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Efficacy of carnosine on activation of caspase 3 and human renal carcinoma cell inhibition

Muthuraman Pandurangan, Bhupendra Mistry, Gansukh Enkhataivan*, Doo Hwan Kim*

Department of Bioresources and Food Science, Konkuk University, Seoul, South Korea

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A B S T R A C T

Carnosine is a natural antioxidant dipeptide that is highly concentrated in muscles and brain. The present study investigated the effect of carnosine cell growth inhibition and activation of the caspase-3 enzyme under cell co-culture system. Renal carcinoma and normal cells were co-cultured to provide three-dimensional views for the experimental analyses. Carnosine inhibited renal cancer cell growth up to 40%, whereas it was 25% in normal cells. Caspase-3 enzyme activity corresponded to the appearance of immunofluorescence in the cytoplasm using the caspase-3 antibody. Caspase-3 enzyme activity gradually increased in renal carcinoma cells in a concentration-dependent manner. The increased immunofluorescence and fluorescent detection of caspase-3 indicated the occurrence of apoptosis. The binding affinity of carnosine with caspase-3 subunit was confirmed by In silico docking study and glide energy was −5.2 kcal/mol. Taking all these data together, it is suggested that the carnosine may be a potential antiproliferative agent in renal carcinoma tumor.

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1. Introduction

Renal cell carcinoma is a most common type of kidney cancer that originates from the lining of proximal convoluted tubule. Renal cell carcinoma is the most frequently occurs in adults [1]. The treatment of renal cell carcinoma is either complete or partial removal of kidneys, and treatment for this cancer remains elusive. Chemotherapy and radiation therapy are highly resistant to carcinoma cells. The survival rate is about 65–90% if carcinoma is burrowed deeper or metastasized [2]. The process of programmed cell death is called apoptosis that plays a vital role in prevention and treatment of cancer [3]. The key apoptotic modifications are nuclear fragmentation, nuclear shrinkage and reduction in cell volume. The condensation of cytoplasm and nucleus could produce membrane bound apoptotic bodies that are phagocytized by macrophages [4]. The loss of apoptosis and uncontrolled proliferation has been known as crucial factors for the tumor development. An excellent anti-tumor agent should prevent the proliferation of tumor cells [5].

Carnosine is a well-known dipeptide, present highly in olfactory bulb and muscles of the animal system [6]. Lean beef, fish, and chicken have been reported as the primary source for carnosine [7]. Carnosine has been recommended 300–600 mg/day of uptake [8]. The enzyme carnosinase liberates free carnosine from food supplementation in our body. Park et al., [9] have reported that the carnosine has been metabolized rapidly following red meat consumption, and carnosine level was found to be zero in blood during the postprandial period. Guiotto et al., [10] have reported the biological function of carnosine such as anti-oxidant, metal-chelation, anti-inflammatory and anti-glycation.

Anti-proliferative effect of carnosine has been reported in animal cells. Yay et al., [11] have been reported the antioxidant property of carnosine in streptozotocin-induced diabetic rats. Carnosine exhibited an antiproliferative effect in human colorectal carcinoma cells through the interference of ATP, ROS, and cell cycle [12]. Tumor cell growth inhibition by carnosine has been reported in NIH3T3-HER2/neu mouse model [13]. Janssen et al., [14] have reported the effect against adverse effects produced by high blood glucose on renal cells.

Tissues are present in complex architecture and highly ordered in an animal, which is three-dimensional in nature with its complex interaction between adjacent cell types. Hence, the complex tissue architecture differs from a one-dimensional in vitro monoculture system [15]. Cell co-culture represents the growth of two different cell types in a common shared medium. The physical contact between these cell types might influence the cell function. Cell co-culture system is widely used for the investigation of angiogenesis, liver function, muscle, immune cell function, blood-brain barrier dynamics and nerve interaction [16,17]. Several studies have been reported the use of three-dimensional cell co-culture system in bio-

* Corresponding authors at: Dept. of Bioresources and Food Science, Konkuk University, Seoul, 143-701, South Korea.
E-mail address: frenzram1980@gmail.com (D.H. Kim).

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logical experiments [18]. Thus, we selected the cell co-culturing system to evaluate the effect of carnosine on renal carcinoma and normal cells.

The present study was aimed to investigate the effect of carnosine on cell viability, and caspase 3 expression in the co-cultured human renal carcinoma (Caki-2) and Madin-Darby canine kidney cells (MDCK) cells. The binding activity of carnosine with a subunit of the caspase- 3 was determined using AutoDock Vina program.

2. Materials and methods

2.1. Materials

Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Welgene (Daegu, South Korea). L-carnosine and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO63178 USA). Fluorescein diacetate (FDA), propidium iodide (PI) and Rabbit polyclonal caspase-3 antibodies (C1815) were purchased from Santa Cruz Biotechnology, Inc. (Delaware Avenue, California, USA). Cell well insert (0.4 μm) was purchased from SPL Life Sciences (Korea). Donkey Anti-Rabbit IgG H&L (FITC) conjugated secondary antibody (GR200554-2) was purchased from Abcam (330 Cambridge Science Park, Cambridge).

2.2. Cell culture

MDCK and Caki-2 cells were obtained from ATCC (10801 University Boulevard Manassas, VA 20110 USA). Cells were kept in the growth medium supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin). Cells were maintained in a CO_2 incubator under standard condition (37 °C and 5% CO_2).

2.3. Co-culture of MDCK and caki-2 cells

Caki-2 and MDCK cells were co-cultured by using transwell inserts with a 0.4 μm porous membrane to separate MDCK and Caki-2 cells. Cells were grown independently in the cell well insert. After 80% of confluence, inserts containing MDCK cells were transferred to 6-well plates containing Caki-2, and inserts containing Caki-2 were transferred to 6-well plates containing MDCK cells [17].

2.4. Two-color flow cytometric analysis of cell viability

Caki-2 and MDCK cells were co-cultured with the use of cell well insert (2.5×10^4 cells/well). Cells were treated with carnosine (1 μM–10 mM) for 24 h. At the end of 24 h, cells in the lower wells were taken and washed with PBS. Cells were incubated with FDA (0.5 μg/ml) for 40 min in the dark place for 40 min. After that, cells were incubated with PI (50 μg/ml) and tubes were kept on ice immediately up to flow cytometric analysis [19].

Cell viability was determined by measuring the intracellular green and red fluorescence using a BD FACS Calibur Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ 07417). The filters such as 625 nm, 530 nm, and 570 nm were used for the detection of red, green and dual fluorescence respectively. The logarithmic amplification of green and red fluorescence signals was used [19].

2.5. Immunofluorescence for caspase-3

Caki-2 and MDCK cells were co-cultured with the use of cell well insert (2.5×10^4 cells/well). Cells were treated with carnosine (1 μM–10 mM) for 24 h. At the end of 24 h, cells in the lower wells were taken and washed with PBS. Cells were fixed and then permeabilized in 0.1% Triton X-100 in PBS for 20 min and then washed twice with PBS. The blocking was carried out with 3% bovine serum albumin (BSA) in PBS for 30 min, and the cells were then incubated with Rabbit polyclonal caspase-3 antibody (Santa Cruz Biotechnology, Inc) for 12 h at 4 °C in PBS-1% BSA. After washing, cells were incubated with a Donkey Anti-Rabbit IgG H&L (FITC) conjugated secondary antibody and then washed thrice in PBS. The coverslips were mounted with a fluorescent mounting medium, and viewed under Confocal Laser Scanning Microscope (CLSM) (1×81^R Motorized Inverted Microscope, Olympus) [7].

2.6. Caspase-3 fluorescence assay

Caki-2 and MDCK cells were co-cultured with the use of cell well insert (2.5×10^4 cells/well). Cells were treated with carnosine (1 μM–10 mM) for 24 h. At the end of 24 h, cells in the lower wells were taken and washed with PBS. Cells were centrifuged, and the supernatant was removed. Then, 200 μl of caspase-3 assay buffer was added to each tube, and the previous step was removed. Then, 100 μl of cell-based assay lysis buffer was added. All the tubes were incubated on a shaker for 30 min at room temperature. All the tubes were centrifuged at $800 \times g$ for 10 min and transferred 90 μl of supernatant to the black 96-well plate. Then, 10 μl of caspase-3 assay buffers and 100 μl of caspase-3 substrate solution were added to each tube. The fluorescent intensity of caspase-3 was measured with excitation at 485 nm and emission at 530 nm (Caspase-3 Fluorescence Assay Kit, Item No-10009135, Cayman Chemical, 1180 East Ellsworth Road Ann Arbor, Michigan 48108 USA).

2.7. Molecular docking

The binding activity of carnosine with a subunit of the caspase-3 was determined by molecular docking (AutoDock Vina version 1.1.2 program) [7]. The crystal structure of the caspase-3 subunit was downloaded from Protein Data Bank (PDB); accession code was 1RE1 (Vaidya et al., 2014). Furthermore, polar hydrogens molecules were added to the structure. A grid box was developed to determine an active site for carnosine binding. The binding site of caspase-3 with carnosine was visualized using PyMol program (Version 1.7.4, Schrodinger LLC, USA).

2.8. Statistical analysis

All the values were expressed as mean ± SD. All the values were obtained from three independent experiments. The difference between control and test was evaluated using Student's *t*-test. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of carnosine on cell viability

In the present study, we have used FDA and PI to differentiate viable and nonviable cells. FDA is a non-fluorescent compound that can freely enter freely and there it is converted into a fluorescent compound by cellular esterase. The fluorescein compound is polar and trapped within the active cells which contain membrane integrity. Whereas, fluorescein diffuses out of the non-viable cells which lack membrane integrity. Thus, the viable cells appear bright green fluorescence. PI is a highly polar and fluorescent compound that can enter the non-viable cells freely where lacks membrane integrity. Thus, the non-viable cells appear as bright red fluorescence [7]. Cell debris and non-viable cells events were separated from the viable cells in the dot plots, which ensures the debris and non-viable cells were not counted in the viable cell window. Co-cultured MDCK and Caki-2 cells were incubated with the FDA for

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