV serotypes are recognized as the causative agents in nearly all cervical cancers. HPV types 16 and 18 are responsible for 50-70% of all cases [2]. In addition, the viruses are extremely prevalent. It has been estimated that women have up to a 79% lifetime risk of becoming infected with the virus [3]. The overall prevalence in the United States was estimated to be 26.8% in a study published in 2007 [4]. This common virus has been well studied in its relationship to cervical cancer but effective treatment modalities for existing pathologies are still being investigated.

There is considerable interest in the phenolic compound, curcumin (diferuloylmethane) which has anti-cervical cancer properties, anti-inflammatory activities, and anti-oxidative properties [5-9]. Curcumin is isolated from the rhizome of tumeric or Cucumina longa Linn, a member of the Zingiberaceae ginger family. The mechanism of action of curcumin involves reduction of inflammation through downregulated cyclooxygenase-2, lipoxygenase, and inducible nitric oxide synthase enzymes [10]. However, its low solubility and poor stability in aqueous solutions [11] and its rapid systemic elimination [12] limits its use in clinical applications [13]. To overcome these limitations, techniques to stabilize curcumin such as conjugation or encapsulation of curcumin into complexes for optimal delivery into cells have been recently reported. Specifically, curcumin has been covalently conjugated to gold nanoparticles (GNPs) [14-15], silica nanoparticles [16], encapsulated in peptide-based nanoparticles [17-18], and polylactic acid nanoparticles [19].

In this study, GNPs were used as part of the stabilizing complex. Past studies did not find GNP cytotoxicity and reported cell viability of up to 98 % [20-23]. The present authors' hypothesis was that the triblock copolymer (TBP) GNPs would enhance cytoxicity of curcumin by improving bioavailability and cell uptake. The main objectives were: (1) to study the cytotoxicity of different concentrations of curcumin on HPV-18 associated HeLa cells derived from human cervical squamous cell carcinoma, and (2) to determine the effectiveness of triblock copolymer GNPs in enhancing delivery of curcumin into HeLa cells.

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Materials and Methods

Curcumin solution was prepared by dissolving curcumin powder in ethanol and vortex-mixing for five minutes. The curcumin stock solution was diluted with Eagle Minimal Essential Medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and 100 µg/ml of primocin. This solution was used in the preparation of the 50 and 100 µM curcumin concentration solutions.

The GNPs were obtained from the laboratory of Christopher Perry and colleagues [24]. The negatively-charges GNPs were embedded within globules of Pluronics (BASF) triblock copolymers. Briefly, the triblock copolymers GNPs were formed by reduction and capping of hydrogen tetrachloroaurate (III) trihydrate (HAuCl4.3H2O) by triblock copolymers (Pluronics L31 and F68) of polyethylene oxide (PEO) and polypropylene oxide (PPO) as previously reported [24-26]. The resulting triblock copolymer GNP micelle contained a hydrophilic corona of PEO and a hydrophobic core of PPO that functioned as a 'cargo hold' (polymer shell) for transporting reagents [27]. The size of each triblock copolymer GNP was approximately 60 nm in diameter. This size was relatively similar to the optimal size of a 50 nm nanoparticle for optimal cell uptake and internalization [22].

The solution of triblock copolymer GNPs (final concentration ten nM) were stored at 4°C until usage. In the treatment groups involving combined curcumin and triblock copolymer GNP, the curcumin solution (50 or 100 µM) was mixed with triblock copolymer GNPs (10 nM), incubated for 30 minutes and visually confirmed by color change before use in the cell cultures.

HeLa cells derived from human cervical squamous cell carcinoma containing HPV-18 integrated at chromosome 8 were obtained. The cells were maintained in Eagle Minimal Essential Medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and 100 µg/ml of primocin. After reaching approximately 100 % confluency, passage of cells was carried out by trypsinization. They were then centrifuge washed (1000 rpm for seven minutes) and the resuspended cells divided into several center-well organ culture dishes (Falcon 3037). The cells were cultured for 24 hours at 37°C in 5% CO_2 in air mixture and examined under the microscope (magnification ×400) for confluency. In all groups, cells that had reached at least 60% confluency were used in the experiments.

The cultured HeLa cells were exposed to either: (1) control medium, (2) 50 µM curcumin, (3) 100 µM curcumin, (4) 50 µM curcumin+TBP-GNP complex, or (5) 100 µM curcumin+TBP-GNP complex. The cells in the five treatment groups were further incubated at 37°C, 5% CO_2 in air for an additional 24 hours. The cells were analyzed for viability, apoptosis or necrosis (Figure 1) using the dual stains fluorescence procedure as described below [28]. The experiment was repeated for a total of six replicates for each group.

The dual stains fluorescence microscopy method has previously been successfully used to distinguish viable, apoptotic or necrotic cells [28, 29]. Briefly, one drop of bisbenzimide (five µL of ten µM, Hoechst 33342) was added into each culture dish containing the HeLa cells incubated in one ml culture medium. After one minute, five µL of propidium iodide (32 µM dissolved in saline) was added to the same culture dish. After another minute, the culture medium with the stains was discarded and pre-warmed culture medium (0.5 ml) was added to each dish. The culture dish was placed on the stage of an epifluorescence UV-microscope set at magnification of 500 x and images of the fluorescent colored cells in each dish were captured on a digital camera. The percentages of viable, apoptotic, and necrotic cells were determined for each treatment group. For the purposes of this study, a viable cell was defined as the capacity of the cell to exclude the fluores-

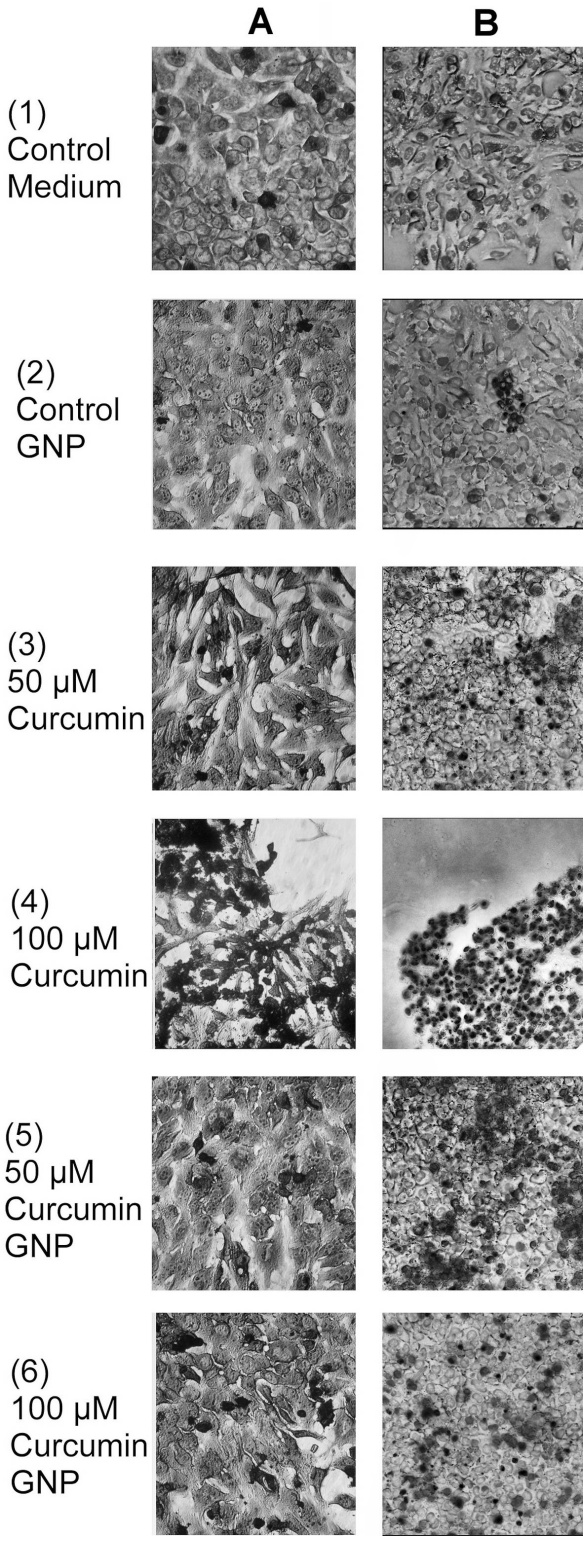

Figure 1. — HPV18-associated HeLa in either: (1) control, (2) Triblock copolymer-gold nanoparticles (GNPs), (3) 50 µM curcumin, (4) 100 µM curcumin, (5) 50 µM curcumin+TBP-GNP, and (6) 100 µM curcumin+TBP-GNP. Spermac-stained cells (left column A, ×400) and dual-fluorescence cells (right column B, ×250). Dark, grey and light-colored cells are necrotic, apoptotic, and live, respectively.

Table 1. — *Comparison of live, apoptotic, and necrotic HPV18-associated HeLa cells after exposure to either: (1) control carrier medium, (2) control GNP only, (3) 50 μM curcumin, (4) 100 μM curcumin, (5) 50 μM curcumin+TBP-GNP complex, and (6) 100 μM curcumin+TBP-GNP complex. Dual-stain epifluorescence analyses were performed on the cells in each group after incubation (37°C, 5% CO_2 in air for 24 hours). The acronym TBP was triblock copolymer while GNP was gold nanoparticle. Values are expressed as mean ± S.E.M.*

Treatment groups (6 replicates per group)	Total cells (n)	Viable Cells (%)	Apoptotic Cells (%)	Necrotic Cells (%)
(1) Control medium	343	81.0±4.4	14.2±4.1	4.8±3.3
(2) Control GNP only	388	69.4±5.5	27.2±5.9	3.4±1.0
(3) 50 μM curcumin	347	64.1±8.9[a]	15.4±10.2	20.5±2.3[a]
(4) 100 μM curcumin	343	2.3±2.2[a]	36.4±11.6[a]	61.3±13.5[a]
(5) 50 μM curcumin+ GNP	350	58.8±7.7[a]	36.9±6.8[a,b]	4.3±1.6[b]
(6) 100 μM curcumin+ GNP	353	74.4±4.8[a,c]	19.3±3.9[c]	6.3±0.9[c]

[a] Different from the control group 1 ($p < 0.05$).
[b] Different from the corresponding 50 μM curcumin group 2 ($p < 0.05$).
[c] Different from the corresponding 100 μM curcumin group 3 ($p < 0.05$).

cent dyes and to display clear coloration. In contrast, an apoptotic (completely blue color) or necrotic (pink-red color) cell was identified by the cell's capacity to absorb bisbenzimide or propidium iodide stain, respectively. Remaining portions of the treated cells were fixed and stained using the Rose Bengal – Janus Green based Spermac stain kit for morphology assessment.

The number of cells needed for each group was determined using the Kelsey calculation for unmatched sample size for case-control studies with 95% confidence and 80% power. Differences in the percentages of apoptotic, necrotic or viable cells in each treatment group were tested using ANOVA and Tukey post-hoc statistics. A value of $p < 0.05$ was considered significant. Data in Table 1 are presented as mean ± standard error of the mean (SEM). Standard error of mean was used as it described accuracy from the population mean in contrast with standard deviation which focused on the spread of data even if inaccurate.

Results

There was a dose-dependent increase in the percentage of necrotic HeLa cells with increasing concentrations of curcumin (Table 1). At the higher concentration of curcumin, the necrotic cells appeared as darkened structures with reduced cell-to-cell contact (Figure 1A, group 3). The nuclei of the cells in this group stained red (dark to black coloration in a grey-scale image) indicating compromised nuclear membranes (Figure 1B, group 3). In contrast to the curcumin only results, the addition of triblock copolymer GNPs blocked the cytotoxic effects of curcumin on HeLa cells. Triblock copolymer GNPs alone had no effect on cell necrosis (3.4 ± 1.0 % versus control 4.8 ± 3.3 % necrosis).

The percentage of apoptotic HeLa cells increased at the higher (100 μM) but not at the lower concentration of curcumin. However, when triblock copolymer GNPs were combined with the lower concentration of curcumin, the percentage of apoptotic HeLa cells increased by two-fold (combined 36.9 ± 6.8 versus curcumin only 15.4 ± 10.2 % apoptosis). At the higher curcumin concentration and presence of triblock copolymer GNPs, an effect on cell apoptosis was not observed.

As expected, in tandem with the finding of a dose-dependent increase in HeLa necrosis with increasing concentrations of curcumin, a dose-dependent decrease in cell viability with increasing curcumin was observed. Percentages of live cells were also lower in the combined curcumin and triblock copolymer GNPs treatment groups when compared with the control group. In contrast, the cells in the group with combined triblock copolymer GNPs and the higher concentration of curcumin (Figure 1A, group 5) had the greatest percent viability (74.4 ± 4.8 versus 2.3 ± 2.2 % live, with and without triblock copolymer GNPs, respectively).

Discussion

Curcumin is a phenolic compound well-known for its anti-cervical cancer properties, anti-inflammatory activities, and anti-oxidative properties [5-9]. In the present study, curcumin was found to be cytotoxic to HPV-18 associated HeLa cell. The process involved apoptotic pathways. The cytotoxicity was consistent with previous published reports [7, 30, 31]. However, the well-known low solubility and poor stability of curcumin in aqueous solutions [11] and its rapid systemic elimination [12] limit its use in clinical applications [13].

To enhance the bioavailability of curcumin, it was proposed that a 'cargo hold' transporting agent such as triblock copolymer GNPs could be used to improve the internalization of curcumin into the HeLa cancer cells. The results showed that the copolymer GNPs were non-cytotoxic to the HeLa cells. Furthermore, past studies did not demonstrate cytotoxicity of GNPs [20-23]. The premise here was that the curcumin molecules would be adsorbed on the copolymer GNP surface and taken into the cells through the receptor-mediated endocytosis pathway (RME). However, the present results did not support the combined use of copolymer GNPs and curcumin. Although, there was increased cell apoptosis at combined copolymer GNPs and low concentration curcumin, an increased in necrosis was not observed. Interestingly, the copolymer GNPs blocked the cytotoxic effect of the higher concentration of curcumin. One possible explanation was that excessive curcumin molecules oversaturated the polymer GNPs resulting in formation of micellar aggregates of the nanoparticles that became too large for cellular uptake. The formation of aggregates was due to curcumin altering hydrophilic PEO and hydrophobic PPO units on the polymer molecules. Alternatively, high concentrations of curcumin might have

caused formation of lamella and cylinders of nanoparticles [27]. Support for the former explanation could be found in a study of polyvinyl pyrrolidone coated GNPs [32]. The authors reported decreased cytotoxicity when the aggregates became too large. Previous studies have emphasized the importance of the size of the nanoparticles in cellular uptake [22, 33]. The studies showed gradual cell uptake with a maximum uptake achieved after four to seven hours. They also showed that a single HeLa cell could take in a maximum of 6160 GNPs at the optimum GNP size of 50 nm [22]. Smaller or larger GNPs were less efficiently transported into the cells. This suggested that the functional use of copolymer GNPs as transporting agents required knowledge of concentration effects of the agent marked for delivery into the cells.

In summary, curcumin was shown to increase necrosis of HeLa cancer cells. Non-toxic triblock copolymer GNPs were effective in increasing cell apoptosis only at the low concentration of curcumin. Combined high concentration of curcumin and copolymer GNP had reduced cytotoxicity possibly due to formation of aggregates. Further studies are needed to continue assessment of other conjugate or polymer nanoparticle combinations and curcumin for the improvement of treatment modalities for HPV-related cervical carcinoma.

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Corresponding Author:
P.J. CHAN, Ph.D., H.C.L.D.
11370 Anderson St., Suite 3950
Loma Linda, CA 92354 (USA)
e-mail: pchann@yahoo.com