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Original Article

Investigation of the Lethal Effect of Purified Arginine Deiminase Purified from *Lactobacillus plantarum* p5 on Murine Mammary Adenocarcinoma and Vero cell Lines

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Abstract

Cancer is one of the most serious diseases facing humanity; accordingly, it is urgent to find a cure that is rarely harmful to the patient as much as possible. It has been approved that arginine deiminase (ADI) can hydrolyze the plasma arginine to citrulline. This hydrolysis activity and reduction in the amount of intercellular arginine suppress lipopolysaccharide-induced nitric oxide synthesis. On the other hand, arginine depletion arrests the cell cycle at the G1 phase; therefore, ADI has been considered a powerful anticancer agent. The current study aimed to investigate the lethal effects of ADI purified from the *Lactobacillus plantarum p5* strain on murine mammary adenocarcinoma and Vero cell lines. Anti-proliferative activity of ADI against murine mammary adenocarcinoma) AMN3) cell line was evaluated after different incubation times (3, 6, 24, 48, and 72 h) of exposure to 1 µg/mL of ADI, compared to Vero (non-cancer cell line) transformed cell line with same conditions. The autophagy process in cancer cells was recognized after three hours of incubation with ADI which was clearly observed in the AMN3 cell line under an inverted microscope. The first stages of the programmed cell death (apoptosis) pathway were only observed in AMN3 cells after 24 h of incubation with ADI, and this process continued with the time until they reached the last stages of apoptosis after 72 h of incubation. The results of the current study showed that the AMN3 cell line was auxotrophic for arginine because it could not produce it in the presence of enzyme which had a robust activity to kill these cancer cells; however, Vero non-cancer cell line survived in the presence of ADI because it had the ability to produce arginine.

Keywords: AMN3, Arginine Deiminase, Autophagy, *Lactobacillus plantarum p5*, Vero

1. Introduction

Murine mammary adenocarcinoma is glandular cancer that represents a malignant neoplasm of epithelial cells with a gland-like pattern. Autophagy occurs as a cellular response to the starvation of some nutrients, which particularly appears as a result of the loss of amino acids. Induction of the autophagy process during the time of starvation is an evolutionarily protected stress reaction in eukaryotes; however, it is likewise related to the cell fate during its interfered connections with apoptosis (1). This phenomenon can be illustrated by the composition of autophagosomes with double membrane forms. Stimulation of autophagy during periods of starvation is an evolutionarily conserved stress response in eukaryotes (2). The use of an inverted microscope is considered a more popular technique for studying autophagy (3). *Lactobacillus plantarum* is a Gram-positive widespread probiotic beneficial bacterium, and it can produce arginine deiminase (ADI) enzyme in the presence of arginine (4). In contrast to normal tissues, some tumors cannot produce arginine from citrulline (5). In fact,

tumor cells do not have the required enzymes, such as argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) which are essential for arginine de novo synthesis in cells. Therefore, exogenous arginine is essential for the growth and proliferation of these cells. ADI inhibits the growth of cells in these types of cancers by exhausting arginine (6), especially in rhabdomyosarcoma and glioblastoma cell lines which were inhibited by very low concentrations of ADI. On the contrary, the transient rat embryo fibroblast non-cancerous cell line is proliferated in the presence of this enzyme with the same concentrations because it could produce arginine (7, 8). One of the most important treatments used for cancer is the induction of the autophagy process by many existing drugs. Several research teams have found cooperation in the activation of apoptosis and autophagy in cancer cells (9, 10). Globocan databases reported that because of cancer diseases, more than 9 from 18 million treated people in the world died in 2018, and these numbers may increase to 70% by 2030. Finding a cure for cancer diseases is very complicated because most treatments also harm healthy tissues; therefore, it is very important to look for long-term safe and non-hazardous treatments for the patient's body.

ADI harms arginine auxotrophic cancer cells; however, it does not damage arginine producer healthy cells. Therefore, in this study, the murine mammary adenocarcinoma cell line was investigated for the mechanism in which ADI, purified from probiotic beneficial bacteria, works to inhibit the proliferation of cancer cells and then kill them, compared to Vero transformed (non-cancer) cell line.

2. Materials and Methods

2.1. Purification of ADI Enzyme

The ADI gene was cloned by polymerase chain reaction (PCR) using M. arginine genomic DNA. The tryptophan codon TGA in the coding region that corresponds to a stop codon in *Escherichia coli* was changed to TGG by site-directed mutagenesis using the overlap extension PCR method. The mutated ADI gene was cloned into a pET32a (+) expression vector (designated pET32a-ADI) and transformed into *E. coli* strain BL21. Recombinant ADI overexpressed as inclusion bodies was denatured with 6 M guanidine HCl, refolded in 10 mM potassium phosphate buffer (pH 7.4) and purified by DEAE- and arginine-Sepharose column chromatography (11).

2.2. Cell Lines

The two cell lines used in this study were provided kindly by the Iraqi Center for Medical Genetics and Cancer Research (ICCMGR) in Iraq.

2.2.1. AMN3

This spontaneous murine cell line (mammary adenocarcinoma) was initiated from mice from the first *in vivo* passage (female BALB/c, passage 75).

2.2.2. Vero Cell Line

This cell line was derived from the kidney of normal adult green (African) monkeys in 1962 (passage 120).

2.3. Media Used for Tissue Culture (Cell Line)

Roswell Park Memorial Institute-1640 (RPMI) culture medium was used for maintaining AMN3 cancer cell line and cytotoxicity assay with and without 20% serum (Fetal calf), respectively. Minimum Essential Medium culture medium was also employed for maintaining Vero transformed cell line and cytotoxicity assay with and without 20% serum (Fetal calf), respectively. The cells were cultured in 50 cm^3 tissue culture flasks under humidified 5% CO2 atmosphere at 37°C in RPMI-1640 medium (Sigma chemical) with 10% fetal bovine serum and penicillin-streptomycin during the experiment.

2.4. Investigation of Pphagocytosis Process in Cell Lines

For the purpose of observation and follow-up of cell lines following 3, 6, 24, 48, and 72 h of treatment with ADI enzyme, a phase-contrast inverted microscope (Olympus, Japan, 400 magnification pour) was used for this purpose by observing cells at 37°C.

2.5. Anti-Proliferative Activity of ADI on Cell Lines

The effectiveness of enzymes on cell proliferation (cytotoxicity) was measured in a microtiter plate (96 wells) according to a study conducted by Xiong,

Teng (12). Briefly, the purified ADI enzyme was diluted by serum-free medium to one µg/ml. Subsequently, the effectiveness of the enzyme was measured after the proper incubation time mentioned in 2.4. Subheading (3, 6, 24, 48, and 72 h). Measurements were prepared in triplicate and compared to their controls (ADI free) using Micro-ELISA reader equipment (Organon Technika, USA) at absorbance 492 nm. Cell viability percentage and rate of cell inhibition were calculated according to Dhyea and Abdul jalill (13).

2.6. Apoptosis Detection Using Mitochondria Bioassay Kit

The detection procedure depends on the potential disruption of mitochondrial transmembrane as a first intracellular incident after the induction of apoptosis genes. A fluorescent-based method was used to differentiate between apoptotic and healthy cells. The cationic stain was utilized which differently fluoresces between cells, and the stain aggregated in mitochondria in healthy cells gives off a red bright fluorescence; however, in apoptotic cells, the stain does not aggregate because mitochondrial transmembrane was altered potentially; accordingly, it was staying as a monomer form, green fluorescing in the cytoplasm. Fluorescence microscopy detects FITC and rhodamine by the band-pass filter.

2.7. Statistical Analysis

A least significant difference (LSD), variance analysis (ANOVA), and a *P*-value of ≤ 0.05 were used for statistical significance.

3. Results and Discussion

3.1. Anti-Proliferative Activity of ADI on Cell Lines

The anti-proliferative activities of purified ADI on both AMN3 and Vero cell lines were measured as described in figure 1 which presented the selective effect of this enzyme on the viability of two cell lines, in which AMN3 cells were affected by decreasing their viability from 100% to 86%, 82%, 38%, 22%, and 3% during the experimental periods of 3, 6, 24, 48, and 72 h, respectively. Statistical analysis showed significant differences when the results of control (Vero cell line) were compared to those of AMN3 at $P \leq 0.05$ with LSD value between times and enzyme concentrations. The cytotoxicity appeared distinctly after 24 h, and it then gradually enhanced to 97% of inhibition rate after 72 h of incubation time. The results indicated the importance of arginine for the proliferation of the AMN3 cell line; therefore, when the cells were starved to this amino acid in the presence of ADI, they could not produce it again, because they were in the shortage of ASS and ASL producer enzymes.

Other researches (7, 8) have also shown the effect of the ADI enzyme on other cancer (Rhabdomyosarcoma and Glioblastoma) cell lines and attributed some of the causes to culture media pH raising by the accumulation of enzyme product NH3 in addition to starvation. On the other hand, the results of studying the anti-proliferative activity of transformed Vero cell line as explained in figure 1 indicated that this non-cancer cell line was not affected by ADI since the viability of the cells were 92%, 89%, 91%, 90%, and 93% following the periods of the experiment (3, 6, 24, 48, and 72 h). The capability of Vero cells to produce arginine was observed in this study. Therefore, Vero cells were not starved to arginine, in addition to the ability of Vero cells in maintaining neutral pH of culture medium in the presence of ADI enzyme.

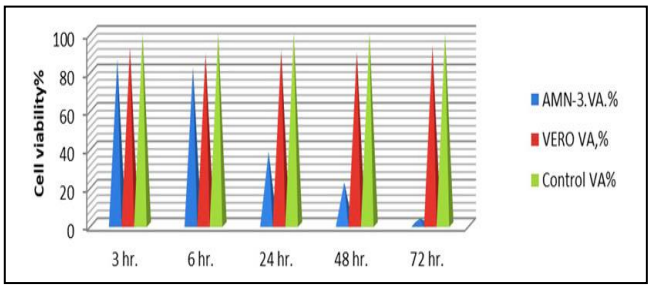

Figure 1. Anti-proliferative activity of purified ADI on both AMN3 and Vero cell lines, compared to control non-treated cells by measuring cell viability (%)

3.2. Investigation of Phagocytosis Process in Cell Lines

When the cancer AMN3 cell line was scanned with an inverted microscope after three hours of incubation, the beginning of the phenomenon of phagocytosis was observed by the production of small circular vesicles (autophagosomes) inside cancer cells, and the number and volume of these vesicles were increased when the cells examined after 6 h. Another phenomenon was observed when the cells were examined after 24 h of incubation with ADI. Some tissue cells began to disconnect and separate from each other and became clusters of cells with different morphological changes and concentrated nuclei for some of them in addition to the presence of decomposed cells.

This phenomenon increased after 48 and 72 h where the numbers of non-decomposed cells have decreased over time. From these results, it can be concluded that the enzyme when converted arginine to citrulline (in culture media) consumed this important amino acid for the proliferation of cancer cells because they have a great need for arginine for protein synthesis by promoting cancer cell growth. Accordingly, they starved for arginine, and the process of autophagy was stimulated in the early hours of exposure. In most cancer cases, the autophagy process results from stress responses (14). As a result of a continued starvation process, the cells are unable to produce arginine in addition to the accumulation of NH3 as an ADI product during the time. This phenomenon was investigated by the enhancement of pH in culture media conditions in the presence of pH indicator and phenol red, which have a stressful effect on cell proteins and enzymes activity. Therefore, the responsible genes for the pathway of programmed cell death were gradually induced over time, and consequently, the cells separated from each other. By changing their forms and disintegrating them into broken cells (15), it can be described that many transmembrane proteins have been implicated in the process of adhesion between cells; therefore, ADI may interfere with a number of these proteins.

When Vero transformed cell line was examined under an inverted microscope after 3, 6, 24, 48, and 72 h of incubation time with ADI, autophagosomes were not observed, and the cells were healthy during the incubation times. In other words, Vero cells could produce arginine by the presence and induction of ASS and ASL genes which express them self-immediately after exposure to the enzyme in this type of cell line. In order to confirm these findings, the cell apoptosis pathway was investigated in this study.

3.3. Apoptosis Detection Using Mitochondria Bioassay Kit

The results of this examination in the treated cancer AMN3 cell line showed the absence of programmed cell death with red cytoplasm regions (apoptosis was not detected after 3 and 6 h of incubation with 1µg of enzyme cells, compared to the control [non-treated; Figure 2] cells which present healthy AMN3 cells with red cytoplasm regions).

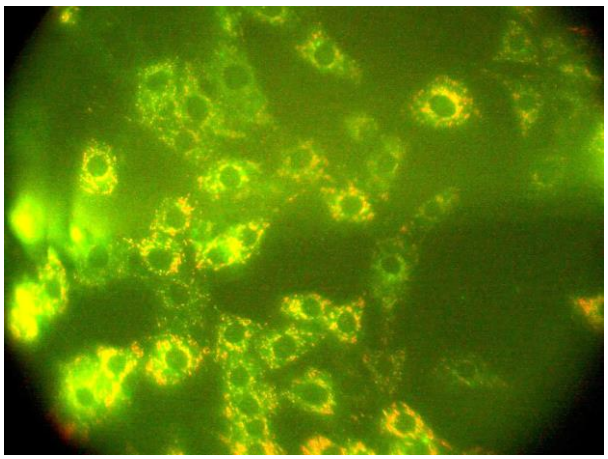

Figure 2. Control (non-treated) AMN3 cell line presenting healthy red cytoplasm areas (M.P=400×) with fluorescence dye (cationic mitocapture reagent)

To discuss these results, it is believed that the cells survive at the early stages of phagocytosis with red cytoplasm regions, and there has been no change in their forms; however, apoptosis pathway was observed when the cells were examined after 24 h of incubation with 1µg of ADI at 37˚C as displayed in figure 3. Accordingly, a few separated clusters of cells with green cytoplasm regions were detected in most fields,

and they were different from control cells. The apoptosis process was increased after 48 h of incubation with different morphological changes and enhancement of decomposed cells, in contrast to healthy red cytoplasm cells which decreased during the time. After 72 h of incubation with ADI, a large number of cells in most fields of the slide reached the final stages of programmed death as demonstrated in figure 4 which exhibits the debris of decomposed cells.

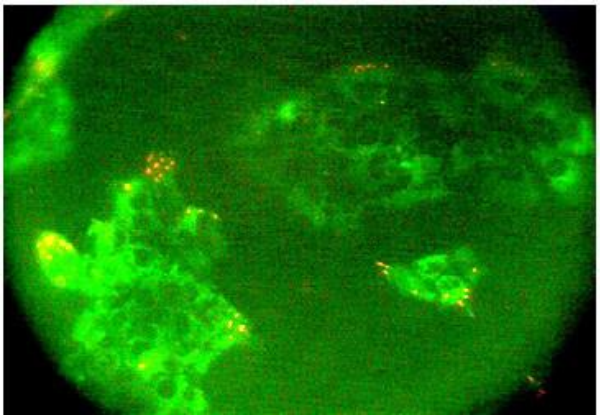

Figure 3. Treated AMN3 cells (after 24hours of incubation) with 1µg/ml of purified ADI enzyme from *Lactobacillus plantarum p5* presenting the number of apoptotic cells with green cytoplasm areas with different morphological changes; however, the number of cells are still healthy with red cytoplasm regions

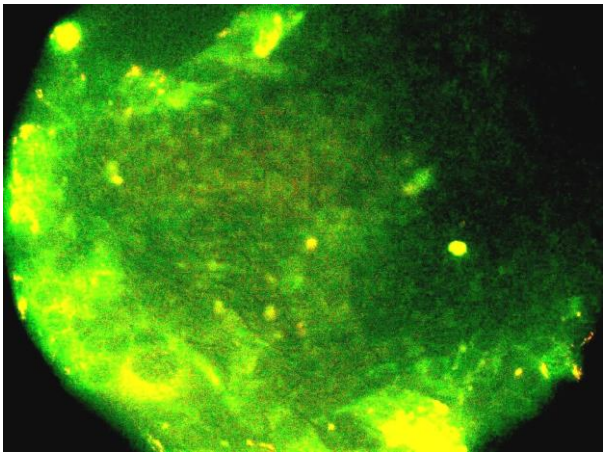

Figure 4. Treated AMN3 cells (after 72 hours of incubation) with 1µg/ml of purified ADI enzyme from *Lactobacillus plantarum p5* presenting apoptotic cells and the debris of decomposed cells

From the results of this study, it can be concluded that ADI enzymes purified from *Lactobacillus plantarum p5* strain have a robust activity in exhausting arginine in culture media and cause *in vitro* starvation for AMN3 cell line; therefore, the autophagy pathway was induced during the first hours of incubation in order to survive the cells. However, these cells could not produce arginine because they have not required the enzymes for de novo synthesis of arginine. All the above-mentioned points lead to the accumulation of NH3 as an ADI byproduct, and consequently, lead to a significant increase in the pH of culture media. Furthermore, all of these events lead to produce inappropriate conditions for the growth and proliferation of cells because they need an abundant amount of arginine for fast malignant evolution. For these reasons, apoptosis was observed after 24 h of incubation in some cells and gradually increased by time, until most cells were destroyed within 72 h.

Some researchers have shown that the presence of ADI in some cancerous tissues (prostate, small-cell lung, pancreatic, retinoblastoma, and leukemia cancer cells) led to induce autophagy pathway by mitochondrial dysfunction. Afterward, this process decreased by inducing the apoptosis pathway (16, 17).

In the transformed Vero cell line, the results demonstrated that these cells were not affected by the presence of ADI during all incubation times. Figure 5 showed that after 72 h of incubation of Vero cells with ADI, most treated cells stilled healthy with red cytoplasm regions and not changed, compared to control (figure 6) non-treated cells. According to the results, Vero transformed cell line had the ability to produce arginine by the presence of ASS and ASL enzymes; therefore, the cells stilled healthy and non-starved. Most researchers confirmed that ADI does not hurt normal cells (12) but only damages cancer cells (18). Since the cancer cells were affected only by the presence of ADI, it can be concluded that the enzyme has a toxic effect only on cancer cells, either transformed cells were safe in the existence of ADI.

Accordingly, it is recommended that the future development of this product be used as an alternative drug instead of treatments that cause damage to natural cells.

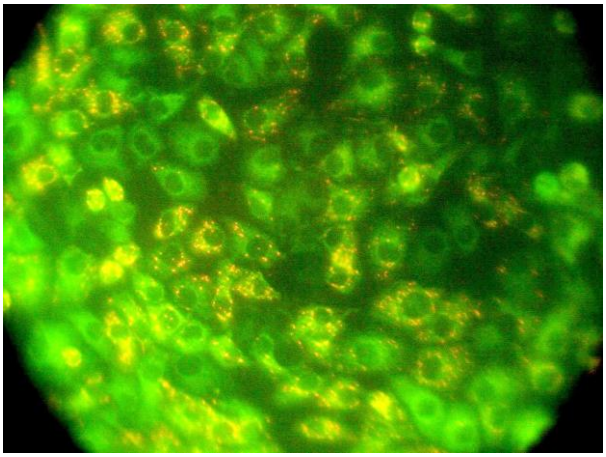

Figure 5. Treated Vero cell line (after 72 h of incubation) with 1µg/ml of purified ADI presenting non-apoptotic healthy red cytoplasm regions

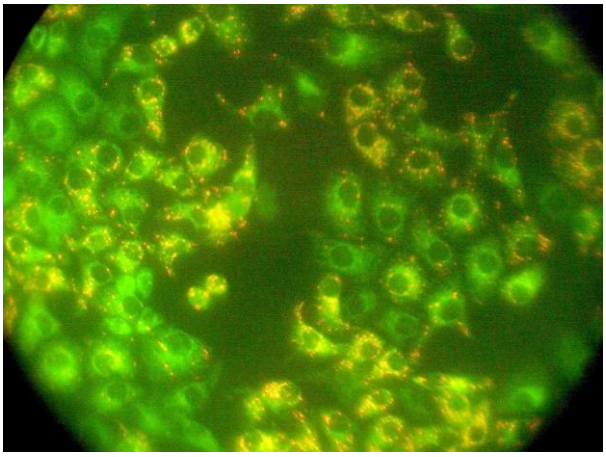

Figure 6. Control (non-treated) Vero cell line presenting healthy red cytoplasm regions

5. Conclusion

According to the results, it can be concluded that the murine mammary adenocarcinoma cell line was auxotrophic for arginine since it could not produce it in the presence of ADI enzyme which had a robust activity to kill these cancer cells. Moreover, Vero non-cancer cell line was not auxotrophic for arginine because it could produce it in the presence of ADI. Therefore, it is hoped to use this enzyme as a cancer treatment agent in the future because it is cytotoxic for cancer cell lines; however, it is safe and does not hurt normal cell lines.

Authors' Contribution

Study concept and design: N. Z. M.
Acquisition of data: N. Z. M.
Analysis and interpretation of data: N. Z. M.
Drafting of the manuscript: N. Z. M.
Critical revision of the manuscript for important intellectual content: N. Z. M.
Statistical analysis: N. Z. M.
Administrative, technical, and material support: N. Z. M.

Ethics

All the procedures was approved by the ethics committee of the Mustansiriyah University, Baghdad, Iraq

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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