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Curcumin-albumin conjugates as an effective anti-cancer agent with immunomodulatory properties

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ABSTRACT

Curcumin (diferuloylmethane) is an active ingredient in turmeric (*Curcuma longa*) with anti-inflammatory, antioxidant, chemopreventive, chemosensitization, and radiosensitization properties. Conjugation of curcumin (Curc) to albumin (Alb) has been found to increase the aqueous solubility of the drug. The current study aimed to prove the safe use of the Curc-Alb conjugate in animals and to demonstrate that it retains drug action both *in vitro* and *in vivo*. Dalton's lymphoma ascites (DLA) cell viability was inhibited by the Curc-Alb conjugate in a dose dependent manner *in vitro*, as evidenced by the MTT assay. Administration of up to 11.4 mg of conjugated curcumin per kg body weight to healthy animals was non-toxic both in terms of lethality and weight loss. Histological analysis of vital organs (kidney, liver and spleen) also did not show toxic effects. Favorable immuno-modulatory activity was observed after continuous administration of sub-acute doses of the conjugate which caused increase in total leukocyte count, platelet count, and viable cell count in bone marrow, and enhanced proliferation of lymphocyte *in vitro* upon culture. *In vivo* studies in the DLA tumor model in mice demonstrated that conjugated drug induces tumor reduction and prevention. Significant tumor reduction was observed when the Curc-Alb conjugate was administered intraperitoneally in DLA-induced mice after 1 day (prevention therapy) and 7 days (reduction therapy) of tumor induction. There was significant reduction in both tumor volume and tumor cell numbers in the treated animals as well as a marked increase in their mean survival time and percent increase in life span. The effect was greater when the conjugate was administered soon after inducing the tumor as compared to when treatment was started after allowing tumor to grow for 7 days. Thus, the results of the present study suggest that curcumin albumin conjugate has immunomodulatory and tumor growth inhibition properties. The study postulates the drug form has the potential to be used as an anticancer agent in affected human subjects.

1. Introduction

Despite progress in the development of strategies for cancer prevention and diagnosis, the disease continues to remain the leading cause of death worldwide. Although chemotherapy is a widely practiced treatment modality, this method has several limitations. Most of the currently used chemotherapeutics suppress the immune system, predisposing cancer patients to secondary infections [1]. Therefore, there is a significant unmet need for novel pharmaceutical regimens with tumor selectivity and specificity, but without side effects. Several molecules under consideration for cancer treatment are the subject of immense *in vitro* research, but many do not reach clinical application due to various problems. One such natural molecule is curcumin.

Curcumin is a diaryl heptanoid [2], which is the principal curcuminoid of turmeric, the popular South Asian spice coming under the ginger family (Zingiberaceae). It is suggested that hydroxyl groups on the benzene rings, double bonds in the alkene part and central β-diketone moiety play a crucial role in the drug actions of curcumin [3]. Several studies have shown that curcumin exerts antioxidant, anti-inflammatory, anti-carcinogenic and chemopreventive actions on many tumor cells [4]. However, the major problem which limits its use as an effective therapeutic agent is its low aqueous solubility and bioavailability [5]. Therefore, this very important anticancer agent needs to be modified to improve its bioavailability. Curcumin has preferential interaction with serum albumins and lipid membranes [6–8]. Liposomes and serum albumins are some of the most commonly used transporting vehicles for drugs, peptides and hormones [9,10].

Previous work in our laboratory demonstrated that conjugation of curcumin to serum albumin (Alb) increases aqueous solubility of the former without affecting its drug action *in vitro* on both cancer cell lines and primary cells in culture [11]. The Curcumin-Alb conjugate could exert anti-tumor effects through direct cytotoxicity and through its effect as an immuno-modulator. However, its safety and anticancer activities were not studied *in vivo*. In this study, we evaluated the effectiveness of the Curc-Alb conjugate in more challenging ascites tumor models so that the use of this drug can advance to clinical trials. Another aspect of this study was to test if the Curc-Alb conjugate has any effect on the immunological response elicited as a consequence of cancer cell death.

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2. Materials and method

2.1. Chemicals

Curcumin, bovine serum albumin (BSA), Sephadex G-25 beads, MTT, DMEM/F12, PHA, Histopaque 1077 were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Antibiotic-Antimycotic, RPMI 1640, and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation, USA.

2.2. Preparation of curcumin albumin conjugate

The curcumin-Alb conjugate was prepared by mixing both at predefined proportions at specified conditions and purifying it using gel filtration chromatography as per the in-house developed method [11]. Briefly, 10 μL curcumin (1 M) in DMSO and 5 mL albumin (50 mg/mL in PBS) were mixed and conjugated at 20–22 °C for 30–60 min. The mixture was then loaded on Sephadex G-25 columns for removing unreacted curcumin from the conjugate which was eluted in the void volume. The Curc-Alb conjugate was identified by measuring the A280:A420 ratio of 1 mL fractions eluted from the column. The protein fractions with minimum spectral peak ratio (~2) were considered to have maximum curcumin bound to albumin. Such fractions were pooled, filtered through a 0.22 μm syringe filter (Millipore, USA) and 1 mL aliquots of the pooled conjugate were lyophilized (Edwards Modulyo, Edwards, UK) and stored at 4 °C. The lyophilized protein was dissolved in water and used for further experiments.

2.3. Quantification of bound curcumin in the conjugate

One mL PBS was added to the lyophilized powder in one vial and the albumin concentration was estimated using the standard Lowry assay. To estimate the concentration of the bound curcumin in the conjugate, 50 μL of PBS was added to another aliquot and the drug was extracted by adding 950 μL of DMSO. The curcumin extracted into DMSO was centrifuged at 1000*g* for 15–20 min and absorbance at 420 nm was measured. The concentration of curcumin in the extract was estimated from a calibration curve generated with 2 μM to 20 μM curcumin in DMSO. From the values of the albumin concentration and extracted curcumin, the quantity of curcumin conjugated per mg protein was estimated.

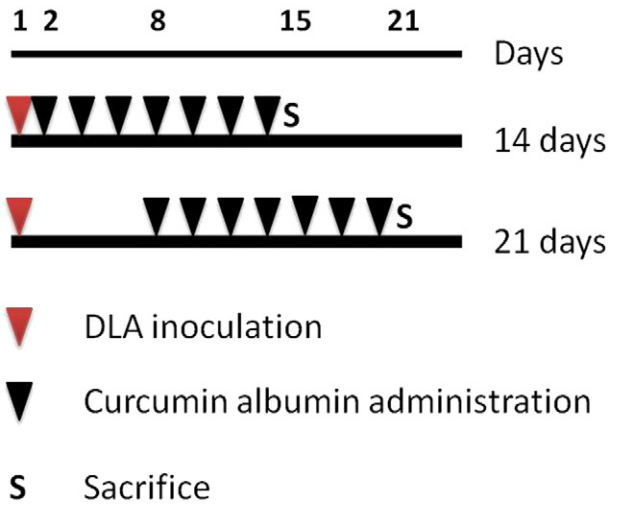

Fig. 1. Experimental design for testing the efficacy of Curc-Alb to reduce cancer growth in DLA mice model.

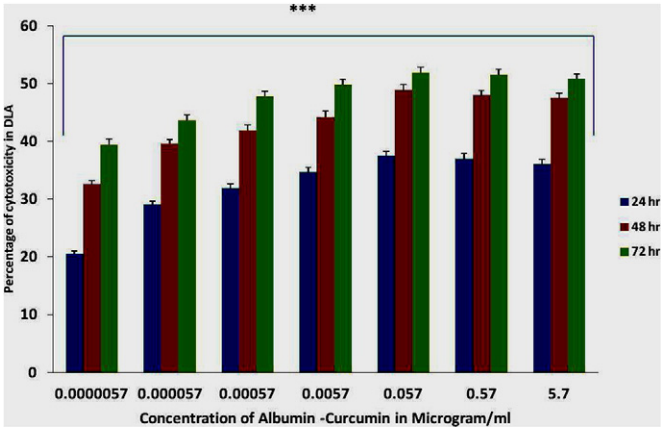

Fig. 2. Graphical representation of cytotoxicity assay of Curc-Alb conjugates using DLA tumor cells. Same number of cultures was exposed to graded concentrations of Curcumin (conjugated) added into medium and assay was done at different periods. Percentage of non-viable cells at each period was calculated; mean ± S.D. (n = 6) is represented in the graph. Statistically significant differences are at *P < 0.05, **P < 0.01, ***P < 0.001; ns = non-significant, as compared with control group.

2.4. Cell lines

DLA (Dalton lymphoma ascites) murine lymphoid cancer cell lines were procured from the Regional Cancer Center, Trivandrum, Kerala, India, and were maintained by weekly intra-peritoneal inoculation of 10^6 cells/per normal mouse.

2.5. In vitro evaluation of Curc-Alb

2.5.1. Determination of cytotoxic activity by MTT assay

Cells (5000 DLA cells/well) were seeded in 96-well, flat-bottom culture plates and incubated for 24 h, 48 h, and 72 h at 37 °C in 5% CO_2 atmosphere. Graded concentrations of Curc-Alb conjugate (0.001–1000 μg/mL) were added to culture well and incubated for various time periods. After completion of incubation, the medium was removed. The wells were washed with PBS, 100 μL of the working MTT dye in DMEM media was added, and they were incubated for 2 h. MTT lysis buffer (100 μL) was added and the incubation continued for 4 h more. The absorbance was measured at 570 nm and the proliferation rate (PR) was calculated using the formulae:

$$PR = \text{Absorbance of Test} \times 100 / \text{Absorbance of Control}.$$

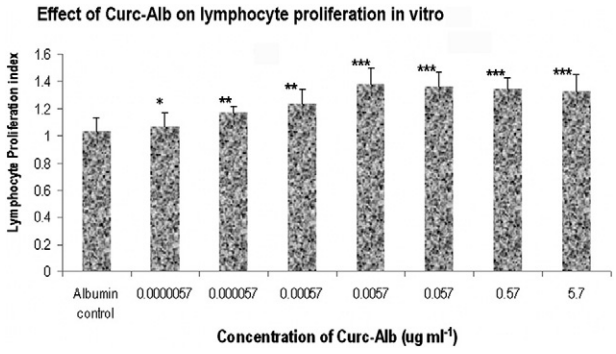

Fig. 3. Graphical representation of lymphocyte proliferation index upon treating with Curc-Alb. Lymphocytes isolated from normal mice was used for proliferation assay. The viable cells in treated wells divided by viable cells in normal control well gives the proliferation index which is presented as Average ± S.D.(n = 6). Statistically significant differences are at *P < 0.05, **P < 0.01, ***P < 0.001; ns = non-significant, as compared with control group.