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Anticancer Effects of *Escherichia Coli*-Derived Outer Membrane Vesicles Against Colorectal Cancer

Marjan Ghiyasvand[1], Ardeshir Hesampour*[1], Hossein Dabiri[2], Javad Arasteh[1]

Abstract

Background: One of the deadliest cancers in the world, colorectal cancer has a dismal prognosis and a poor response to therapy. It was suggested that outer membrane vesicles (OMVs) produced by *Escherichia coli (E. coli)* are a powerful inducer of inflammation in intestinal epithelial cells. This research aimed to determine the anticancer potential of *E. coli*-derived OMVs using a colorectal cancer model.

Methods: Five distinct *E. coli* strains were collected for this study. Their OMVs were then isolated and characterized using dynamic light scattering (DLS) and scanning electron microscopy (SEM). The effects of *E. coli*-derived OMVs on colorectal cancer were evaluated in vitro and in vivo using a colorectal tumor model in nude mice.

Results: Obtained results showed that *E. coli* probiotic strains released spherical-shaped vesicles ranging from 5 to 200 nm. *E. coli*-derived OMVs showed that in the untreated group, a large portion of the tumor tissue continued to grow, with only a few cells undergoing apoptosis. Conversely, the OMV-treated group exhibited a higher number of apoptotic cells, highlighting the anticancer effects of E. coli-derived OMVs in colorectal cancer.

Conclusion: These results demonstrated that *E. coli*-derived OMVs can be employed as a potential treatment for colorectal cancer with minimal adverse effects. Mechanistic studies indicate that these vesicles may promote apoptosis and inhibit cell proliferation, supporting their therapeutic potential.

Keywords: CT-26, Colorectal cancer, *E. coli*, OMVs.

Introduction

Colorectal cancer (CRC) is a prevalent gastrointestinal malignancy and a significant cause of morbidity and mortality (1-4). Currently, CRC has the third-highest new cancer incidence rate in the world (4, 5). Approximately 600,000 deaths and over one million new cases are reported annually, which poses a significant threat to the global economy (6). By 2030, it is projected that there will be over 2.2 million new cases and 1.1 million deaths annually (7). Approximately 20% of patients present with *de novo* metastatic disease, and 25-30% of patients with stage II/III illness will have a recurrence within 5 years following curative intent surgery (8). A variety of risk factors for colorectal cancer have been identified, including age, obesity, smoking, alcohol use, a family history of colorectal cancer, and inflammatory bowel disease (9, 10). It has recently been proposed that the development of this illness may involve gut bacteria, including commensals and pathogens (3, 7, 11). At present, chemotherapy, surgery, and radiotherapy are the three primary treatments for colorectal cancer. Furthermore, certain species of intestinal flora and their biological products have been administered with

1: Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran.
2: Microbiology Department, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
***Corresponding author: Ardeshir Hesampour; Tel: +989123017118; E-mail: Ardeshir.Hesampour@iau.ac.ir.**
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unexpected success in cancer therapy, particularly immunotherapy (12). Previous studies have substantiated the anticancer properties of the microbiota, as well as their metabolites and oncogenic toxins (13, 14). This is exemplified by the ongoing investigation of bacterial outer membrane vesicles (OMVs) from gram-negative bacteria, which has lasted for more than fifty years (15). OMVs are non-replicative particles that are naturally occurring and have a size between 30 and 250 nm. OMVs contain a variety of bacterial cargoes, such as proteins, peptidoglycan, lipopolysaccharide, nucleic acids, and bacterial toxins, which are comprised of various immunostimulatory components (16-20). OMVs derived from *Escherichia coli (E. coli)* are thought to play a range of roles in bacterial-host interactions, including immune response modulation, gut homeostasis, intestinal epithelial barrier integrity control, and horizontal gene transfer (21-23). These properties provide a distinct and unique advantage in the development of vaccinations, targeted medicine administration, clinical tumor therapies, and cancer diagnostics (24, 25). Given the significance of colorectal cancer detection and therapy, the current research sought to determine the anticancer effects of *E. coli*-derived OMVs on colorectal cancer. We created a colorectal tumor model in nude mice and investigated whether *E. coli*-OMVs may provide anticancer effects in vivo.

Materials and Methods

Bacterial strain and E. coli-derived OMV preparation

Five different and non-repeating probiotic strains of *E. coli* were procured from Histogenotech company located in Tehran, Iran. The strains were cultivated using MacConkey and Eosin Methylene Blue Agar (Qlab, Canada), and they were incubated for 48 hours at 37 °C. To identify distinct *E. coli* strains, common biochemical tests, such as lactose fermentation, citrate, MR, VP, and indole were carried out. Every strain was kept at -70 °C in a 20%–25% BHI medium.

To isolate OMVs, *E. coli* isolates were sub-cultured in BHI medium (Merck, Darmstadt, Germany), and the broth was incubated for 72 hours at 37 °C. After incubating the bacteria for 72 hours, 0.5 liters of BHI medium was centrifuged at 18,000 g for 15 minutes at 4 °C to isolate the bacteria, and the resultant supernatant was filtered through standard bacteriological filters (0.45-μm) (Corning, USA). Outer membrane vesicles (OMVs) were then extracted by ultracentrifugation (Beckman, CA, USA) operating at 200,000 g for three hours at 4 °C (26). For further research, extracted OMVs were kept at -80 °C after being cleaned in 10 milliliters of sterile distilled water.

Scanning electron microscopy (SEM) analysis

A scanning electron microscope was used to evaluate the morphology of *E. coli*-OMVs. The OMVs were preserved using 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA). Dried samples were coated with gold using a sputter coater (SBC-12, KYKY, China) by a physical vapor deposition process after washing with PBS. The SEM (KYKYEM3200, KYKY, China) was used to examine the generated samples.

Particle size analysis of OMVs by dynamic light scattering (DLS)

DLS was employed to quantify the size distribution of OMVs. In summary, the diameters of various OMVs were determined using Zetasizer 3000 HSA (Malvern, Worchestershire, UK) after the OMVs were diluted to a concentration of 0.1 mg/mL in milli-Q water.

Therapeutic efficacy in vivo

Six male BAB/c mice (6-8 weeks old, weighing 25 ± 2 g) were provided by Pasteur Institute in Tehran, Iran. The Ethics Committee for Animal Care at Islamic Azad University's Central Tehran Branch authorized all experimental protocols. All animals were kept in a conventional animal-grade room with specific pathogen-free settings. The temperature was maintained at 23 ± 3 °C, the relative humidity at 50 ± 10%, and the light cycle at 12 hours daily. Colorectal cancer

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(CRC) induction was achieved by subcutaneously administering 5×10^5 CT26 cell lines suspended in 100 µl PBS into the abdominal region. Following 14 days of tumor development, the mice were randomly allocated into two groups (n = three per group). The control groups received either PBS alone or nothing. Every three days, mice received intratumoral injections of 5 µg/mL *E. coli*-OMVs (100 µl each animal). After 14 days of *E. coli*-OMV treatment, the mice's colon tissues were taken for histological and biochemical analysis.

Histological Evaluations

Upon conclusion of the 14-day experiment, the mice were killed, and their cancerous tissues were euthanized, and preserved in a 10% neutral buffered formalin solution. Following dehydration, the tissues were fixed in paraffin for one hour and sectioned into 5 µm thick slices using a microtome. The portions were then deparaffinized in a 90 °C oven for twenty minutes and rinsed with xylene. The tissue was hydrated in a sequence of progressively diluted ethanol solutions for microscopic inspection. The tissue slices were then stained with hematoxylin and eosin (H&E) dye for 3-5 minutes and analyzed using an optical microscope (LABOMED).

Statistical Analysis

All data were presented as mean ± SD. Statistical analysis was conducted using GraphPad Prism (version 6; San Diego, USA) and SPSS 16.0. Group comparisons were conducted using one-way ANOVA followed by Tukey's HSD post-hoc test. A p-value less than 0.05 is deemed significant.

Results

E. coli strains released spherical OMVs

Under the biochemical and microscopic analysis, gram-negative rods were found to have a positive response to the MR test, a negative reaction to the VP and citrate reactions, and a positive Indole reaction. SEM and DLS were used to characterize the isolated OMVs; over 90% of the OMVs were found to be in the 5-200 nm size range (Fig. 1). Thus, the polymorphic morphological nature of OMVs was supported by scanning electron microscopy observations (Fig. 2).

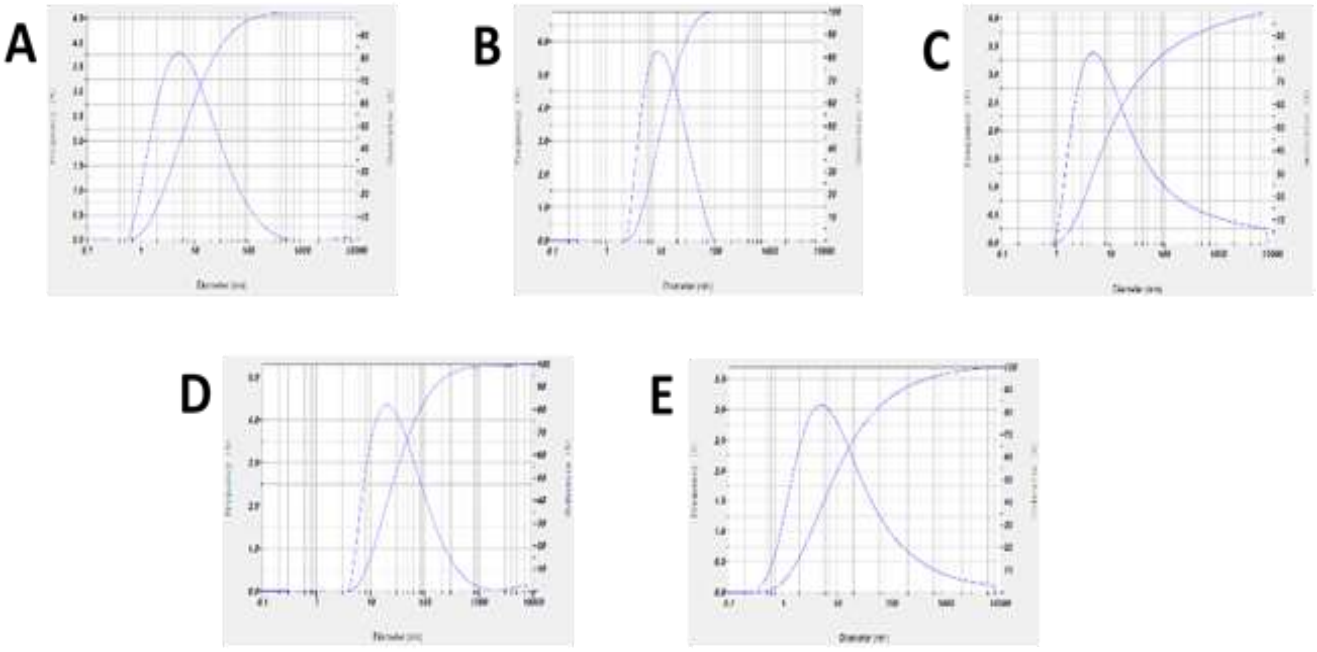

Fig. 1. Physicochemical characteristics of *E. coli*-derived OMVs based on DLS. Size distribution is determined based on the intensity of OMVs in the ultracentrifugation technique. DLS confirmed nano-sized OMVs in a range of about 5–200 nm, A. OMV1, B. OMV2, C. OMV3, D. OMV4, E. OMV5.

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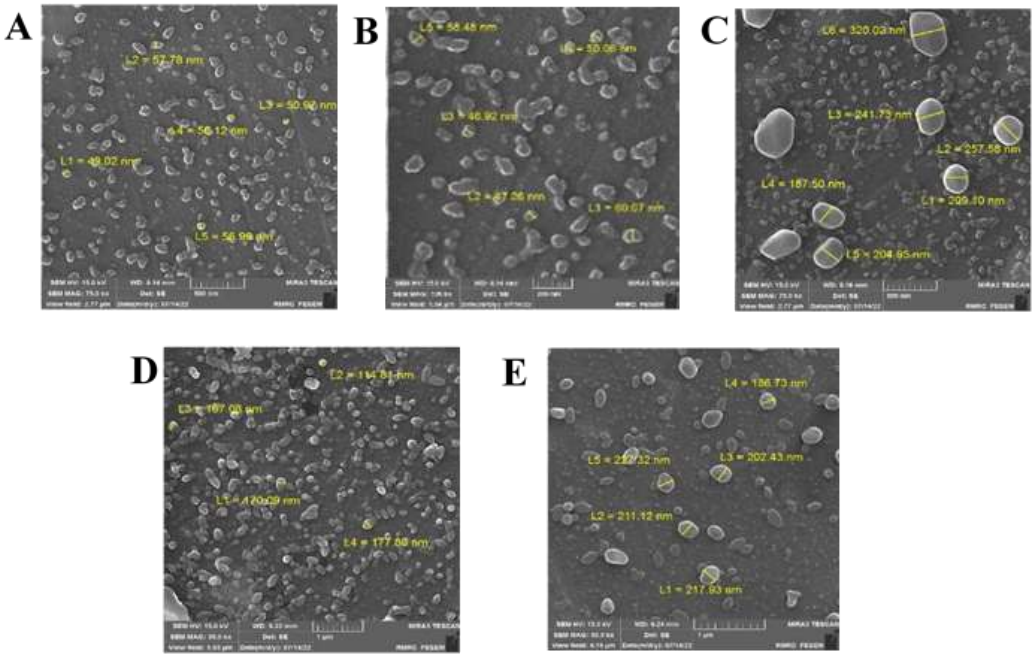

Fig. 2. Scanning electron microscopy (SEM) of *E. coli*-derived OMVs showed the polymorphic vesicles in different sizes. A. OMV1, B. OMV2, C. OMV3, D. OMV4, E. OMV5. (SEM at magnification: ×40 kX).

Histopathology Experiment

The study conducted histological assays to investigate the effect of OMVs on CRC and explore the potential therapeutic benefits of OMVs. Figure 3 shows a transverse section of mouse colorectal tumor tissue stained with H&E. Different areas of the tumor were evaluated, including the peripheral and central parts of the tumor. In each of the regions, areas with high proliferation cells, apoptotic cells, and necrotic cells were visible. Hence, the size of the tumor, the amount of angiogenesis, and the blood supply to the tissue were visible in different areas of the tumor.

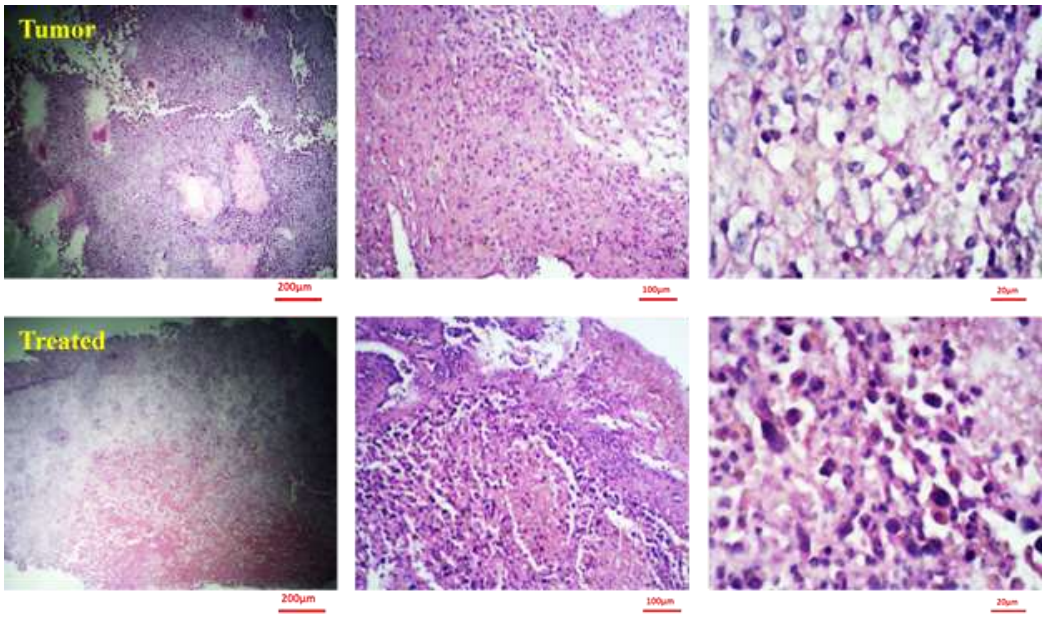

Fig. 3. Histopathological assessment of metastasis in the tumor tissue of mice with colorectal cancer in control group (up) and treatment group with OMVs (down).

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In the peripheral area of untreated tissue, most of the cells that have high proliferation were mostly in the mitosis phase, which indicated the high activity of tumor cells. In this area, the amount of angiogenesis and blood supply to the tissue increased significantly. Consequently, in terms of the high activity of the cells, the tumor size increased. On the other hand, in the center of tumor, we observed a necrotic area, which is due to a lack of oxygen supply to the tissue, and decreased angiogenesis, and the nucleus of the cells was destroyed.

Based on the observations made in the tissue, it was found that in the untreated group compared to the treated group, a large part of the tumor tissue was growing and few cells were in the process of apoptosis, therefore it was placed in grade 1 (Fig. 4). In other words, cell death caused by a lack of blood supply or the effect of any type of treatment was the lowest in this group.

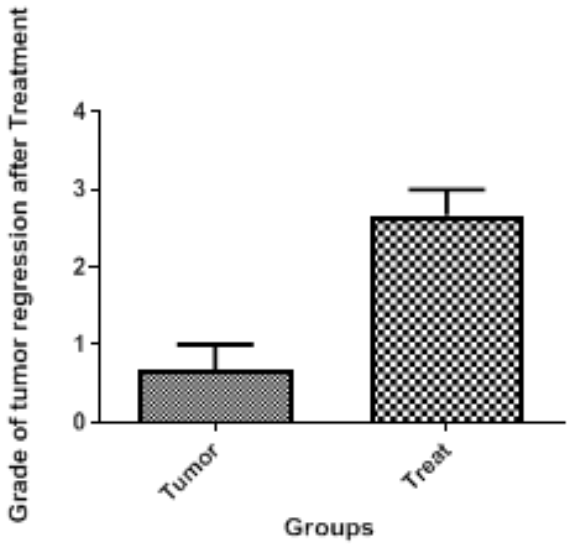

Fig. 4. Grade of tumor regression after Treatment.

Discussion

Globally, CRC is the third most common cancer and the third major cause of cancer-related mortality (27, 28). Despite advances in research for novel therapies, CRC treatment continues to present a number of therapeutic hurdles. Some of these issues include late diagnosis and resistance to chemotherapy, which make effective treatment difficult, as well as the significant risk of developing metastatic diseases. In recent years, OMVs have gained a lot of interest as new cancer therapeutic options (24, 29). They have significant benefits as anticancer immunotherapies and drug-targeted treatments due to their numerous properties, particularly their high levels of immunogenic components (30, 31). Numerous studies have investigated bacterial outer membrane vesicles for the treatment of colorectal cancer. Nevertheless, akin to other novel therapy methodologies, further prospective trials are necessary in this domain. This section examines the anticancer effects of *E. coli* probiotic strain-derived OMVs on CT-26 cells in a colorectal cancer animal model. We isolated OMVs from several non-repetitive probiotic *E. coli* strains. We showed that OMVs have a spherical shape with a diameter ranging from 5 to 200 nm. Similar to our findings, Park et al. conducted a study regarding the lethality effects of *E. coli*-derived OMVs in mouse models. Their investigation demonstrated that the outer membrane vesicles (OMVs) isolated from the supernatant of cultivated *Escherichia coli* were distinguished by their spherical, bilayered morphology. According to DLS analysis, 75.0% of the isolated vesicles had a diameter ranging from 25 to 50 nm (32). A study by Kulkarni et al. indicated that the OMVs extracted from *E. coli* ranged from 50 to 80 nm (33). Furthermore, Imamiya et al. used ultracentrifugation to isolate OMVs from *E. coli* strains, with transmission electron microscopy revealing that the OMVs were around 100 nm in diameter (34). The data indicated that using various techniques to isolate OMVs does not influence their size or shape. The histological results of this study showed that, in the untreated group, a large part of tumor tissue was growing, and few cells were in the process of apoptosis. That is why it was placed in grade I. On the other hand, the group treated with OMVs was placed in grade III. This means that more cells were in the process of apoptosis. The results highlight the emphasis of the anticancer effect of OMVs in colorectal cancer. Consistent

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with our findings, Jin et al. (35) developed a human neuroblastoma subcutaneous xenograft tumor model in nude mice to investigate the potential anticancer effects of *E. coli* outer membrane vesicles in vivo. Their findings illustrate the anticancer properties of OMVs, with no significant side effects seen. Therefore, another study conducted by Lanxi (36) investigated the effect of *E. coli*-OMVs on the breast cancer cell line (4T1) in vivo. Their results showed that OMV can increase cells in the G0-G1 phase and stop the cell cycle. Their results showed the importance of OMV derived from *E. coli* in inhibiting breast cancer cells.

In conclusion, we propose the use of *E. coli*-OMVs as a therapeutic intervention for CRC with few side effects. Our mechanistic investigations indicate that these nanoscale vesicles may reduce cellular growth and enhance the process of apoptosis. Additional pertinent research is required to validate the effectiveness and safety of the OMVs approach for CRC.

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Conflicts of Interest

The authors declare that they have no competing interests.

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