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Interleukin-10 as a marker for response to dendritic cells-dribbles immunotherapy in hepatocellular carcinoma, a mice model

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Abstract

Cancer immunotherapy is a promising strategy in cancer management, including hepatocellular carcinoma (HCC). This experimental study aimed to evaluate interleukin-10 (IL-10) as a biomarker for monitoring the response of tumor-derived autophagosomes vaccine in inducing antitumor immunity in HCC induced mice. It was conducted on 56 BALB/c mice; divided into 20 normal and 36, cancer induced with human liver cancer cell line (HepG2) cells. The latter group was subdivided into a positive control group (n=6) and a treated group (n=30), that was subdivided into 3 subgroups: (A) treated with dendritic cells (DC) vaccine only, (B) treated with vaccine named Dribbles only, and (C) treated with DC plus Dribbles. Serum IL-10 was assessed after immunotherapy. The mean percentage of tumor volume reduction in mice vaccinated by DC plus Dribbles was significantly superior to DC and Dribbles groups (p= 0.013, and p= 0.043, respectively). There was a statistically significant difference in IL-10 levels between different immunotherapy groups (p= 0.0003). As the mean IL-10 level was 19.50 pg/ml for the positive control group, 13 pg/ml for Dribbles group, 10 pg/ml for DCs group and 3.50 pg/ml for DCs plus Dribbles group. We conclude that DC-Dribbles vaccine has a remarkable efficacy superior to either Dribbles alone or DC alone in the decline of HCC development and survival improvement. IL-10 is a predictive biomarker for response after immunotherapy.

Keywords: Hepatocellular carcinoma, Immunotherapy, Dendritic cells, Interleukin 10.

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Introduction

Liver cancer is estimated to be the sixth most frequently diagnosed cancer and the fourth major cause of cancer-associated deaths globally, constituting 841,000 new diagnosed cases and 782,000 deaths yearly.[1] Unfortunately, the majority of patients with hepatocellular carcinoma (HCC) are diagnosed at an advanced stage of the disease or have impaired liver and are treated with local, regional, or systemic therapy. However, within 5 years, most of them experience recurrence. So,

the new multiple treatment regimens for HCC, including immunotherapy is needed.[2, 3]

A process that is carried by the cellular machinery to remove dysfunctional cellular components through lysosomes degradation and autophagosomes is called Autophagy.[4] Accumulating evidences suggest that autophagy plays a vital role in antitumor immunity through cross-presentation of tumor antigens.[5] Dendritic cells (DCs) have the machinery for antigen processing and presentation on MHC class I and II, despite expressing an eminent surplus of MHC molecules permitting the presentation of numerous antigenic peptides simultaneously and induction of T-cell immune responses.[6] "Dribbles" is a term used to describe autophagosome-rich blebs of tumor cells that can be prepared from the human liver cancer cell line Hep-G2 cells by autophagy inducer and lysosome inhibitor [7]. Several studies in cancer immunotherapy reported that Dribbles vaccines enhance antitumor immune responses through acting as an effective antigen source.[8, 9, 10]

Interleukin 10 (IL-10) is an immunoregulatory cytokine produced by a diversity of immune cells involving DCs, that can inhibit T cell activation. Whereas DCs functions are in return strictly controlled by IL-10.[11] Moreover dendritic cell maturation and differentiation can be prevented by IL-10 though downregulation of MHC class II and costimulatory molecules, thus inhibiting antigen priming of naïve T cells.[12] Furthermore, some cancer cells may suppress host immunity through blocking DCs functions by secreting IL-10.[13] The serum IL-10 also may prove to be a helpful marker in the management of several malignancies.[14] In HCC patients, elevated serum levels of IL-10 have been linked with poor prognosis.[15, 16] Therefore, in this work, we assessed the serum level of IL-10 as a biomarker for the response of tumor-derived autophagosomes vaccine named Dribbles in reducing tumor volume and improving of survival of HCC induced mice.

Materials and Methods

Study design

This experimental (Interventional) study was conducted at the Immunology Research Laboratory, Animal Care Center, Cell Culture Research Center in the Department of Biochemistry, Faculty of Medicine, Zagazig University and Nile Center for Experimental Research, Mansoura, during 2019-2020.

The study included 56 BALB/c mice, 6- to 8-week-old, weighing about 22 gram each. They were purchased from the animal care center of VACSERA (Holding Company for Biological Products & Vaccines) and maintained under specific pathogen-free conditions. Mice were divided randomly into two groups, 20 normal control mice and 36 cancer induced mice as shown in Figure 1. Mice in each group were observed for eating, drinking, and excretion and behavior during the experiment. They were maintained under optimal light, temperature, humidity, and specific pathogen-free conditions.

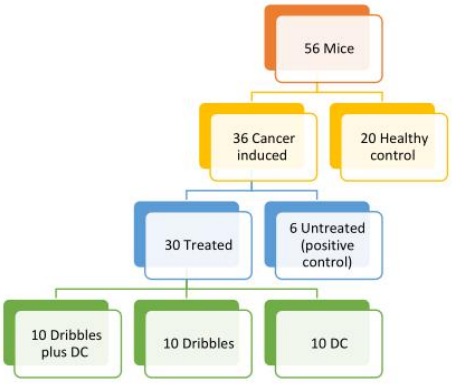

Figure 1. A flowchart representation of the studied groups. Healthy (negative control) (20 mice) and cancer induced (36 mice). Cancer was induced by subcutaneous inoculation with 2.8×106 humanized HepG2 cells in the lower right flank. On day 7 after hepatocellular carcinoma induction, immunotherapy was started in treated group with either DC (1×106 DCs) (10 mice) only or Dribbles (200µg Dribbles) only (10 mice) or Dribbles plus DC (10 mice). Meanwhile, positive control group (6 mice) treated with phosphate buffered saline.

Methods

-HCC induction

HCC was induced in 36 BALB/c mice by subcutaneous inoculation with 2.8×106 humanized HepG2 cells in the lower right flank with insulin syringe. The HepG2 cell line was obtained from “Nile Center for Experimental

Research". Tumor volume was estimated at regular intervals by a Vernier caliper and calculated according to formula:
Volume = (Width2 × Length) /2 17.

-Immunotherapy schedule

Immunotherapy was initiated on day 7 after HCC induction in mice. Immunotherapy was injected subcutaneously into both flanks at two doses one week apart.

-Immunotherapy preparation

Three types of vaccines were prepared: DCs vaccine, Dribbles vaccine and DCs plus Dribbles vaccine.

-DCs vaccine

Peripheral blood mononuclear cells (PBMCs) from healthy donors, were separated by Ficoll (Lymphoflot; Biotest, Dreieich, Germany) density gradient centrifugation. Every two days, the PBMCs were nourished with fresh RPMI medium (Catalog. no. R8758, Sigma, USA) containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (catalog no 215-GM, R&D Systems, USA) and IL-4 (catalog no 204-IL, R&D Systems, USA). On day six, maturation was achieved by toll-like receptors (TLRs) agonist, resiquimod (R848) (Catalog no. 4536, TOCRIS, UK) for an additional two days. The expression of CD80, CD86 and HLA-DR on DCs compared to the freshly isolated monocytes was evaluated (data not shown).

-Dribbles vaccine

HepG2 cells were kept in Dulbecco's Modified Eagle Medium (DMEM) complete medium (Gibco, ThermoFisher Scientific, USA) containing 10% fetal bovine serum and treated with rapamycin (500 nM) and chloroquine (10 µM) for 24 h in CO2 incubator. The resultant suspension was cleared by centrifugation at 300 xg for 10 min and the supernatant was then recentrifuged at 10,000 xg for 30 min followed by washing with phosphate buffered saline and recentrifuged again as previously done 18. Dribbles were then aliquoted into tubes and kept frozen at -80°C until used. The total protein was measured by bicinchoninic acid assay according to the manufacturer's protocol (catalog no 23227, ThermoFisher Scientific, USA). Autophagosomes formations were detected by Flow Cytometry (BD Pharmingen, USA) using 250 µl solution of Cyto ID stain (ab139484, Autophagy Detection Kit, Enzo Life Sciences, USA).

-DCs plus Dribbles vaccine

The mature DCs (1x106) were loaded with autophagosomes (Dribbles) from Hep-G2 cancer cell line. They were co-cultured in 6-well plates for an additional 24 h at 37°C and 5% CO2.

-IL-10

Blood samples were collected from the mice eyeballs, after they were anesthetized, according to the specified time points. Then serum was isolated and stored frozen at −80°C for later use. The serum levels of IL-10 were determined using enzyme linked immunosorbent assay (ELISA) kits (RayBiotech, Inc, USA) based on the manufacturer's instructions. A wavelength of 450 nm was used to read the absorbance using an ELISA reader (Stat Fax 303 Plus, USA) and the results were expressed in pg/ml.

Statistical Analysis

The collected data were analyzed using the Statistical Package of Social Services (SPSS) version 24. The Shapiro-Wilk test was used to test normality of quantitative results. Mann-Whitney U (MW test) and Kruskal Wallis test (KW test) were used for comparing numerical variables. The Freidman test was used for comparing numerical variables between paired data more than 2 settings (before and after treatment). Kaplan Meier (KM) curve was plotted for survival assessment using the Graphpad Prism 8.0 (Graphpad software, San Diego, CA). The results were considered statistically significant when the significant probability was less than 0.05 ($p < 0.05$).

Results

Autophagosome formation was detected by flowcytometry

The results in Figure 2 describe the flow cytometry-based analysis of Hep-G2 cells. Control cells displayed low fluorescence signal intensity. In the samples treated with

Rapamycin and chloroquine for 24 hours, the Green Detection Reagent signal (Cyto-ID dye) increased, representing a rise in autophagic vesicles with microtubule-associated protein light chain 3 (LC3-II) in Hep-G2 cells.

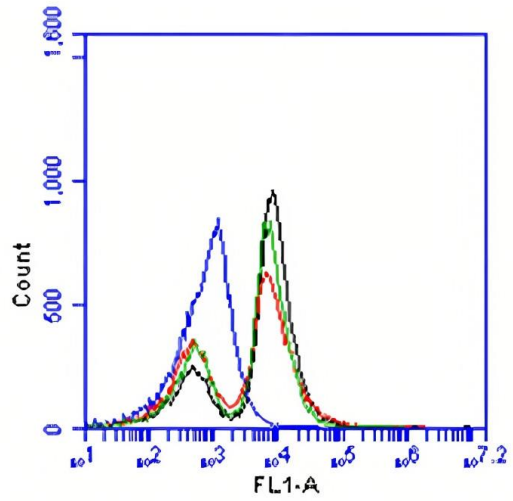

Figure 2. Detection of autophagosome by flow cytometry. Histogram overlapping of all groups (blue: uninduced, red: Rapamycin, green: Chloroquine, black: combination of Rapamycin and Chloroquine). These results indicated that the combined use of two drugs on cultured HepG2 cells efficiently induced autophagy and the accumulation of autophagosomes.

Immunotherapy suppresses HCC growth in BALB/c mice

Analysis of the rate of the mean growth during the immunotherapy demonstrated that tumors in the positive control group were growing more rapidly compared with tumors in the treated group (Figure 3). The mean of percentage of tumor volume reduction in the group of mice vaccinated with DCs plus Dribbles was significantly superior to that in other types of vaccines, DCs and Dribbles groups, (p=0.013, and p=0.043, respectively). While there was no statistically significant difference in the mean of percentage of tumor volume reduction between the DCs and Dribbles groups (p=0.586) as shown in Figure 4.

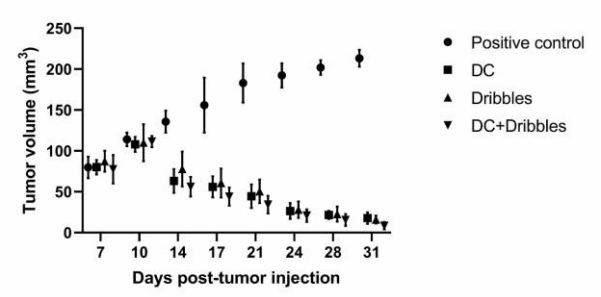

Figure 3. Tumor volume plots for hepatocellular carcinoma implanted subcutaneously in BALB/c mice. The main graph presents the mean and average tumor weights for the tumor induced groups. Tumor volume of tumor-bearing mice was monitored every 3 days. Data is from three independent experiments.

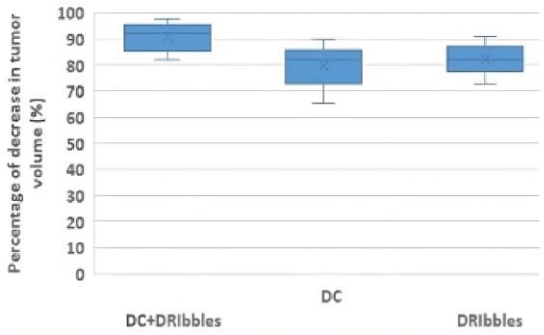

Figure 4. Boxplot analysis showing the percentage of decrease in volume of hepatocellular carcinoma among the treated groups. The median (middle quartile) marks the mid-point of the data and is shown by the line that divides the box into two parts. The middle "box" represents the middle 50% of scores for the group (IQR), lower and upper whiskers represent lowest and highest data)

Immunotherapy improves Survival rate in HCC induced BALB/c mice

The percentage of survival in the untreated positive control group (placebo) was 50% (three mice died out of six) while in the three treated groups it was 100% (no deaths). Figure 5 reveals that the mean survival time for the untreated positive control group (placebo) was 19.17 days (range 15.6-22.7 days), while it was longer for the three treated groups as follows; [30 days (29.4-30.6) for DCs, 31.16 days (30.6-31.7) for Dribbles and 30.2 days (29.5-30.9) for DCs plus dribbles].

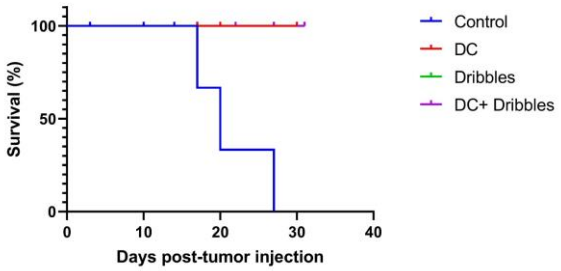

Figure 5. Kaplan Meier curve for overall survival of BALB/c mice bearing hepatocellular carcinoma. Immunotherapy was injected subcutaneously into both flanks two doses one week apart on day 7 after hepatocellular carcinoma induction. Dotted lines indicate 95% confidence intervals. Log-rank =10.79 (p = 0.0129).

Il-10 is a prognostic biomarker for the response to immunotherapy in HCC

The mean IL-10 serum level was 19.50 pg/ml for the positive control group, 13 pg/ml for Dribbles group, 10 pg/ml for DCs group and 3.50 pg/ml for DCs plus Dribbles group. The Kruskal-Wallis H test showed that there was a statistically significant difference in the serum levels of IL-10 between the different immunotherapy groups (p= 0.0003).

Discussion

Most HCC patients are inoperable due to late diagnosis at the metastatic stages. Chemotherapy in advanced HCC is an unsatisfactory with a response rate of < 10% and minimal improvement in survival.[19] There is crucial need of novel effective treatment approaches as the treatment efficacy of the present therapeutic modalities in reducing mortality of HCC is limited.[20] Emerging immune therapies are under investigation. These include DCs vaccination, immune-modulator strategy, and immune checkpoint inhibition. The HCC immunotherapy was studied by diverse clinical trials with initial promising results.[21]

This experimental study assessed the efficacy of immunotherapy in HCC induced mice regarding tumor volume reduction and improving the survival rate. Furthermore, we compared the effectiveness in different vaccine preparation. In the current study we generated mature DCs *in vitro*, from PBMCs.

DCs are professional antigen presenting cells which have the ability for boosting tumor-specific T cell responses.[22] Mature DCs were used in vaccination, and assessed in various clinical trials.[23] Evidence indicates that mature DCs are more effective in producing therapeutic effect in cancer immunotherapy using DCs because applying immature DCs can induce tolerance rather than immunity.[24, 25.]Moreover, mature DC is more efficient in migration to the lymph nodes as well as expression of the central chemokine receptor 7 and apoptosis inhibition.[26]

In this study autophagosomes (Dribbles) were isolated from HepG2 cell line after treatment with rapamycin and chloroquine. Those antigen-containing vehicles were characterized by LC3-II, which was examined by flow cytometry. Dribbles vaccine was used alone or cocultured with mature DCs. Due to the excellent immunogenicity of Dribbles, they were readily taken up by the antigen presenting cells, the presence of C-type lectin domain family 9A (CLEC9A) receptor on DCs and CLEC9A ligand on autophagosomes responsible for their phagocytosis. Tumor proteins in Dribbles are phagocytosed and degraded by DCs, then processed and presented on surface with MHC I to effector cells to enhance antitumor immunity.[27] Dribble-DCs immunotherapy could efficiently activate T cells, which secrete interferon-γ and inhibit tumor growth.[9]

Current immunotherapy schedule was started after 7 days of subcutaneous HCC tumor induction in mice, at this point, the mice were randomly distributed into four groups. The tumor volume was measured twice a week throughout the experiment in each group. Regarding tumor volume reduction, the results of DC-Dribbles vaccine were significantly superior to Dribbles vaccine alone or DCs vaccine alone. Meanwhile, there was no statistically significant difference in the percentage of tumor volume reduction between DC and Dribbles groups. Our findings showed that Dribbles-DCs immunotherapy had the greatest therapeutic effect on tumor regression. Our results agreed with data reported by experimental trials on different types of cancer.[9, 27, 28]

In China, Dong et al., 2018, studied the efficacy of Dribbles, Dribble-DCs or Lysate-DCs vaccines in oral squamous cell carcinoma induced mice. They reported that Dribble-DCs immunotherapy was significantly better than Dribbles and tumor cell lysate-loaded DCs in inducing antitumor response.[9] Another agreement with our results was achieved in experimental studies on lung carcinoma, melanoma and sarcoma models.[27, 28] In our study, the overall survival varied significantly between the placebo control and the treated groups. Similarly, Su et al., 2013, observed that the tumor growth was significantly controlled by treatment with DCs-Dribbles, compared with phosphate buffered saline controls or DCs therapy alone.[29]

IL-10 has arisen as a significant immune response inhibitor and a contributor to human disease.[30] The elevation of IL-10 in HCC that is produced by regulatory B, T cells, tumor-associated macrophages, and DCs,[31] leads to an impairment of the tumor-specific immune response in HCC patients.[12] On multivariate analysis, serum IL-10 was reported to be an independent prognostic marker, showing the crucial physiologic impact of this cytokine in the HCC disease process.[15] Therefore, in the current study, we anticipated that the level of serum IL-10 could be considered a biomarker for the response to immunotherapy in HCC mice models. We found that IL-10 serum level was significantly lower in the dribble plus DCs immunotherapy group when compared to other groups.

Consistent with our result, some studies evaluated the use of Il-10 level as a biomarker after different therapeutic interventions for HCC and other tumors.[15,32] Shan et al., 2022, recorded that after trans arterial chemoembolization, patients with decreased plasma IL-10 levels had promising outcomes.[32] Moreover, Chau et al., 2000, determined that the level of IL-10 after HCC tumor resection was reduced.[15] However, Nordin et al., 2020, revealed that the serum level of IL-10 was slightly reduced but non-significant following cancer nano therapy in breast cancer mouse model compared to the untreated group.[33] It was observed that in HCC patients, IL-10-mediated reduction of circulating DC subsets was related to tumor immune evasion.[12] In support of this notion, a previous study reported that DC generated from IL-10 knockout mice (IL-10-/-DC) vaccine was demonstrated to promote persuasively both therapeutic and protective immunity against the tumor.[34] The lack of IL-10, a powerful immunosuppressive cytokine, could account for the preeminence of IL-10-/-DCs immunologically 11. When compared to control wild type DCs, the IL-10-/-DCs were able to express MHC class II and class I molecules on their surface with greater frequency, which improved the efficiency of how the antigen was presented to the helper T cells and cytotoxic T lymphocytes, respectively.[34]

On the other hand, Wu et al., 2021, documented that the administration of Zotarolimus immunosuppressant drug on A549 human lung adenocarcinoma cell line implanted in BALB/c nude mice could increase serum IL-10 levels.[35] They attributed their results to the anti-inflammatory response which is regulated by IL-10. IL-10 prevents the tumor development by reducing vascular endothelial growth factor, IL-1b, tumor necrosis factor alpha, and IL-6.[36] According to a research study, an IL-10 shortage results in an increase in the production of pro-inflammatory cytokines, which aid in the establishment of tumors in mice.[37] The discrepancy between their results and our finding could be accounted for by the different types of carcinomas. Moreover, their study protocol is distinct as they used an immunosuppressive therapy for treatment of lung carcinoma. Antagonizing the immunosuppressive effects of IL-10 may be a promising protocol for HCC immunotherapy.[15]

In conclusion, our data indicated that DCs plus Dribbles vaccine has a remarkable efficacy superior to either Dribbles alone or DCs alone in reduction of HCC growth and survival improvement. Furthermore, Il-10 could be considered a valuable biomarker for monitoring the response to immunotherapy in HCC.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AAE, NMA and AAA. The first draft of the manuscript was written by AAG and TEM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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.

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Ethical approval

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee (ZU-IACUCC/3/F/54/2018).

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