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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ISSN: 1929-2260 / E-ISSN: 1929-2279/18

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

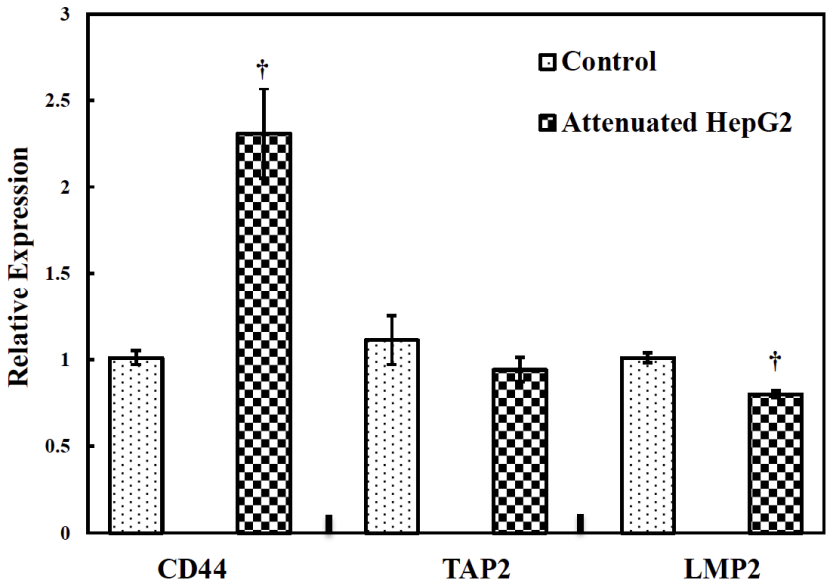

Figure 1: Effect of HepG2 treatment with RNase A on the relative expression of CD44, TAP-2 and LMP-2 genes. Bars are mean ± SE (n=15). † p<0.001 versus untreated HepG2 cells (Control).

(128.71%; p<0.001), and in contrast a slight significant downregulation was recorded in the expression of LMP-2 gene (20.79%; p<0.001), compared to untreated pulsed HepG2 cells (Figure **1**).

Effect of HepG2 Attenuation on Dendritic Cell Markers

Dendritic cells pulsed by RNase A-treated HepG2 cells showed a significant elevation in some DC immunophenotypic markers, including $CD83^+$ (35.69%; p<0.01) and $CD83^+CD86^+$ (43.17%; p<0.01), whereas the relative count of mature DCs ($CD86^+$) was significantly reduced (30.77%; p<0.01), compared to untreated pulsed HepG2 cells (Figure **2**).

Effect of HepG2 Attenuation on T-Lymphocyte Markers

Ribonuclease treatment of HepG2 cells induced significant reduction in the relative count of T-helper lymphocytes ($CD3^+CD4^+$) (39.47%; p<0.01), cytotoxic T lymphocytes ($CD3^+CD8^+$) (34.99%; p<0.05), double positive T cells ($CD4^+CD8^+$) (49.74%; p<0.01), as well as NK cells ($CD56^+$) (52.50%; p<0.001), compared to untreated pulsed HepG2 cells (Figure **3**).

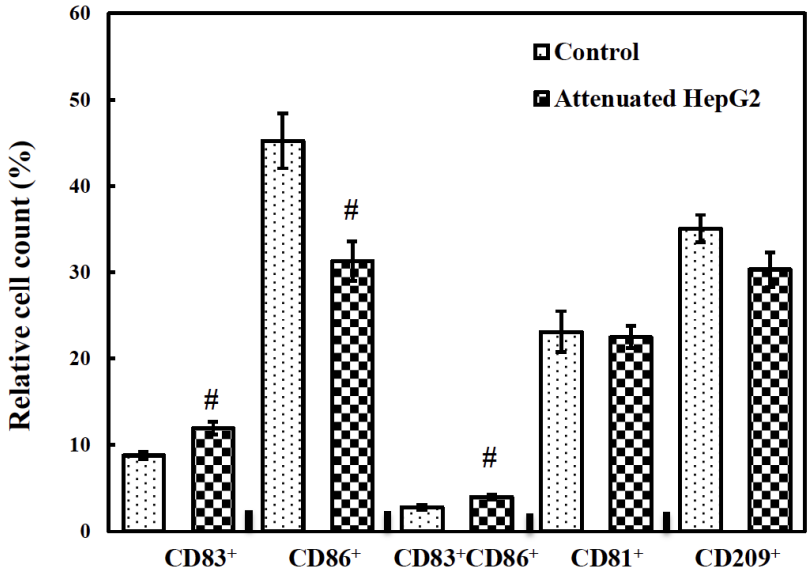

Figure 2: Effect of HepG2 treatment with RNase A on dendritic cells immunophenotype characterization. Bars are mean ± SE (n=15). # p<0.01 versus untreated HepG2 cells (Control).

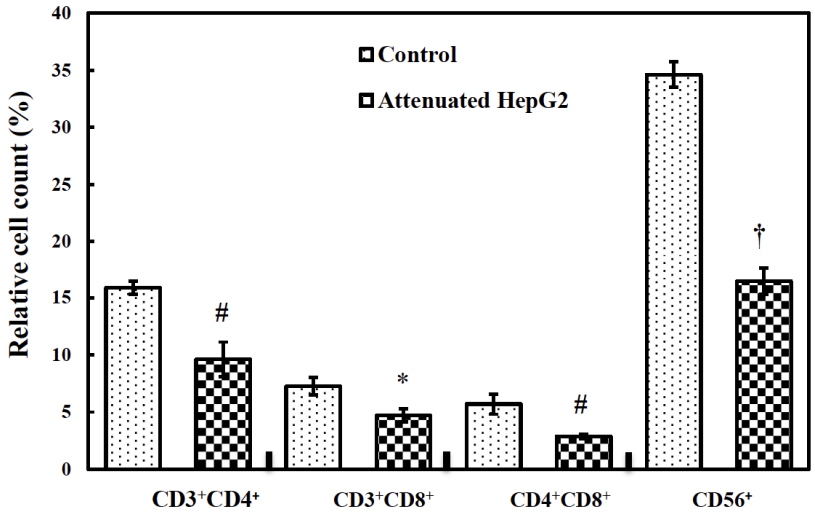

Figure 3: Effect of HepG2 treatment with RNase A on T-lymphocytes immunophenotype characterization. Bars are mean ± SE (n=15). * p<0.05; # p<0.01; † p<0.001 versus untreated HepG2 cells (Control).

Effect of HepG2 Attenuation on B-Lymphocyte Markers

Pulsing immune cells by RNase A-treated HepG2 cells caused a significant decrease in the relative count of the total B-lymphocytes ($CD19^+$) (44.07%; p<0.001) as well as activated B-lymphocytes ($CD19^+CD38^+$) (68.91%; p<0.001), and in contrast a significant increase was observed in the relative count of activated T-lymphocytes ($CD19^-CD38^+$) (52.26%; p<0.01), compared to untreated pulsed HepG2 cells (Figure **4**).

Effect of HepG2 Attenuation on LDH and IL-12 Levels

Treatment of HepG2 cells with RNase A resulted in a significant increase in LDH (95.97%, p<0.001) and IL-12 (23.73%, p<0.01) levels in the culture media supernatant, compared to untreated pulsed HepG2 cells (Figure **5**).

DISCUSSION

As tumor cells are immunogenic, they must acquire a plethora of molecular mechanisms to avoid destruction by CTLs and NK cells [21]. Conventional changes of HLA expression in malignant cells include total or allele specific loss of classical HLA-I expression (to prevent tumor recognition and rejection by CTLs) and the induction of non-classical HLA-I and HLA-II expression, potentially due to an immune selection process that enables the initiation of malignant lesions with an HLA-altered phenotype, which will be

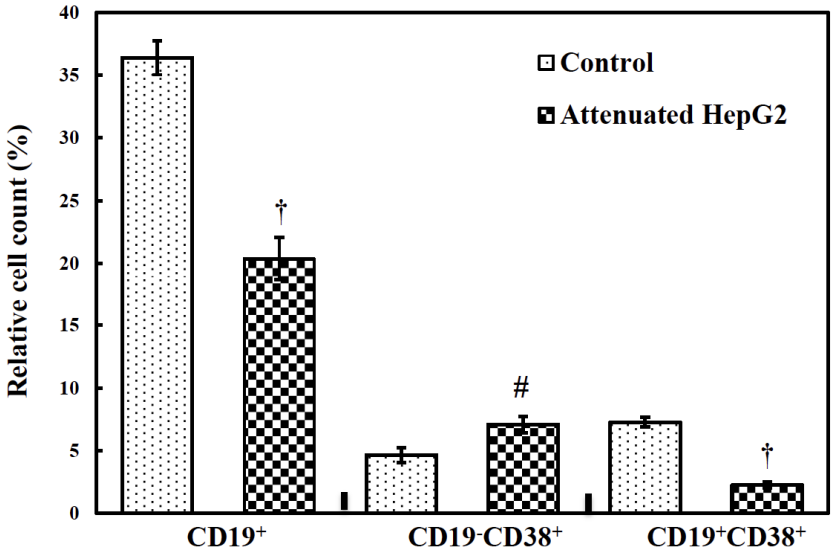

Figure 4: Effect of HepG2 treatment with RNase A on B- and T-lymphocytes immunophenotype characterization. Bars are mean ± SE (n=15). # p<0.01; † p<0.001 versus untreated HepG2 cells (Control).

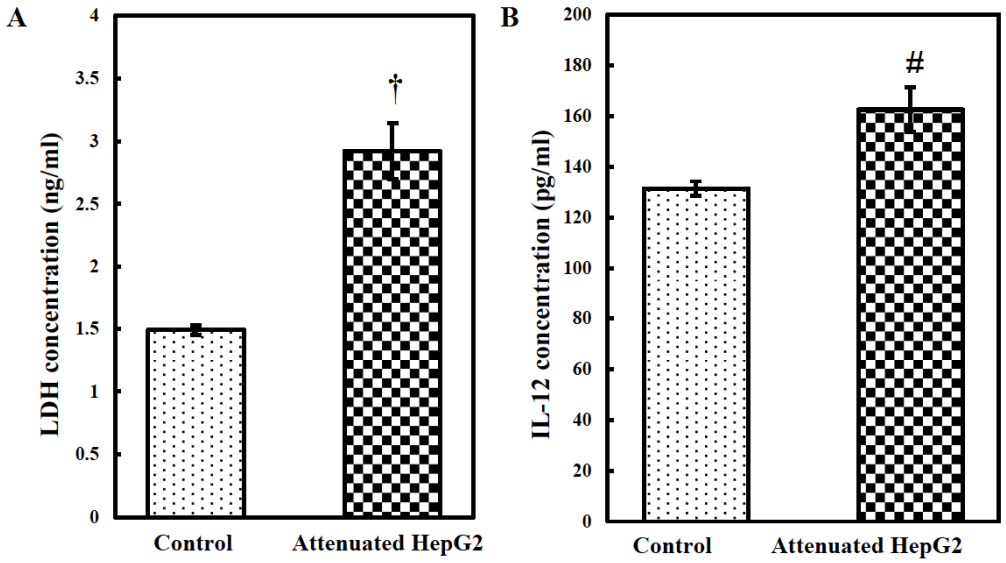

Figure 5: Effect of HepG2 treatment with RNase A on LDH (**A**) and IL-12 (**B**) levels in the supernatant of culture media. Bars are mean ± SE (n=15). # p<0.01; † p<0.001 versus untreated HepG2 cells (Control).

necessary to consider when designing novel immunotherapies [22].

HLA expression is crucial for the generation of adaptive immunity, as tumor antigens are presented in an HLA-restricted manner to T-cells, activating them and controlling immune crosstalk [23]. Altered HLA expression on the tumor cell surface has been described in a variety of human tumors [24]. These alterations result in different HLA-altered phenotypes, including the neo-expression of non-classical HLA-I molecules like HLA-G, which primarily function as inhibitor ligands for immune-competent cells [25]. HLA-G expression by the tumor prevents immune responses by a variety of strategies, including the prevention of cell lysis by CTLs and NK cells, the induction of tolerant myeloid DCs, and the induction of anergic or immunosuppressive $CD4^+$ and $CD8^+$ T-cells [26]. Moreover, alterations in HLA-II were reported to function in immune escape by impairing the antigen-presenting capability of peripheral blood monocytes in patients with acute leukemia [27].

The literature implies several methods for attenuating cancer cells in order to enhance immune sensitization, which include functionalized poly-lactic-co-glycolic acid nanoparticles [28], knockdown genes [29] and sensitization using polythylene glycol [30] that resulted in enhanced activation and maturation of DCs and activated proliferation of lymphocytes. Palmer et al. [31] and El Ansary et al. [32] pulsed autologous DCs propagated *ex-vivo* using lysates from a liver cell line (HepG2) and reported that this technique is a safe and well tolerated method for the treatment of advanced HCC patients. Similarly, the cell lysate of heat-shocked HepG2 cells was used to pulse matured DCs co-cultured with autologous $CD4^+$ and/or $CD8^+$ cells and was found to improve the cellular immune response [33]. Transfection of autologous DCs with heat shock protein-70 mRNA *ex vivo* and subsequent injection in HCV-related HCC patients showed a complete response in most of the patients as demonstrated by the enhancement of tumor killing activity of the cytotoxic T-lymphocytes [34]. Xing et al. [35] pulsed DCs and lymphocytes with dead HepG2 cells that resulted in an increased level of DC and CTL markers. Similarly, immature DCs (imDCs) transfected with total RNA from HepG2 cells resulted in a better differentiation and maturation of DCs in addition to the elicitation of specific T-cell responses that specifically recognize HCC cells [36, 37].

Attenuation of HepG2 cells using RNase A by the current method activated subsets of DCs ($CD83^+$ and $CD83^+CD86^+$) and T-lymphocytes ($CD19^-CD38^+$), gene expression of CD44, as well as LDH and IL-12 levels. In contrast to our expectation, immunosuppression of $CD86^+$ DCs, T-cells (including $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD4^+CD8^+$), B-cells (including $CD19^+$ and $CD19^+CD38^+$), NK-cells ($CD56^+$), as well as LMP-2 gene expression was observed. The data obtained from the current results suggest that RNase probably altered the expression of TAAs, classical and/or non-classical HLA on the surface of tumor cells, even though our aim target was to reduce the immune suppressive role of HepG2 cells using RNase A

vaccination without altering the expression of cell surface antigens. Such alteration may be in part responsible for this bizarre situation where stimulated DCs and T-cells were detected in a swarm of inhibited immune cells.

In conclusion, the current study showed that RNase A treatment of HepG2 cells and subsequent pulsation to allogenic DCs and lymphocytes induced a paradoxical effect on antigen presentation. Further investigations are required to study human leucocyte antigen (HLA) profiling, which will assist in explaining the probable role of RNase A in modulating antigen expression on the tumor cell surface.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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Received on 26-02-2018 Accepted on 20-03-2018 Published on 26-03-2018

DOI: http://dx.doi.org/10.6000/1929-2279.2018.07.01.3