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EVALUATION OF T-CELL CYTOTOXICITY IN THE TREATMENT OF MALIGNANT BRAIN TUMORS BY DENDRITIC CELL VACCINES IN VITRO

By

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ABSTRACT

Background: Glioma is the mostly occurred primary tumor in our central nervous system. As the chemotherapy and radiotherapy has failed to safely boost the efficacy of malignant glioma treatment during the last twenty years, there is an urgent need to find alternate new therapeutic protocols, and the immunological therapy is one of them. Dendritic cells (DCs) gained great attention in this field of research.

Objective: To determine T lymphocytes (CTL) cytolytic activity, when stimulated by immunological product consisted of the fusion between glioma-DCs to be suggested as adjuvant in glioma therapy.

Patients and method: This was a descriptive study conducted on 14 high grade glioma patients at Neurosurgery Department; Al-Maadi Military Hospital between 2018 and 2020. By using chemical fusion method, cultured glioma cells and DCs acquired from mononuclear cells of peripheral blood were fused. The processed glioma products were divided into 3 categories: (A) Suspension of fused glioma with CTL-effectors; (B) Suspension of co-culture glioma and DC cells with CTL-effectors, and (C) Lymphocytes only categorized as control. CTLs cytotoxicity effects on tumor cells were measured using MTT colorimetric analysis Ex-vivo.

Results: Cytotoxic effects of CTLs activated by fused cells compared with co-cultured CTLs group and un-sensitized lymphocytes was shown to be significantly high. The potency level of CTLs fused category elevated tremendously (P-Value less than 0.01).

Conclusion: The fusion method of dendritic cell based immune-therapy has highly potent antitumor effects via inducing CTLs actions.

Keywords: Immunotherapy, Lymphocytes, MTT assay.

INTRODUCTION

Malignant brain tumors are one of the most serious kinds of cancer. Glioblastoma multiforme is ranked as the most frequent of these incurable tumors, accounts nearly about 50% from brain glioma and 25%from brain malignancies in grownups *(Davis et al., 2010).*

Surgical treatment, chemotherapy and conventional radiotherapy are the classically used therapies for gliomas. However, all are failed to achieve

complete healing in majority of cases and the relapse is inevitable, also with bad prediction. Improving and updating the clinical tools for curing and avoidance of malignant glioma relapse after traditional treatment is mandatory in neurosurgical field *(Zhong et al., 2012)*. Immunotherapeutic is one of the most important candidates in the new proposed and possible managements for glioma. Due to its main contribution in immune system modulation and cross presentation of antigens, DCs gain more attention in research of immune based therapeutics for malignant glioma *(Banerjee et al., 2010)*.

The fusion tumor vaccine which is known as dendritoma, in addition of being the newly synthesized antigen from the original tumor, it efficiently displays the new antigens and stimulates CTLs, opening a novel research area as the results are encouraging *(Fan and Zhu, 2011)*.

The development of anti-glioma effector cytotoxic T lymphocytes sensitized in reaction to the gene vector IFN-β and DCs presentation, has showed effective immunotherapy techniques against glioma *(Nakahara et al., 2010)*. The discovered plans for immune based therapies for glioma is to focus on natural killer T cells *(Dhodapkar et al., 2011)*. The researches on animals like mouse for comparison the intra-tumor injection of DCs combined with pre-irradiated cells of glioma in their brains, showed that brain tumor-bearing mice lived longer *(Kikuchi et al., 2010)*. All of these observations suggest the therapeutic benefits of injecting DC and irradiation glioma cells into tumors. Comprehensively, immunological therapy by using DCs in animal models and clinical trials has been proven to be safe for enhancing the local immunity in the brain *(Okada et al., 2013)*.

The main goal of successful therapeutic course for malignant brain tumor is to target and eradicate leftover tumor cells after surgery. Given the widespread nature of glioma, finding and killing every residual tumor cell is a challenging feat. However, the therapeutic techniques that hire the body's own immune system to fight residual tumor cells hold the hope. Immunotherapy for cancer treatment has the privilege that the immune effectors are able to specifically target and destroy tumor cells that are invisible to traditional treatment modalities, meanwhile stimulating long-lasting immune monitoring against cancer cells *(Pachter et al., 2010)*. Traditionally dendritic cell-dependent immunotherapy has utilized the tumor lysis products or tumor derived peptides to stimulate anticancer immunity. The findings of previous researches testing the CTLs activity on dendritoma or Dendritic cells stacked with apoptotic cells of glioma showed that it has elevated killing activity and proliferative capacity versus dendritoma prepared from monocyte derived DCs fused with self-tumor cells *(Sloan and Parajuli, 2010)*. *Koido et al. (2013)* concluded that giving cancer patients fused DCs with neoplastic cells is a safe immunotherapeutic intervention technique that produces effective tumor-specific T-cell immune responses Ex Vivo.

The current study aimed to investigate the anticancer effects of cytotoxic T cells incubated with a fused

glioma - DCs vaccination as adjuvant therapy for glioma.

PATIENTS AND METHODS

This was a descriptive study that conducted on 14 high grade glioma patients from the Department of Neurosurgery, Al-Maadi Military hospital during the period from 2018 to 2020. Of the 14 patients, four patients were diagnosed as glioblastoma multiforme, seven patients as astrocytoma and three patients as oligo-dendroglioma.

Glioma was removed aseptically from the patient. RPMI1640 was used to wash tissues. The tumor was cut into small pieces to start digestion by trypsin, followed by filtration with mesh of steel having varying sizes to prepare the suspension of glioma cells. After centrifugation and two times wash of the suspension, it was suspended again in culture medium for propagation, and subculture was done while seen the cells covering the bottom.

Lymphocyte separation medium (Ficoll-paque) was used to centrifuge 100 ml of anticoagulated peripheral blood mingled with same volume of phosphate buffered saline. Following washing and culture, the layer of mononuclear cell was discarded cautiously, centrifugated, and reconstituted in a complete cell growth medium. After 2 hours of incubation, non-adherent T lymphocytes were collected, and stored in tank of liquid nitrogen, for further use in collecting of purified CTLs lymphocytes. Complete cell growth medium was added to adherent T cells which contain conc. 1µg/ml for rh IL-4and rh GM-CSF. Three days later, the starting volume of the complete culture medium with cytokines was reduced to half and exchanged. Six days later, we added 50 ng/ml TNF-αto the culture within the media, and ten days post-harvest DCs were gathered. On day six and day ten HLA-DR, CD86 phenotypic testing was done respectively.

DCs were cultured for seven days, then CD86-FITC was used for staining and mingled coequally with PKH-26 stained glioma cells at a 2:1 ratio in a cell culture tube. The supernatant was removed after centrifuging the cell mixture. After one minute of incubation, 0.001L of polyethylene glycol solution with concentration of fifty percent was gradually supplemented. To stop the fusion, centrifugation of the suspension then upper layer was eliminated. The cell pellet was re-suspended in a cell growth medium. After thirty-six hours culturing, flow-cytometry was utilized to capture the final fused cells.

A Nylon wool column was used for collection and elution T-lymphocytes which previously collected non-adherent lymphocytes. For preparation of DCs, Glioma tumor cells were co-cultured with DCs in a ratio (3:1) respectively for 36 hrs. After that, DCs were mixed with T-cells in a percentage of 1:10 for three days. At the end, by co-culturing CTLs with the fusion cells at a ratio of 1:10 for three days, the tumor-specific T-cells were produced. Following maturation, the specialized cytotoxic T lymphocytes were extracted as non-clung cells, then washed and moved to a 96-well plate for cytotoxicity test.

The suspensions of tumor cells were divided into 3 main groups which included: (A) activated CTLs by fused cells; (B) activated CTLs by co-cultured

cells of glioma with DCs and (C) A control group consisting of lymphocytes. The percentage of plating cytotoxic T lymphocytes on the 96 well plate were adjusted to 10: 1, 20: 1, 40: 1 and 80: 1 (CTLs : glioma cells) respectively, with three replicates. Target glioma cells and effector cells separately were considered two negative control groups. The optical densities of all groups were calculated by OD after 3 days in culture via MTT assay, which was a colorimetric assay used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence), and 20 µl MTT was dropped in each well, after then, the 96 well-plate was incubated for another four hours. A total amount of 150 µl dimethyl sulfoxide (Sigma-Aldrich) was then poured in each well. The culturing plate was gently agitated at 37°C for 10 min. Spectrophotometer was used for measuring absorbance (A) values at 570 nm, and used to calculate the percentages of cytotoxicity. Data was demonstrated as the mean of three ODs. The following formula was used to compute the effector cell mortality rate: [O.D1-(O.D3-O.D2)] / O.D1 × 100, where O.D1: glioma cell optical density; O.D2: CTLs optical density; O.D3: target cells after CTLs reaction optical density.

Statistical analysis:

The Statistical Package of the Social Sciences (SPSS 25.0) statistical tool was used to perform the statistical analysis. Numerical values were suggested as mean +standard deviation (SD).

RESULTS

Of the fourteen patients, there were four patients of glioblastoma multiforme (GBM), seven patients of astrocytoma, and three patients of oligo-dendroglioma.

To obtain adherent cells, peripheral blood mononucleated cells (PBMC) were cultivated for 2 hours **(Figure 1A)**. The cytokines were added to cultures with adherent cell and then the culture was continued for another 24 hours. Cell aggregation was detected during the culture time, because multiple colonies altered to the current large phenotype, producing suspended aggregates after culturing for six days. Meanwhile, the edge of cells in colonies showed ruffles which were the characteristic of DCs morphology **(Figure 1B)**. Following the addition of TNF-α, cells appeared irregular under the microscope, transitioning from an aggregated to a dispersed condition. Lastly, the typical DCs were shown to have apparent surface expansion bulging **(Figure1C)**. Flowcytometric analysis revealed that mononuclear cells lacked the expression of cluster of differentiation 86 and MHC class II cell surface receptor HLA-DR **(Figure 1D)**. Both CD86 and HLA-DR expression were found to be low after culturing in conditions containing both GM-CSF and IL4 **(Figure1E)** as this corresponds to the immature state of DC phenotype. Oppositely, after adding TNF-α, there was a greater expression of both molecules CD86 and HLA-DR **(Figure 1F)**, an indication of maturity and activation of DCs.

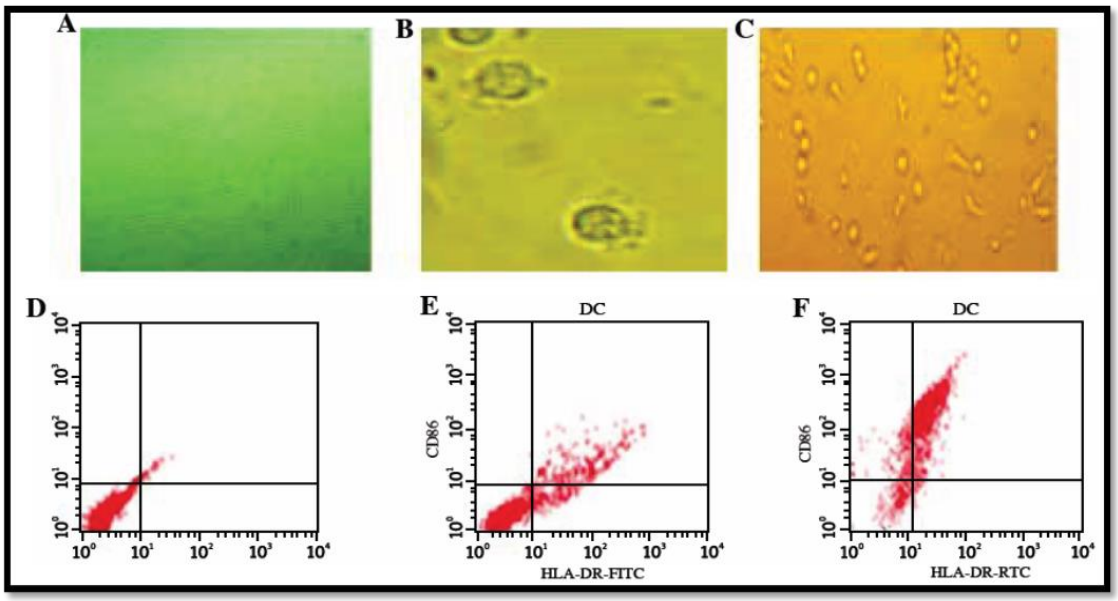

Figure (1): Dendritic cell morphology and phenotypic detection.

Microscopically, initial cells of glioma were shown to be spherical suspension scattered in culture medium. The cells turned into oval and became adherent to the culture dish's sides after 4 hours. After 48 hours of culture, the cells number decreased with a low density, although cell generation was robust. The sizes and shapes of tumor cells were diverse, showed to be trigonal, as a polygon or irregular with minimal cell bulge. Following ninety six hours cultivation, the density of cells elevated markedly, and the morphology was still diverse and similar to the pathology of glioma. These stable cellular shapes kept for ten days of incubation **(Figure 2A)**.

Following similar conditions, FITC-labeled CD86 pre-stained DCs appeared green. Also, stained glioma cells appeared yellow by bright-field. At a wave length 570 nm, all cells were seen red **(Figure 2B)**. The cell membranes showed to be in contact after a period of 24hour DCs-glioma fusion incubation by using an inverted contrast microscope. Fusions may occur in different forms as multiple glioma fused to multiple DCs; multiple glioma cells fused with a single DC; or multiple glioma cells fusions as a cluster. As seen under fluorescent microscope after 36-hour fusion incubation, DCs were green colored but fusion cells were colored yellow **(Figure 2C)**.

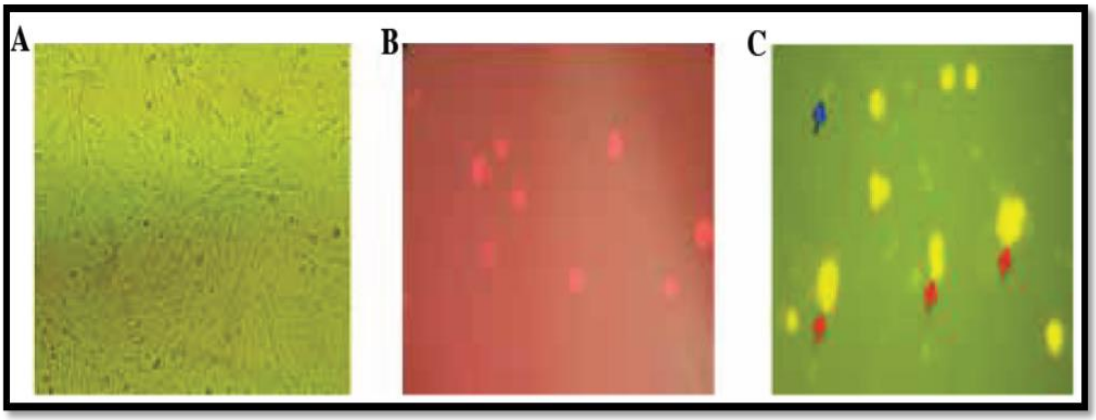

Figure (2): **A)** Cultivated glioma cells After 10 days, s; **B)** Stained glioma cells (PKH26-red staining); **C)** Fusion of glioma cell and DCs, where DC appeared green(blue dart); fused cells were yellow colored (red darts).

The cytopathic effects of cytotoxic T lymphocytes, stimulated by glioma cells fusion, highly increased than the effects caused by the CTLs group stimulated by co-cultured glioma cells and with un-sensitized T lymphocytes (P.Value<0.01). However, the ratio of cytotoxic activity elevated in both of fusion cells and cocultured CTLs groups **(Figure 3)**.

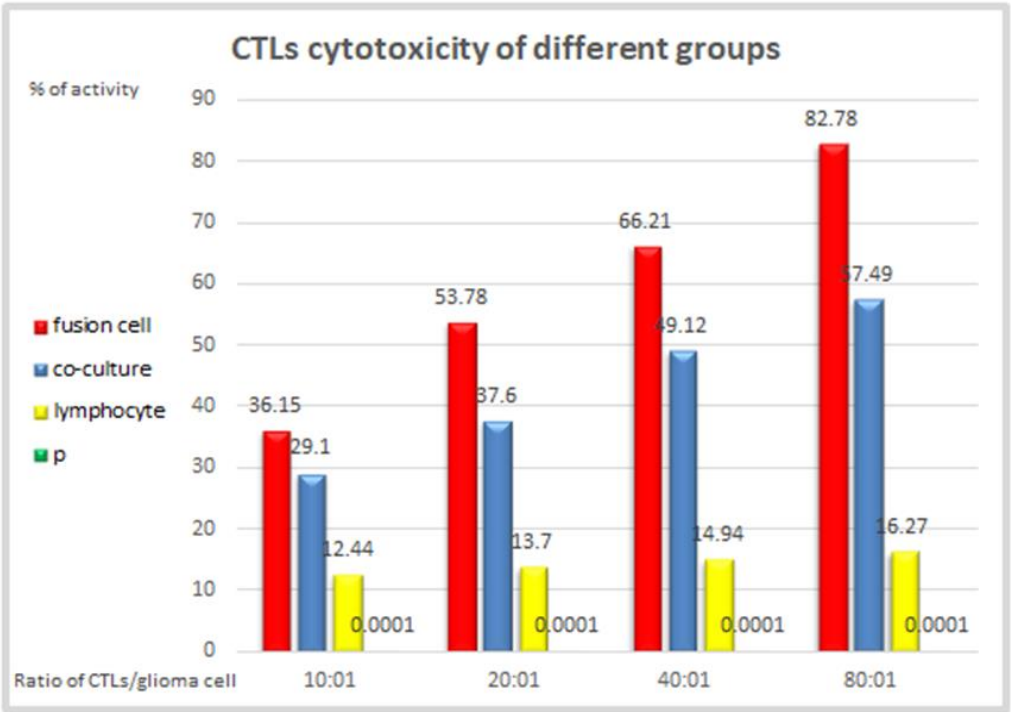

Figure (3): The fused group efficiently increases CTLS activity.

DISCUSSION

Dendritic cells are proved to be the core of both arms immune system, innate and adaptive immunity. Many researches have been conducted to look for their differentiation, derivation and its roles in modern immunotherapies. Dendritic cells can be originated from diverse of cellular sources, like CD34+ primogenitor in the bone marrow, pluripotent BM stem cells and cord blood, from circulating monocytes in the peripheral blood. Different techniques using cocktails of cytokines are used to shift the differentiation toward DCs *(Wolfl et al., 2011)*.

Monocytes-derived DCs are the mostly utilized type of DCs for clinical research. Their differentiation from PBMCs was by the help of IL4 and GM-CSF *(Chiang et al., 2011)*. The immunogenicity of the cells of glioma is potentially low as naturally. Several T cells have been discovered to invade inside glioblastoma as shown in different studies *(Wolfl et al., 2011* and *Waziri et al., 2012)*. Lymphocytes around glioma are frequently used as a sign for good prediction *(Dunn et al., 2011)*. DCs are commonly considered to be the most potent APCs, with a potential capability to activate T lymphocytes initially.

As DCs stimulation and function directly impact the type of immune responses, the function of DCs needs more research to define its therapeutic capabilities *(Zhou et al., 2011)*. Different studies proved that the dendritic cells can induce tumor-specific CTLs for the treatment of glioma. Furthermore, DC vaccines have been utilized as part of the treatment regimen in patients with minor lesions after the traditional treatment because its effect is affordable and stimulates the body immunity *(Yu et al., 2010)*.

The number of DCs in different body tissues is extremely low but dispersed vastly. So, it is important to setup a mature DCs culture protocols to get enough number of dendritic cells for further researches *(Liu et al., 2011)*.

Implementation of new protocols for isolation and culturing of Dendritic Cells ex vivo is expensive under specific precautions *(Li and Chen, 2013)*. Peripheral blood derived DCs culturing give many benefits; a) the sample obtained simply; b) the donor pool is widely available; c) the stage of maturation is controlled easily. As a Consequence, the most ideal way to obtain dendritic cells is through the peripheral blood. So in our research, we used the patient peripheral blood as a donor of DCs. PBMC was separated by Density gradient centrifugation technique. Due to their higher gravities than lymphocytes, RBCs and granulocyte were separated in low fraction; at the separator medium interphase, mononuclear cells were settled. The used separation technique was appropriate for a little blood volume. Dendritic cells origins, phenotypes and actions are variant and diverse. DCs perform a vital function in both activating T cells and developing peripheral and central immune tolerance *(Li et al., 2011)*.

Depending on the maturation state of dendritic cells, there will be corresponding immune-modulatory actions *(Shi et al., 2012)*. Whereas activation of T lymphocytes and initiation immunological

response occur by mature DCs, the immature DCs will process antigens robustly and inhibit T lymphocyte activation; finally, T lymphocyte will be poorly primed *(Yang et al., 2011)*.

Ex vivo researches revealed that iDCs may prevent the production of antigens specialized T lymphocytes *(Xia et al., 2010)*. Accordingly, investigations have shown that a higher level of DCs maturation is connected with the generation of effective antitumor-specific cytotoxic T cells *(Shinozaki et al., 2010)*.

Many cytokines supplied in a two steps combinations technique have been shown in studies to stimulate DCs collected from peripheral blood; the starting adding of IL-4 and GM-CSF activate PBMC to enhance the development of immature DCs. whereas adding of tumor necrosis factor-α will promote dendritic cells maturation. In the current research, lymphocyte separation medium was used to collect mononuclear cells derived from the peripheral blood of affected patients. Both Interleukin 4 and GM-CSF are used in culture to skew the growing and differentiation of mononuclear cells to toward DCs, whereas hinder the formation of neutrophils and macrophages. To increase the maturation of DCs we added a Tumor necrosis factor-α, so DCs stimulate a powerful capability to induce T cells. By using microscopy the idealistic visual morphology of DCs showed stellate-like projections of the cells which progressively grew toward maturation and differentiation. Moreover, DCs Flowcytometric characterization shows high presentation of CD86 and HLA-DR phenotypes indicating DCs maturation *(Wen et al., 2011)*.

Optimizing the production of immunotheraputics against tumors by varied techniques can improve and increase the activity of dendritic cells. The term heterozygote Dendritoma refers to the outcome of fusions between different types of glioma antigens and autologous dendritic cells as a modification on DCs maturation. The antigens specific to the tumor and co-stimulatory actions with Dendritic Cells was ideally presented by the fusion technique. Dendritoma is able to effectively deliver numerous antigens specific to the tumor through functional molecules recognized by triggering the response of immunity against selective and intended tumor toxicity (e.g. MHC class I-II molecules and other co-stimulatory signal molecule). Depending on the existing results the use of Dendritoma is feasible for treating malignancy *(Kjaergaard et al., 2010)*.

Tumor-DCs fusions as a vaccine not solely shows full antigens of the tumor cells, however it furthermore provides many capabilities of presentation of antigens and stimulation of cytotoxic T lymphocyte *(Fan and Zhu, 2011)*, consequently the immune response is enhanced. The primacy in the cell fusion technology is that it provides triggering of an effective immune response because the fusion cells present all specific tumor antigens. Given the fact that the most of the antigen classes identifying human cancer cells are not yet known, fused tumor cells could be a simple and effective way for developing an anticancer vaccination. Both polyethylene glycol approach and the electric fusion technique are two current fusion technologies that can be used in clinical studies. Electric fusion is the most efficient fusion process

currently available with efficacy 1 to 2 grades greater than chemical fusion *(Zhu et al., 2011)*. The polyethylene glycol approach on the other hand, is a simple, cost-effective, and practical procedure *(Shi et al., 2010)*.

CONCLUSION

This study has showed that, activating the cytotoxic T lymphocyte (CTLs) by a vaccine compromised of DCs-glioma cell fusion, have efficient antitumor actions. These findings show that developing a safe and effective fusion vaccine could be a potential clinical therapy for glioma patients.

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تقييم سمية الخلايا التائية فى علاج أورام المخ الخبيثة بواسطة لقاحات الخلايا التغصنية خارج الجسم

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خلفيـــة البحـــث: الـــورم الـــدبقي هـــو أشـــهر الاورام الأوليـــة فـــى الجهـــاز العصـــبي المركـــزي. ونظـــرًا لفشـــل العـــلاج الكيميـــائي والعـــلاج الإشـــعاعي فـــي تعزيـــز فعاليـــة عـــلاج الـــورم الـــدبقي الخبيـــث بأمـــان خـــلال العشـــرين عامًـــا الماضـــية، كانـــت هنـــاك حاجـــة ملحـــة لإيجـــاد بروتوكـــولات علاجيـــة جديـــدة بديلـــة وكـــان العـــلاج المنـــاعي أحـــدها. اكتسبت الخلايا المتغصنة إهتمامًا كبيرًا في هذا المجال البحثي.

الهـدف مـن البحـث: تحديـد نشـاط الخلايـا اللمفاويـة التائيـة عنـد تحفيزهـا بواسـطة منـتج منـاعي يتكـون مـن الانـدماج بـين الـورم الـدبقي، والخلايـا المتغصـنة، ليـتم إقتراحـه كعلاج مساعد في علاج الورم الدبقي.

المرضـى وطـرق البحـث: أجريـت هـذه الدراسـة الوصـفية علـى أربعـة عشـر مريضـاً بـالورم الـدبقى عـالى الدرجـة بقسـم جراحـة المـخ والاعصـاب بمستشـفى المعـادى العسـكرى فـى الفتـرة مـن 2018 الـى 2020 بإسـتخدام طريقـة الانـدماج الكيميـائي حيـث تـم دمـج خلايـا الـورم الـدبقي المـزروع و الخلايـا التغصـنية المستخلصـة مـن الخلايـا وحيـدة النـواة للـدم. وتـم انـدماج الخلايـا الدبقيـة الأوليـة المسـتزرعة مـع الخلايـا التغصـنية تحـت ظـروف حضـانة مـن البـولي ايثيلـين جلايكـول. وقـد تـم تعيـين معلـق لخلايـا الـورم الـدبقى علـى ثـلاث مجموعـات لتشـمل: (1) مجموعـة الخلايـا الليمفاويـة التائيـة السـامة والتـى تـم تنشـيطها بالخلايـا المندمجـة، (2) مجموعـة الخلايـا الليمفاويـة التائيـة السـامة والتـي تحفزهـا الخلايـا المحضـنة، (3) مجموعـة الخلايـا اللمفاويـة كمجموعـة ضـابطة مرجعيـة فقـط، والتـي لـم تحفـز بالخلايـا التغصـنية. وقـد تـم تقيـيم

كفاءة الخلايا الليمفاوية التائية السامة للخلايا على خلايا الورم الدبقى بواسطة استخدام اختبار صباغ التترازوليوم.

نتائج البحث: قدرة وكفاءة سمية الخلايا التائية المنشطة بواسطة الخلايا المندمجة أعلى بكثير من الخلايا التائية الغير منشطة (المجموعة الضابطة)، وأعلى أيضاً من مجموعة الخلايا التائية المنشطة بواسطة الخلايا المحضنة بدون اندماج.

الاستنتاج: طريقة الاندماج فى العلاج المناعي القائم على الخلايا المتغصنة لها تأثيرات قوية للغاية مضادة للأورام من خلال تحفيز نشاط الخلايا الليمفاوية التائية.

الكلمات الدالة: العلاج المناعي، الخلايا الليمفاوية، طريقة صبغ التترازوليم.