Biochemical Study on Activated Immune Cells Isolated from Human Hepatocellular Carcinoma

Thesis *Submitted by*

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Declaration

This thesis has not been submitted for

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Dedication

I would like to dedicate this thesis to whom I am greatly indebted.

..... To my father's spirit

..... To my mother

..... To my brothers and sister

..... To my wife

..... To my daughter "Sara"

(The merciful, supportive and beloved persons in my life).

..... To every member in my family for his endless love, support and concern.

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HepG2 Attenuation Induced by RNase A Modulates Gene Profiling and Immunophenotypic Characterization of Some Immune Cells Operating in Cancer Vaccine

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Abstract: Hepatocellular carcinoma (HCC) ranks as the third leading cause of cancer death. Attempts to induce an effective immune response against cancer by immunotherapeutic intervention, including activation of dendritic cells (DCs), were established. The present study was undertaken to investigate the attenuation of HepG2 cells using ribonuclease enzyme A (RNase A) as a possible biological factor to sensitize allogenic DCs and lymphocytes isolated from Egyptian HCC patients. Attenuation of HepG2 cells resulted in a significant increase in activated DC and T-lymphocyte markers, upregulation of CD44 gene expression and increased lactate dehydrogenase as well as interleukin-12 levels. In contrast, a significant decrease in mature DCs, B-cells, T-helper, cytotoxic T-cells, and NK-cells, as well as LMP-2 gene expression was recorded. In conclusion, the attenuation of HepG2 cells with RNase A and subsequent pulsation to allogenic DCs and lymphocytes caused a differential immune response. Further studies are recommended to explain the role of RNase A in modulating antigen expression on the tumor cell surface.

Keywords: Antigen presentation, dendritic cells, HepG2, immunotherapy, ribonuclease A.

INTRODUCTION

Hepatocellular carcinoma (HCC), the most frequent primary liver malignancy and one of the most common malignancies worldwide, is considered as the sixth most common cancer type and the third cause of cancer-related death in the developed countries [1]. It attracts a significant attention due to its aggressive nature and low response rates to different treatments. Even though chemotherapeutic or radiotherapeutic interventions are used in the clinic for the treatment of HCC, the survival benefit is limited, and other interventional approaches are therefore pursued, such as dendritic cell (DC)-based immunotherapy [2].

Tumors alter the immune homeostasis by suppressing T-cell activation and effector function and simultaneously activating suppressor pathways to prevent T-cell mediated killing [3]. The immune checkpoint molecules have been demonstrated to be excellent targets for cancer immunotherapy, which groups a variety of techniques directed to induce strong anti-tumor immune responses by increasing the activities of T-cells, B-cells and dendritic cells (DCs) [4]. Dendritic cell enhancement of antigen presentation is considered as one of the best immunotherapeutic strategies that strongly activate low-affinity effector T-cells and break the natural tolerance towards endogenous tumor associated antigens (TAAs) [5].

Ribonucleases/RNases are pyrimidine-specific endonucleases that have the function of forming smaller RNA fragments through the transphosphorylation and hydrolysis of the cytosine (C) or uracil (U) residue of RNA [6]. Some members of this RNase family exhibit angiogenic, neurotoxic, antitumor, or immunosuppressive activities [7]. The remarkable antitumor activity of RNases is linked to their ability to destroy RNA and not to genotoxicity, and therefore, they are a second line of cancer chemotherapeutics [8].

The current study was designed to investigate the potential role of RNase A, as a vaccine, to attenuate HepG2 cells and enhance antigen presentation through the subsequent culturing of RNase-treated HepG2 cells with allogenic monocyte-derived DCs and lymphocytes isolated from peripheral blood mononuclear cells (PBMCs) of HCC patients *ex-vivo*. In order to achieve the goal of the study, molecular markers including the expression of some genes involved in antigen presentation and T-cells priming, as well as immunophenotypic markers were evaluated.

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MATERIALS AND METHODS

Blood Sampling

Blood samples were obtained from fifteen newly diagnosed HCC patients chosen from the outpatient clinic (2015-2016) at the Egyptian National Cancer Institute (Cairo University, Giza, Egypt). All experiments were approved by the Institutional Ethical Committee of the Egyptian National Cancer Institute (IRB No.: IRB00004025 and Approval No.: 201516031.3) and informed consents were obtained from all subjects according to the Helsinki Declaration.

Chemicals and Cell Line

Human hepatoma cell line (HepG2, ATCC® HB-8065.1™) was cultured in RPMI-1640 medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biochrom AG, Berlin, Germany) in a humidified 37°C, 5% CO_2 incubator. Bovine pancreatic ribonuclease A (RNase A, A797A) was provided from Promega (WI, USA). Recombinant human interleukin-2 (rhIL-2), interleukin-4 (rhIL-4), interleukin-6 (rhIL-6), granulocyte monocyte-colony stimulating factor (rhGM-CSF) and human tumor necrosis factor alpha (rhTNF-α) were purchased from Bio Basic Inc. (Ontario, Canada). Paraformaldehyde was obtained from Sigma Aldrich (St Louis, MO, USA) and trypsin was purchased from Biowest Inc. (Nuaillé, France).

Isolation, Preparation and Storage of DCs and Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Hypaque density gradient centrifugation [9] (Biochrom KG, Berlin, Germany) and then cultured in a complete RPMI-1640 medium (Sigma Aldrich, St Louis, MO, USA) containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biochrom AG, Berlin, Germany) in 25 cm^2 cell culture flasks that were stored horizontally for 4 h in a humidified 37°C, 5% CO_2 incubator [10]. The medium suspension containing non-adherent cells (lymphocytes) was transferred into a sterile falcon tube and centrifuged at 1800 rpm for 5 min. Cell pellets were cultured in cell culture flasks containing complete RPMI-1640 medium enriched with rhIL-2 (20 IU/ml) and rhIL-6 (10 IU/ml) [11]. Flasks were stored horizontally for 6 days in a humidified 37°C, 5% CO_2 incubator and semi-refreshment of the culture medium, including the cytokines, was performed every three days. The medium suspension containing propagated lymphocytes was transferred into a sterile falcon tube and centrifuged at 1800 rpm for 5 min. Pelleted lymphocytes were washed twice with PBS. The lymphocyte cell pellet was resuspended in 1 ml complete RPMI-1640 medium and cell viability was determined using 0.4% trypan blue exclusion by counting in a hemocytometer. Lymphocyte suspensions were centrifuged at 1800 rpm for 5 min, cell pellets were preserved in a 1 ml cryotube containing complete RPMI-1640 medium supplemented with 10 % DMSO and then stored at -80°C. After the removal of the medium suspension containing non-adherent cells (lymphocytes), the flasks were washed twice with PBS, adherent cells (monocytes) were cultured in a complete RPMI-1640 medium containing 10% FCS, rhGM-CSF (800 IU/ml) and rhIL-4 (500 IU/ml). Flasks were stored horizontally for 6 days in a humidified 37°C, 5% CO_2 incubator and semi-refreshment of the culture medium, including the cytokines, was performed every three days. After incubation, non-adhered and loosely adhered cells (non-viable dendritic cells) were removed by washing the flasks twice with PBS and viable immature dendritic cells (imDCs; well-attached cells) were harvested after adding trypsin for 30 sec followed by the addition of complete RPMI-1640 medium. The cell suspension was transferred into a sterile falcon tube and harvested imDCs were washed in PBS twice, centrifuged at 1800 rpm for 5 min and the separated cell pellet was resuspended in 1 ml complete RPMI-1640 medium. Immature DCs (imDCs) were then stained with 0.4% trypan blue and counted with a hemocytometer. The suspension of imDCs was centrifuged at 1800 rpm for 5 min and cell pellets were preserved in a 1 ml cryotube containing complete RPMI-1640 medium supplemented with 10 % DMSO and then stored at -80°C.

Treatment of HepG2 Cells with RNase A

A number of 5×10^3 HepG2 cells was cultured in complete RPMI-1640 culture medium containing 5 µl RNase A (10 mg/ml) for 12 h at 37°C and 5% CO_2 atmosphere, followed by cell trypsinization and fixation with 2% cold and freshly prepared paraformaldehyde in phosphate buffered saline (PBS) for 10 min. Fixed HepG2 cells were washed thrice with PBS and then centrifuged at 1800 rpm for 5 min. Cell pellets were suspended in 2 ml complete RPMI-1640 medium.

Study Design, Activation and Maturation of DCs

Propagated imDCs were activated by culturing in a six-well plate in complete RPMI-1640 media containing

Table 1: Primers Used for Real-Time Polymerase Chain Reaction

Gene	Primer sequence	Accession nb
CD44	**F:** 5'-AGAAGGTGTGGGCAGAAGAA-3' **R:** 5'-AAATGCACCATTTCCTGAGA-3'	KR709963
TAP-2	**F:** 5′- GCAGAATCTGTACCAGCCCAC-3' **R:** 5′- GGGCCTGCTCGCACTGC-3'	NM_000544
LMP-2	**F:** 5'-CTCCACTTTACAGATGCAGA-3' **R:** 5'-ACTTGGTGACTGTTGACTCC -3'	X66401
GADPH	**F:** 5′-ACCCACTCCTCCACCTTTGAC-3′ **R:** 5′-TGTTGCTGTAGCCAAATTCGTT-3′	AF261085

rhGM-CSF (400 IU/ml), rhIL-4 (250 IU/ml) and rhTNF-α (10 ng/ml) for 24 h in a humidified 37°C, 5% CO_2 incubator in the presence of intact HepG2 cells (5×10^3) (group 1; Control) and RNase-treated HepG2 cells (5×10^3) (group 2; Attenuated HepG2) at a ratio of one-fold HepG2 cells to ten-folds imDCs. After the incubation period, propagated lymphocytes were added to the previous cocktail of cells (at a ratio of 10:1 lymphocytes to mature DCs) in addition to rhIL-2 (50 IU/ml) and rhIL-6 (50 IU/ml) for 72 h. All non-attached cells (mature DCs, lymphocytes and unattached HepG2 cells) were transferred into a sterile falcon tube and centrifuged at 1800 rpm for 5 min. The cell pellets were then suspended in 2 ml complete RPMI-1640 media then subdivided into two aliquots. The first one was instantaneously used to measure immune cells immunophenotyping, while the other aliquot was preserved at -80°C for mRNA extraction. The liquid culture media supernatant was separated and preserved at -20°C for the quantitation of interleukin 12 (IL-12) and lactate dehydrogenase (LDH) levels.

Immunophenotypic Analysis of Immune Cells

Immunophenotyping analysis was done in EPICS® Flow Cytometer (Beckman Coulter Inc., CA, USA) using monoclonal antibodies against CD3, CD4, CD8 and CD56 (DakoCytomation, Münster, Germany), CD19, CD38, CD81, CD83, CD86 and CD209 (eBioscience, CA, USA) [12].

Estimation of Interleukin-12 (IL-12) and Lactate Dehydrogenase (LDH) Concentrations

The levels of LDH (a cell death marker) [13] and IL-12 [14] were measured in the culture media supernatant using ELISA kits provided from Cloud-Clone Corp. (Houston, TX, USA) and R&D Systems Inc. (Minneapolis, MN, USA), respectively.

Molecular Analysis of Selected Genes

Total cellular RNA was extracted from all non-attached cells (DCs, lymphocytes and HepG2 cells) using an RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Extracted RNA was quantified by NanoDrop One (Thermo Fisher Scientific, WI, USA) and was reversely transcribed by a cDNA Reverse Transcription Kit (Applied Bioscience, CA, USA). Real-time polymerase chain reaction (RT-PCR) quantitative estimation of CD44, TAP-2 and LMP-2 gene expressions was done by RT^2 SYBR® Green ROX™ qPCR Mastermix (QIAGEN, Hilden, Germany) in a mini PikoReal™ (Ver.2.2) machine (Thermo Scientific, Finland). Primers used in the amplification process [15-18] (AlphaDNA, Montreal, Canada) were listed in Table **1**. Relative gene expressions were calculated using the comparative threshold cycle method [19].

Statistical Analysis

The Shapiro-Wilk's test for normality ($p>0.05$) was used to examine the normality of obtained data [20]. Equal variance between the groups was first checked using the Levene's test for homogeneity of variances and statistical analysis of the difference between the mean values of both groups was carried out using Student's T-test. A value of $p<0.05$ was considered statistically significant for all tests. SPSS statistical software 24.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all analyses.

RESULTS

Effect of HepG2 Attenuation on Selected Genes Expression

Treatment of HepG2 cells with RNase A and subsequent pulsation to dendritic cells produced a significant upregulation of CD44 gene expression