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行政院衛生署九十五年度科技研究計畫

樹突細胞腫瘤疫苗治療：臨床試驗後之轉譯醫學研究

研 究 報 告

執行機構：中國醫藥大學

計畫主持人：楊文光

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湯曉君

執行期間：95 年 01 月 19 日至 95 年 12 月 19 日

行政院衛生署「95 年度建立轉譯醫學臨床研究計畫」成果報告審查表

執行機構	中國醫藥大學		主持人	楊文光
計畫名稱	樹突細胞腫瘤疫苗治療：臨床試驗後之轉譯醫學研究			
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※成果報告審查綜合意見

1. 本計畫為由在長庚所進行之一個腦瘤(GBM)臨床治療的試驗(以 dendritic cell 作為手術及放射或化療後之輔助治療)，計畫主持人由其中建立 GBM 細胞株及病人之周邊淋巴細胞作各種免疫學檢測，初步可認為其結果可以接受，但未來其研究方向應更明確。
2. 已依計畫完成研究。初步成果顯示樹突細胞腫瘤疫苗療法可能用以治療 Glioblastoma multiforme。
3. 本計畫主要探討自體樹突細胞腫瘤疫苗治療與Treg之抑制抗癌免疫性之相關性，並研究放射治療和化療對 Treg 或 CD8 之影響。計畫之執行多項（計有十項）指標均已達成，其結果顯示：(1)其研究室自行配製之樹突細胞腫瘤疫苗具有療效，並以 IL-12 之表現確立其品質。(2)此疫苗之治療在腫瘤切除後，儘快與放射療法合併使用。研究成果相當不錯，宜繼續推動策劃並改進癌症免疫療法，並將研製樹突細胞腫瘤疫苗之製造技術與配方儘快申請專利。
4. 本計畫個案數太少，雖可見到結果，但只 proof of concept。未來如要申請延續型計畫，應有統計意義的個案數。

總評：

■ 同意結案
□ 依審查委員意見修正後同意結案
□ 其他：

審查委員	劉滄浯、胡幼圃、林志六、鄧哲明、許須美

目　　錄

中文摘要

本轉譯醫學研究計畫的最終目的是研究一些可以使用於目前無法治療的人類癌症以減少死亡率、延長存活期和提升病人的生活品質。我們以「惡性腦膠質胚原癌」(Glioblastoma multiforme)簡稱 GBM 為樹突細胞腫瘤疫苗輔助療法為主要對象，因為這種病人在診斷後的存活期平均是 8~14 個月，2 年的存活率幾乎為零。雖然我們已經完成第一期的臨床試驗，成果很好，但也令我們認出一些重要轉譯醫學的問題需要進一步研究以改進以後之第二期、第三期的臨床試驗。

本計劃有兩個重點目標:(甲)探討自體樹突細胞腫瘤疫苗治療和 T 調控淋巴細胞(Treg)之抑制抗癌免疫性的相關性，並研究如何增進疫苗配製的方法以減低 Treg 之免疫抑制作用;(乙)探討放射線治療和化學治療藥劑對侵入腫瘤之 Treg，或特殊抗癌免疫 CD8 淋巴細胞的影響，以並探討對放射治療或化療後殘留而再發的 GBM 腫瘤細胞是否仍然會被特殊抗癌免疫細胞 CTL 殺傷或是不再受特殊 CTL 之免疫作用。

一年來的研究成果包括:：(1)研究活細胞 CFSE 螢光染法以探測細胞的成長或受抑制的技術平台； (2)證實我們所配製的樹突細胞腫瘤疫苗會刺激特殊抗癌 CD8 免疫淋巴細胞(CTLs)，並拮抗 Treg 免疫抑制的作用，由 FOX-P3 RNA 表現之變化可知； (3)樹突細胞腫瘤疫苗刺激 CTL 功能的主

要關鍵因素是 interleukin-12 之產生；(4)GBM 腫瘤細胞會產生刺激 Treg 細胞之生長，但是如果事先對腫瘤細胞加以處理促使其產生 Hsp 和 MHC 則可以增進抗癌 CTL 之生長，並以壓制 Treg 之作用；(5)抗 GBM 之新藥 Temozolomide 會毒死某些 GBM 病人之腫瘤細胞和免疫細胞，但對另外 GBM 病人之腫瘤與淋巴細胞則沒有抑制效用；(6)大部分 GBM 腫瘤細胞會被治療劑量的放射線所殺傷，但其中的「癌幹系細胞」則具相對地不受放射線性的傷害而存活下來且繁殖成治療無效的腫瘤細胞；(7)大部分的抗癌 CTL 會受放射線影響，變成無能或「記憶」狀態，相反地，Treg 則會被低劑量之放射線刺激生長；(8)經 1000G 放射線處理後存活下來並會快速繁殖的 GBM 腫瘤細胞與其原來之腫瘤細胞仍然同樣會受特殊抗癌 CTLs 之殺傷；(9)Topo I 抑制藥物 NS38 和 CPT 會毒殺 GBM 腫瘤細胞也會對 Treg 調控淋巴細胞產生很有意義之抑制性， NS38 似乎是經由其殺傷淋巴細胞的機制，而 CPT 則主要會降低 Treg 之功能而具較少細胞殺傷作用；(10) COX-2 抑制藥物 Celecoxib 在體內的最高濃度(16~20 μM)並不會毒殺 GBM 腫瘤細胞，但卻會改變腫瘤細胞的性質，進而刺激 Treg 細胞的性質。

上述研究成果的意義如下：其一，可以證實我們配製的樹突細胞腫瘤疫苗的確有療效性，但可以用 IL-12 之表現為基準以確定其品質；其二，樹突細胞腫瘤疫苗法應該在腫瘤切除後盡速進行，可以和放射治療合併應用

或在治療之前進行則可以提升療效；其三，值得繼續研究人類腫瘤細胞之體外培養技術，特別注重「癌幹系細胞」之培養和分析，以及體外大量擴張抗癌特殊性 CTL 和 Treg 細胞之生長，並且建立細胞技術平台，以發現調控抗癌免疫機能與免疫調控機能之藥物(如 CPT?)，最後可應用於人類重要疾病之治療。

中文關鍵詞:抗癌免疫細胞，免疫調控 T 細胞，惡性膠質腦瘤，免疫調裡藥物，放射線癌治療

Summary

For the ultimate purpose of developing novel therapeutic approaches to decrease the mortality rate, to prolong the survival period and to improve the life quality of patients suffering from currently incurable human cancers of high mortality and short survival, this translational medicine research project is to address some important questions raised from our previous phase-1 clinical trial, "Autologous Dendritic Cell Tumor Vaccine Adjuvant Therapy of Malignant Brain Gliomas". In this clinical trial, the survival period of the most lethal glioblastoma multiforme (GBM, WHO grade IV gliomas with mean survival period of 8-14 months after onset/diagnosis) were evidently increased in some but not all patients. It is therefore important to understand the reason(s) why our DC-tumor vaccine therapy did not work in these GBM patients, so that planning of the phase-2 and phase 3 clinical trials could consider these reasons for improving the therapeutic protocol. .

Thus, the two specific aims of this translational medicine research project are: (A) To determine the possible controlling or stimulating effects of autologous dendritic cell-tumor vaccine therapy on Treg suppressor cells and, to search for additional preparative procedures to prevent undesirable Treg stimulation; and (B) To determine the sensitivity of GBM-tumor infiltrating Treg and tumor-specific cytotoxic T lymphocytes (CTLs) (relative to GBM tumor cells) to radiation and chemotherapeutic agents and to determine whether cancer cells treated but not killed by radiotherapy or chemotherapy become more susceptible or more resistant to tumor specific CTLs.

Patient's GBM tumor cells and sutologous T lymphocytes, including tumor infiltrating Treg and CTLs, were used for in vitro experiments to answer the

questions raised in the specific aims.

We have virtually achieved the two specific aims proposed in this project for the past one year. Highlights of the progress are: (1) A vital cell-staining method, namely, CFSE-labeling immunocytofluorometric procedure, has been developed for quantitative measurement of cell growth or cell killing of GBM tumor cells and lymphocytes in vitro, as a general technical platform; (2) We have found that dendritic cell-tumor vaccines, which we prepare with additional procedures to stimulate and stabilize the maturation state of dendritic cells, can in general stimulate tumor-specific CTLs and counteract or inhibit the Treg immunosuppressive activity; (3) The major factor of dendritic cell-tumor vaccine to counteract the immuno-suppressive function of Treg lymphocytes is the production of interleukin-12; (4) GBM tumor cells apparently produce factors to stimulate autologous CD4(+)/CD25(+)/CD45RO(+)/Fox-p3 RNA(+) Treg immuno-suppressor lymphocytes and to inhibit CD8(+) lymphocytes. But, if GBM tumor cells are first treated to express heat-shock protein (90-95 Kd Hsp) and major histo-compatibility complexes (MHCs), the Treg effect can be overcome by strong stimulation of tumor-specific CD8(+) CTLs; (5) Susceptibility of GBM tumor cells as well as autologous lymphocytes to chemotherapeutic drug, temozolomide, vary among different patients; (6) Therapeutic doses of ionizing radiation can kill most GBM tumor cells. However, the "cancer stem line" cells of GBM are relatively radio-resistant and may survive to subsequently re-populate the radio-resistant GBM tumors; (7) Tumor infiltrating Treg lymphocytes are less susceptible to ionizing radiation than tumor infiltrating CTLs, which however are often not killed but rendered "inactive" (into memory cells?) by exposure to ionizing radiation or

chemotherapeutic agents; (8) The radio-resistant GBM tumor cells that survive 1000 cG radiation are equally sensitive as their parental radiation-sensitive GBM tumor cells to cytotoxic and inhibitory effects of tumor-specific CTLs; (9) We have found that topoisomerase inhibitors, NS38 and CPT, while similarly exert cytoxicity on GBM tumor cells, also can inhibit CD4(+)/CD25(+)/CD45RO(+)/Fox-p3 RNA(+) Treg immuno-suppressor lymphocytes by different inhibitory mechanisms, NS38 by severe killing Treg cells whereas CPT by inhibiting the Treg suppressor functions (but not killing Treg cells); and (10) We found that a COX-2 inhibitor, celecoxib, at therapeutic doses was not cytotoxic to GBM tumor cells but appeared to decrease the immuno-suppressive activities of GBM tumor cells.

The major implications of the results accomplished by this translational medicine research project are that our dendritic cell maturation procedures should be enforced, if not improved, to ensure the high production of interleukin-12 , that the dendritic cell-tumor vaccine adjuvant therapy for GBM patients should start right after surgical resection and before or simultaneously with post-surgical radiotherapy and that we should continue to search for chemotherapeutic agents (such as CPT?) that can temporarily inhibit Treg cells for simultaneous application with cancer immunotherapy. In addition we should continue to improve our procedures to culture GBM tumor cells and characterize GBM "cancer stem line" cells, to expand the in vitro growth of Treg immunosuppressor T lymphocytes and tumor-specific immune CTLs and discover the chemotherapeutic drugs that can modulate of these important regulatory and immune lymphocytes for eventual clinical application to treat human diseases.

Keyword: tumor-specific cytotoxic lymphocyte, T regulatory lymphocyte, malignant gliomas, immune modifier drugs, cancer radiotherapy

Descriptions of Technical Progress

INTRODUCTION

Previous studies of immunotherapeutic approaches in animal tumor models have led to the idea that dendritic cells (DC), the most potent "professional" antigen-presenting cells in the body, are likely useful for immunotherapy of human cancer[1-3] {Dhodapkar, 2000 #32}. Initial clinical trials of DC-based tumor vaccine therapy produced promising data of 30% or better response rates in B lymphoma patients treated with DC-idiotypic immunoglobulin vaccine[4] and in malignant melanoma patients vaccinated with DC-tumor cell lysate[5]. However, according to a recent review, no firm conclusion regarding efficacy could be drawn from the reported results of most concurrent or subsequent clinical trials of DC-based immunotherapy in various human cancers (Crammer et al 2004 Cancer Immunol Immunotherap) because of variations in DC preparation, choice of antigens, vaccination protocols, means of immune assessment, criteria of clinical responses, and interpretations of different clinical trials, although minimal or no adverse effects were experienced by the treated cancer patients[6].

High grades gliomas are usually, if not always, fatal despite the standard procedure of surgical resection and radiotherapy. According to a recent prospective study[11], the mean survival periods of WHO grade IV gliomas (GBM) and grade III gliomas (anaplastic astrocytoma) after diagnosis were 45 and 87 weeks, respectively. Also, a meta-analysis of 12 clinical trials showed that chemotherapy could only increase the first-year survival from 40% to 46% with life extension of 2 months[12]. Novel therapeutic approaches are therefore urgently

needed and can be evaluated by small-scale clinical trials because of the almost 100% mortality and less than 2-year survival period of GBM. Recently, such clinical trials of DC-based tumor vaccine therapy for GBM have been reported to obtain promising results[13-16]. However, so far only very small fractions of the DC vaccinated GBM patients have shown favorable survival responses, while factors related to the failure in most cases are largely unknown.

Since the end of 2002, we have conducted a clinical trial of autologous DC-based tumor vaccine adjuvant therapy aiming at post-surgical residual tumor cells of glioblastoma multiforme and anaplastic astrocytoma. On the basis of our previous translational research, we modify the procedures of DC-tumor vaccine preparation to include stimulation of cultured glioma cells (Chen et al Apoptosis 1998) to express major histocompatibility complexes and heat-shock proteins before use for antigen processing by phagocytic mdDCs, induction of the antigen-exposed "immature" mdDCs to mature for CCR-7 expression, stable viability and interleukin-12 production, and use of serum-free medium and/or patient's own heat-inactivated platelet-containing plasma for cell cultures and vaccine preparation. And all procedures were carried out in barrier for-clinical-use (cGMP) laboratory facilities. Further, we have escalated the DC dosage and vaccination number to several folds of those used by other reported clinical trials. In a current report, we present clinical data of 16 patients, followed up to 2-5 years, to show the apparent adverse effects and likely survival benefits of our vaccine therapy, with statistical analysis for possible factors associated with unsatisfactory responses to the vaccine therapy in some GBM patients. Also, we have isolated the tumor infiltrating lymphocytes from tumor specimen of re-operated GBM patients and found them to comprise

mostly immunosuppressive T regulatory cells prior to the DC-tumor vaccine therapy whereas tumor-specific CD8(+) T lymphocytes during and after the vaccination course (to be published in a separate paper).

Thus, the two specific aims of this translational medicine research project are: (A) To determine the possible controlling or stimulating effects of autologous dendritic cell-tumor vaccine therapy on Treg suppressor cells and, to search for additional preparative procedures to prevent undesirable Treg stimulation; and (B) To determine the sensitivity of GBM-tumor infiltrating Treg and tumor-specific cytotoxic T lymphocytes (CTLs) (relative to GBM tumor cells) to radiation and chemotherapeutic agents and to determine whether cancer cells treated but not killed by radiotherapy or chemotherapy become more susceptible or more resistant to tumor specific CTLs.

MATERIALS AND METHODS

Tumor cell culture. Primary cell cultures of glioma cells were established from surgical specimen of 20 glioblastoma multiforme (WHO grade IV gliomas) patients. After preparing dendritic cell-tumor vaccines for use in the phase I clinical trial, individual patients' culture glioma cells were stored frozen in liquid N2 freezer. For the present study, some of the frozen aliquots of these glioma cells were thawed. The 100-mm dishes were plated each with about ten millions cells in 3 ml of Eagle's non-essential amino acids-containing minimal essential medium (GIBCO) with addition of 40 g /ml gentamycin, 1 g/ml EGF (R&D), 1 g/ml b-FGF (R&D), 1 unit/ml insulin (Elli-LiLy), 5 mg/ml human serum albumin (Plasbumin-25, Bayer Corp. Elkart, USA) and 2% heat-inactivated autologous platelet-containing plasma (collected at leukapharesis), After overnight incubation and subsequently every 2 days, the dishes were removed of non-attached cells/clumps (which were saved from third day on for culturing the tumor-infiltrating lymphocytes), added fresh growth medium and maintained at the initial cell density of about 30% (by trypsinization and combining dishes of tumor cells, if necessary). Upon growth to near confluency, the cultures were split 1 to 3. After three passages or yield of at least $2x10^8$ cells, the tumor cells were harvested for direct use or, until use, storage in AIM-V medium (GIBCO) containing 10% DMSO /7% HSA under liquid N2. Sources of reagents were EGF and b-FGF (R&D, Minnesota), insulin (Elli-LiLy), human serum albumin (Plasbumin-25 USP, Bayer Corp. Elkart, Indianna), DMSO (Sigma, St. Louise), AIM-V and MEM (GIBCO)

Peripheral blood monocytes and lymphocytes. Leukapharesis was performed

in the CGMH Blood Unit usually one or two days before craniotomy or, in some cases, after recovery from craniotomy, to separate peripheral blood monocytes for derivation of dendritic cells by modified procedures of the published methods[7,8]. Briefly, peripheral blood mononuclear cells, totally 2-10x10^9 in number, were collected from each patient, further purified by Ficoll-Hypaque density gradient centrifugation, washed twice with Hank's balanced salt solution, suspended at 2x10^7/ml in AIM-V containing 2% heat-inactivated autologous plasma and placed on culture dishes for 2 hours at 37^0 C in a 5% CO_2 incubator. After removal of floating cells (mostly lymphocytes), the dishes with attached monocytes were added fresh AIM-V medium containing 25ng/ml of recombinant human granulocyte macrophage-colony stimulation factor (GM-CSF) (Immunex, Seattle) and 25 ng/ml of recombinant human interleukine-4 (IL-4) (R&D, Minnesota). Stock solutions of GM-CSF and IL-4 at 25 g per ml of 5% human serum albumin in normal saline (clinical grade) were stored in aliquots of 0.2 ml at -80^0C. The medium was replenished at day 1, 3 and 5. Phagocytic "immature" dendritic cells harvested and pooled on day 7 were qualitatively assessed for decrease of CD14 phenotype and increase in CD86, CD40 and tartrate-resistant acid phosphatase expression by multi-color cytofluorometic analysis (Janckila,Yang et al 2003 J Histochem Cytochem). The pooled DCs were used directly or stored in liquid N2 until vaccine preparation. Anti-human CD phenotype monoclonal antibodies with different fluorescent labels were purchased from Becton Dickinson/Pharmingen Corp.

The lymphocytes free of the 2-hour attached monocytes were pooled and centrifuged at 1200 rpm 10 min. The cell pellets of mostly lymphocytes were stored frozen in aliquots under liquid N2 until use. T lymphocytes were isolated

by positive separation procedures using anti-CD3, anti-CD4 and anti-CD8 antibody-coated magnetic beads according to the commercial suppliers' instruction. To expand the T lymphocytes, the separated T lymphocytes were suspended at 2 x 10^6 cells/ml of growth medium [AIM-V medium containing 40 ug/ml gentamycin, 50 U/ml interleukin-2 and incubated at 37C in 5% CO2 atmosphere with medium changes every 2-3 days, maintaining at the same cell density. Two procedures were used for rapid expansion, one by adding anti-CD3 OK-T3 monoclonal antibody at 0.2 ug/ml once a week for a growth period of 3 weeks and the other was used to expand small T cell numbers by addition of 3000 cG (cesium gamma source) irradiated autologous or isologous peripheral blood mononuclear cells maintaining at cell density of 2-3x10^6 cells per ml, which could expand the T lymphocytes 300 to 1000 folds in a 3-week period. The expanded T lymphocytes were stored in 1.5-ml aliquots of 10 million cells (in 10% DMSO AIM-V medium containing 7.5% human serum albumin) under liquid N2.

Isolation of tumor infiltrating T lymphocytes. The medium of primary monolayer glioma cell cultures was changed every other days for 10-days. From second medium change on, the medium containing the lymphocytes detached from the tumor cell monolayer was pooled and the lymphocytes collected and, if necessary, separated by ficoll-hypaque density centrifugation, for subsequent growth using the lymphocyte growth expansion procedures described above.

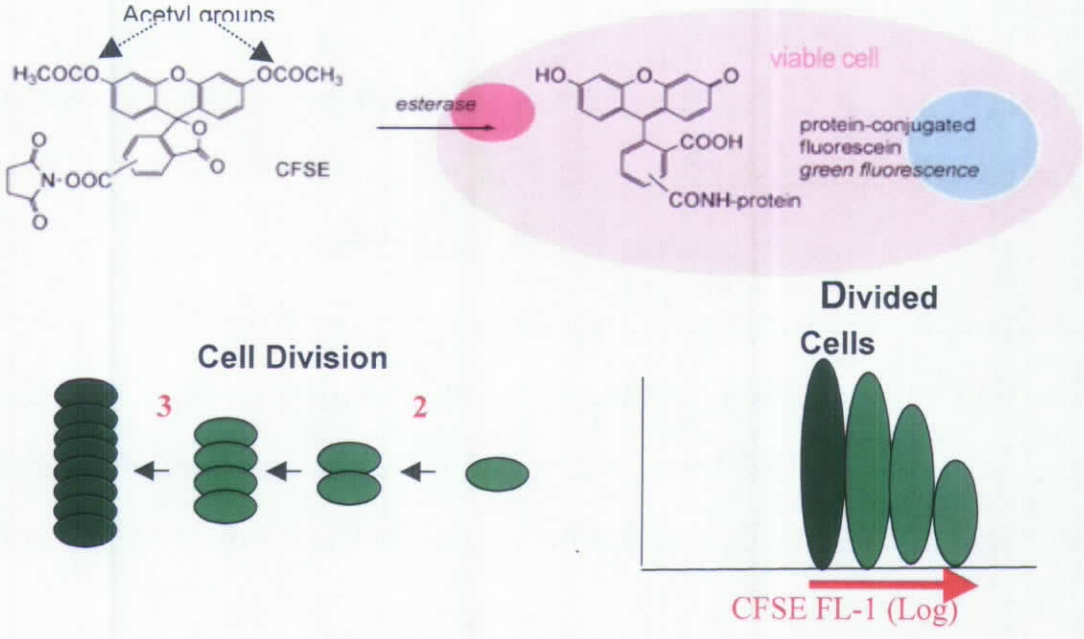
FACS analysis was performed using anti-human CD marker monoclonal antibodies labeled with different color fluorescent-tags including FITC, phycoerythrine and other fluorescent dyes (Becton Dickinson, Inc, Pharmgen, DAKO or Molecular Probe). Lymphocytes were stained with the fluorescent

antibodies according to the standard staining procedures. Multi-color fluorocytometric analysis was performed in BD Vantage and analyzed using the BD softwares.

RT-PCR and qPCR were performed using the standard procedures provided by the commercial suppliers. RNA extraction was performed with PBS-washed fresh cells by the trizol procedure and initial reverse transcription was carried out using oligo dT as the primer. The cDNA products were aliqouted and stored at -80C and subsequently amplified by using specific pair of oligonucleotide primer. For Fox-p3 RT-PCR a pair covering the 3' terminal portion and a pair covering exon-2 to exon-3 were employed for detectiong the total isoforms of Fox-p3 RNA and the isoform without exon-2 deletion, respectively.

Vital cell-staining method, CFSE-labeling immunocytofluorometric procedure, has been developed for quantitative measurement of cell growth or cell killing of GBM tumor cells and lymphocytes in vitro, as a general technical platform. Briefly, washed cells were suspended in phosphate buffer saline containing 1-5 uM of CFSE (Molecular Probe) and incubated at 37C for 5-10 min, washed 1x with 7% HSA, 3x with 1% HSA, and used for experiments or cryopreserved in liquid N2 frezer until use. The principle of CFSE vital staining method for studying cell growth or cell inhibition is illustrated (Figure 1).

Detection of Cell Proliferaton by CFSE Vital Staining



Modified from Lyons and Parish 1994

Figure 1- Principle of vital cell staining by fluorescent CFSE-labeling of GBM tumor cells or various human T lymphocytes for studying individual cell growth and/or inhibition (e.g. cytotoxic effects of CTL on GBM tumor cells). Individual cells can be analyzed and quantitated by multi-color immuno-cytofluorometry.

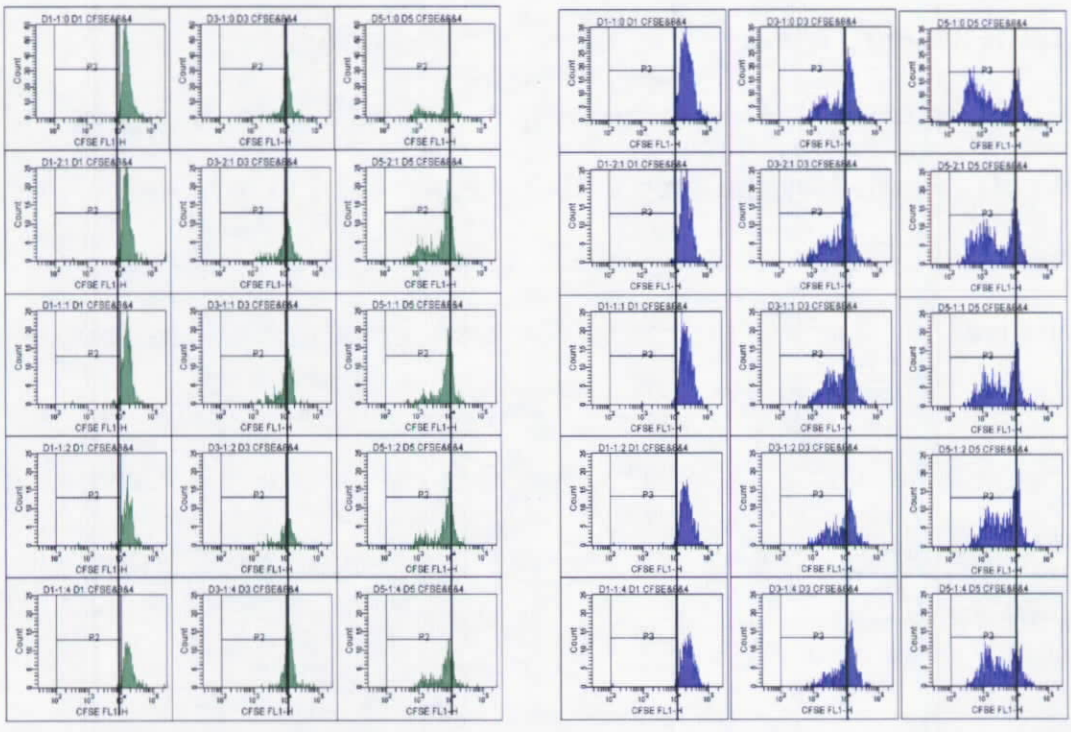


Figure 2- FACS histograms of CD8 (+) and CD4(+) T lymphocytes co-cultured at various cell ratios (in 5 columns at 1:0. 1:0.5; 1:1: 1;2 and 1:4 of lymphocytes/tumor cell ratios) for 1, 3 and 5 days (in rows) with autologous GBM tumor cells of GBM#12 patient. The results show that CD8(+) T lymphocytes (left panel, green) were more susceptible than CD4(+) T lymphocytes (right pane, blue)l to immuno-suppressive effects of autologous GBM tumor cells

RESULTS

The major results are summarized in the following

1. **Decrease of Treg lymphocytes in post-vaccination TILs of GBM patients revealed by the Treg molecular marker Fox-p3.** We have found that dendritic cell-tumor vaccines, which we prepare with additional procedures to stimulate and stabilize the maturation state of dendritic cells, can in general stimulate tumor-specific CTLs and counteract or inhibit the Treg immunosuppressive activity. This was demonstrated by the decrease of Fosp3 RNA expression in tumor infiltrating T lymphocytes after DC-tumor vaccine therapy (Figure 3).

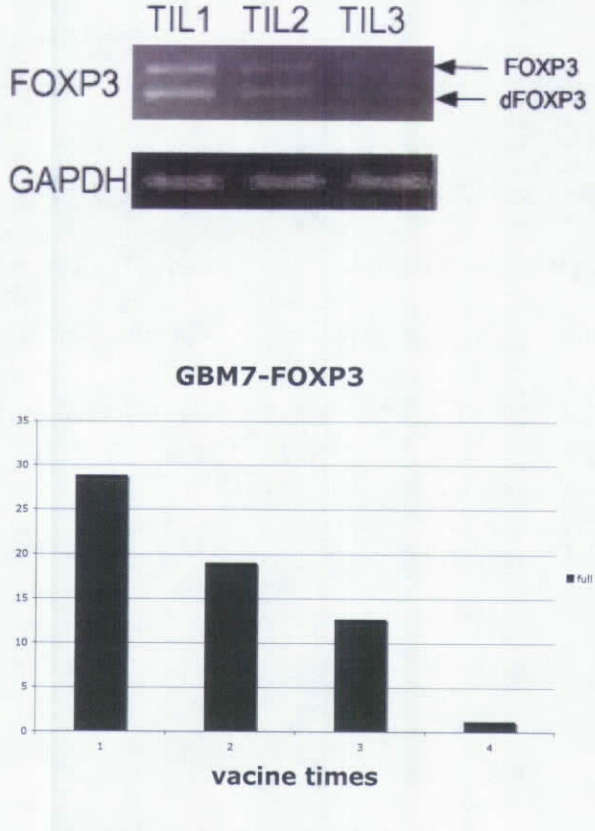


Figure 3 –RT-PCR (upper panel) and qPCR (lower panel) analyses of Fox-p3 RNA contents of tumor infiltrating T lymphocytes isolated from GBM patient #7 before (TIL1), after 8 vaccination (TIL2) and 2 months after

completion of 10 vaccination. The results show continuous decrease of Fox-p3 RNA content in TILs following autologous dendritic cell-tumor vaccine therapy.

2. **In vitro treatment of GBM tumor-infiltrating Treg lymphocytes with interleukin-12 induces changes in Fox-p3 expression.** The major factor of dendritic cell-tumor vaccine to counteract the immuno-suppressive function of Treg lymphocytes appears to be the production of interleukin-12, as demonstrated by in vitro experiments (Figure 4).

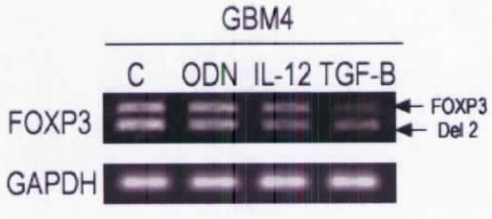


Figure 4 – RT-PCR analysis showing effects of cytokine treatment (for 4 days) on the Fox-p3 RNA content of tumor-infiltrating T lymphocytes (TILs) of GBM patient #4. The pre;iminary results show that interleukine-12 inhibited the expression of both the full-length and exo-2 deled Fox-p3 RNA isoforms whereas TGF-b1 affected the ratio of two isoforms.

3. **Immuno-suppressive effects of GBM tumor mediated through modulation of growth and functions of regulatory T lymphocytes.** It has been long recognized that malignant gliomas are among the most immuno-suppressive human cancer diseases. We have found that GBM tumor cells apparently produce factors to stimulate autologous

CD4(+)/CD25(+)/CD45RO(+)/Fox-p3 RNA(+) Treg immuno-suppressor lymphocytes (Figure 5) and to inhibit CD8(+) lymphocytes (Figure 2). But, if GBM tumor cells are first treated to express heat-shock protein (90-95 Kd Hsp) and major histo-compatibility complexes (MHCs), the Treg effect can be overcome by strong stimulation of tumor-specific CD8(+) CTLs.

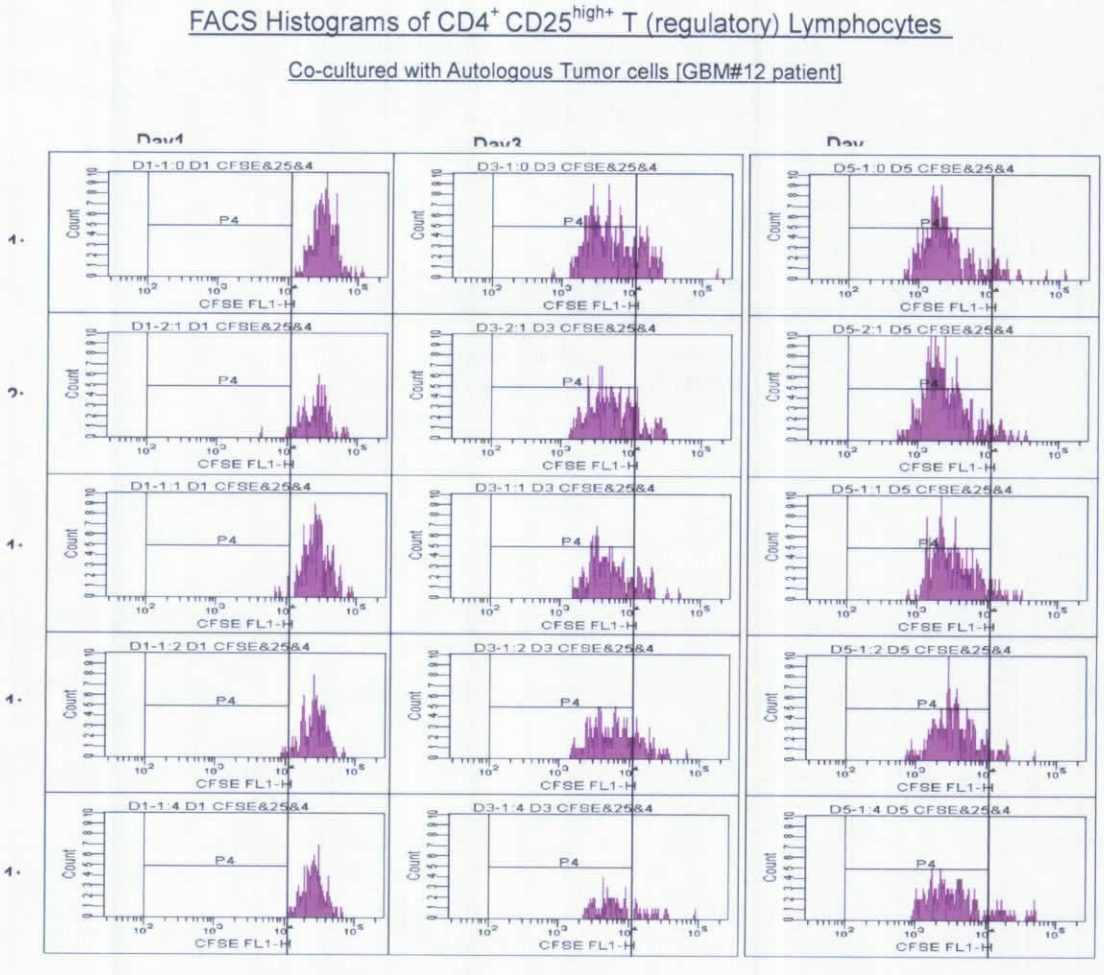


Figure 5 – FACS analysis of CD4(+)/CD25(+)high/CD45RO(+) T lymphocytes co-culture at ratios 1:0, 1:0.5, 1:1, 1:2 and 1:4 (Lymphocytes: tumor, in 5 columns) for 1, 3, and 5 days (in 3 rows) with autologous GBM tumor cells. The results show that the growth of Treg lymphocytes was

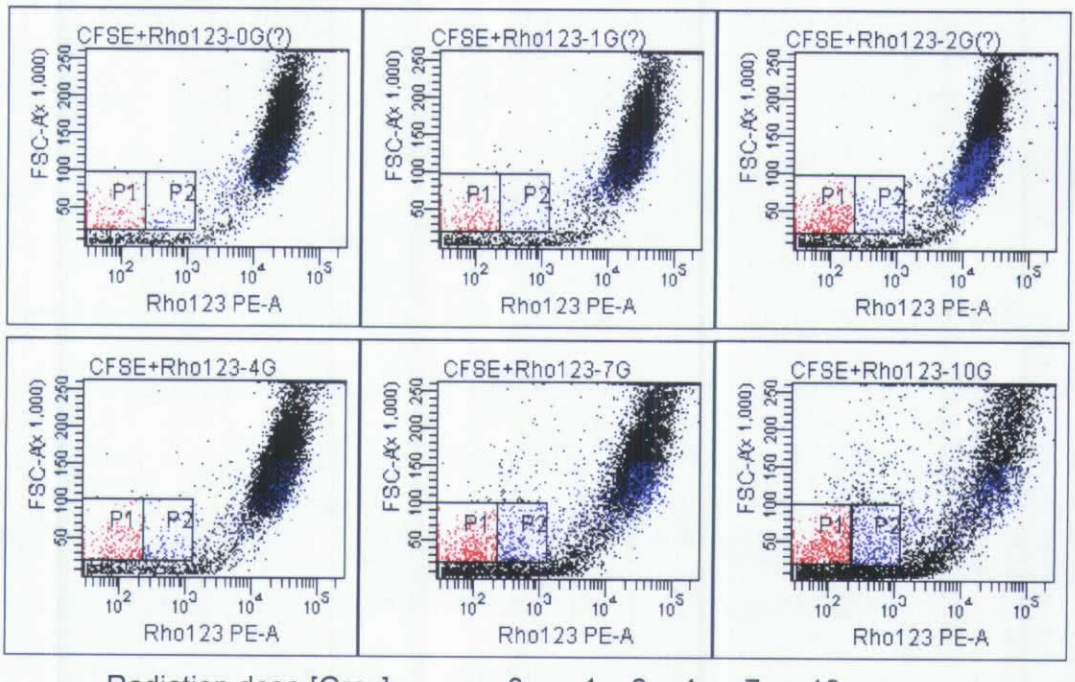
stimulated.

4. **Indiividual susceptibility of GBM tumor cells as well as autologous lymphocytes to chemotherapeutic drug temozolomide among different GBM patients.** Susceptibility of GBM tumor cells as well as autologous lymphocytes to chemotherapeutic drug, temozolomide, vary among different patients.Tomozolomide at the therapeutic doses of 56 nM inhibited the growth of GBM tumor cells as well as lymphocytes from GBM#13 patients, only moderately those from GBM#1 patient, but not those from GBM#16 patient (data not shown). The content and enzyme activities of MGMT (methyl guanosine methyl transferase) of the tumor cells and lymphocytes of these GBM patients remain to be examined.

5. **"Cancer stem cells" of GBM are relatively resistant to ionizing radiation.** Therapeutic doses of ionizing radiation in the range of 700-1000 cG can kill most GBM tumor cells. However, the "cancer stem line" cells of GBM are relatively radio-resistant and may survive to subsequently re-populate the radio-resistant GBM tumors. It has been reported that "GBM cancer stem cells" are similar to normal neural stem cells isolated from subventricular zone (SVZ) of adult cerebraum in the ability to form neurospheres in define culture conditions in vitro. Also, we have used the rapid expulsion of fluorescent dyes such as rhodamine-123 as a working criteria for detecting the apparent stem cell property, which presumably represents a high activity of the cell membrane ABC (G2?). Using this criteria for FACS analysis, we found that percentage of "side populations" increased in the surviving portions of neuroblastoma and

GBM tumor cells after exposure to ionizing radiation at 400-1000 cG. (Fuigure6). However, other parameters such as CD133 and nestin immuno-phenotypes remain to be investigated. Preliminary results showed that the GBM cell clones that survived the repeated 1000 cG ionizing radiation have higher neurosphere forming cells than the parental GBM tumor cells (GBM#13 patient).

Effects of x-irradiation on the re-populated "side populations" (low rhodamine123 retention) of TE671 neuroblastoma cells



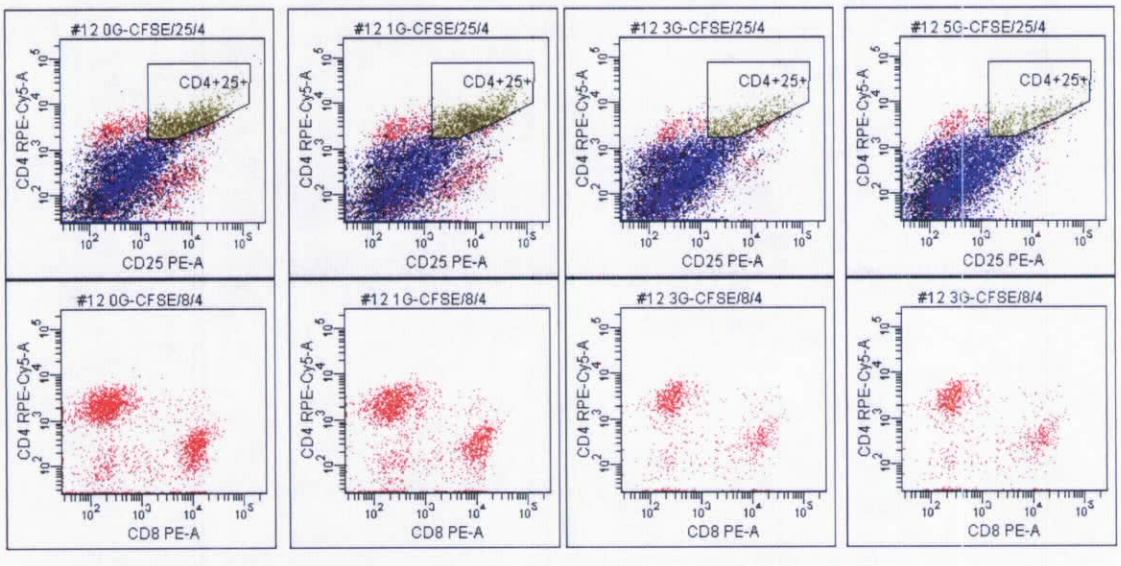
Radiation dose [Gray]	0	1	2	4	7	10
SP1 cells (%)	1.2	1.6	2.8	1.8	3.7	4.8
SP2 cells (%)	0.5	0.6	1.0	0.9	2.3	3.5

Figure 6 – Increases in the percentage of "side population" cells in the survival cell population of neuroblastoma TE671 tumor cells following exposure

to various doses of ionizing radiation (1, 2, 4, 7 and 10 cG). The "side population " cells, designted as SP1 and SP2, were measured by FACS analysis of relatively low cell- associated rhodamine-123 fluorescence.

6. **Different sensitivities of GBM tumor-infiltrationg T lymphocyte subtypes to ionizing radiation.** Tumor infiltrating Treg lymphocytes are less susceptible to ionizing radiation than tumor infiltrating CTLs, which however are often not killed but rendered "inactive" (into memory cells?) by exposure to ionizing radiation or chemotherapeutic agents.

Relative resistance of CD4(+)CD25(+) Treg cells to ionizing radiation



Dose	Total cell #	CD4(+)	CD8(+)	CD4(+)CD25(+)	CD4(+)CD25(-)	CD8(+)CD56(+)
0	3.22M	21.2%	9.1%	10.8%	8.0%	1.0%
1G	2.29M	20.8%	7.1%	12.4%	6.1%	0.8%
3G	1.84M	15.5%	3.1%	9.2%	3.0%	0.5%
5G	1.46M	12.2%	2.3%	4.6%	2.8%	0.4%

*Immuno-fluorocytometric analyses performed 108 hours after radiation exposure

Figure 7 – CD4(+)CD25high(+) Treg cells are more radioresistant then

CD4(+)CD25(-)low and CD8(+) T lymphoctes. The CD*(+)CD56(+) NKT sub-type appeared to be also radio-resistant' which needs to be confirmed because of small cell numbers (low percentage) in the isolated GBM#12 TIL(1) T lymphocyte population.

7. **Similar susceptibility of radiation-survivors and relatively resistant GBM tumors are similarly sensitive to immune CTLs as the parental GBM tumor cells.** The radio-resistant GBM tumor cells that survive 1000 cG radiation are equally sensitive as their parental radiation-sensitive GBM tumor cells to cytotoxic and inhibitory effects of tumor-specific CTLs.

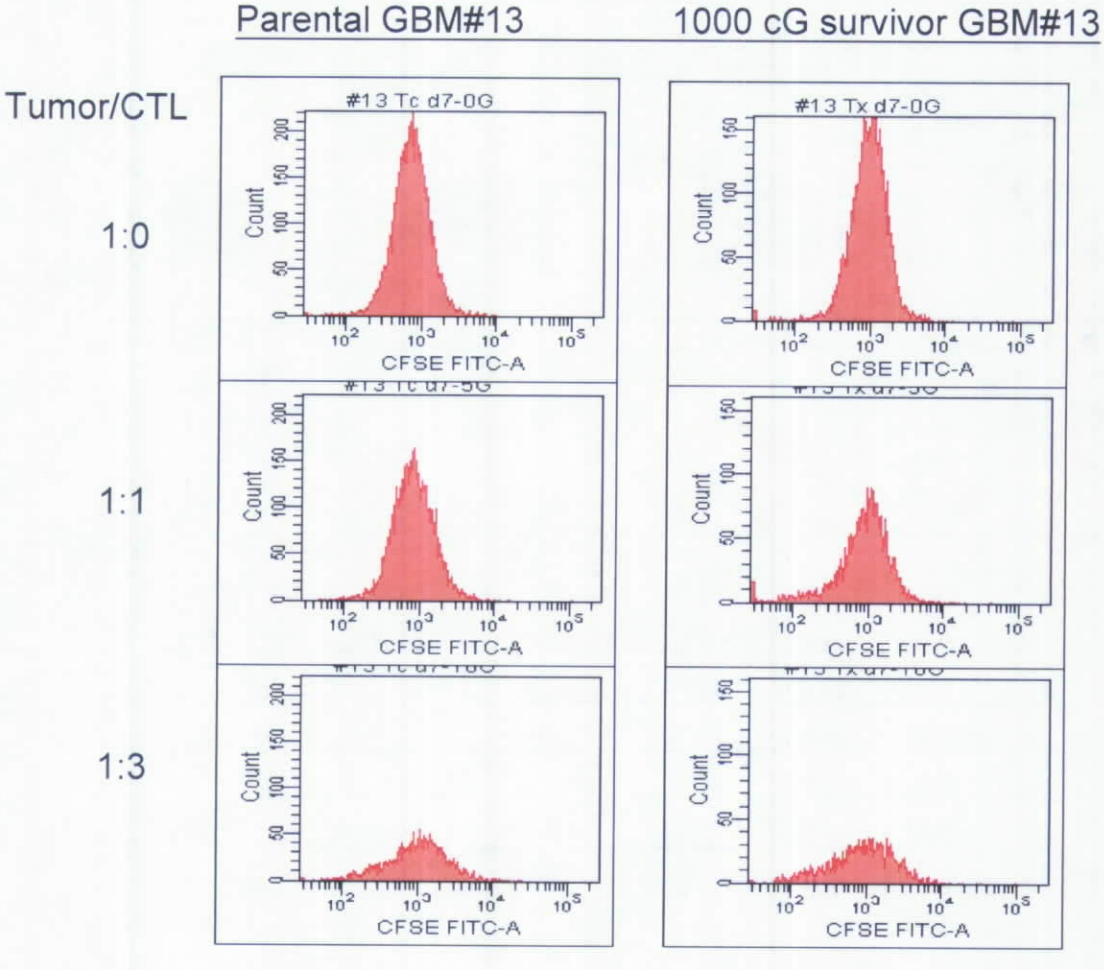


Figure 8 – Cytotoxicity of immune CTLs to the 1000-cG survivor tumor cells (Tx) and the parental tumor cells of GBM#13 patient showing similar susceptibility to immune attack. -

8. **Chemotherapeutic modulation of Treg immunosuppressor T lymphocytes by topoisomerase I inhibiting drugs CPT and NS38.** We have found that topoisomerase inhibitors, NS38 and CPT, while similarly exert cytoxicity on GBM tumor cells, also can inhibit CD4(+)/CD25(+)/CD45RO(+)/Fox-p3 RNA(+) Treg immuno-suppressor lymphocytes by different inhibitory mechanisms, NS38 by severe killing Treg cells whereas CPT by inhibiting the Treg suppressor functions (but not killing Treg cells).

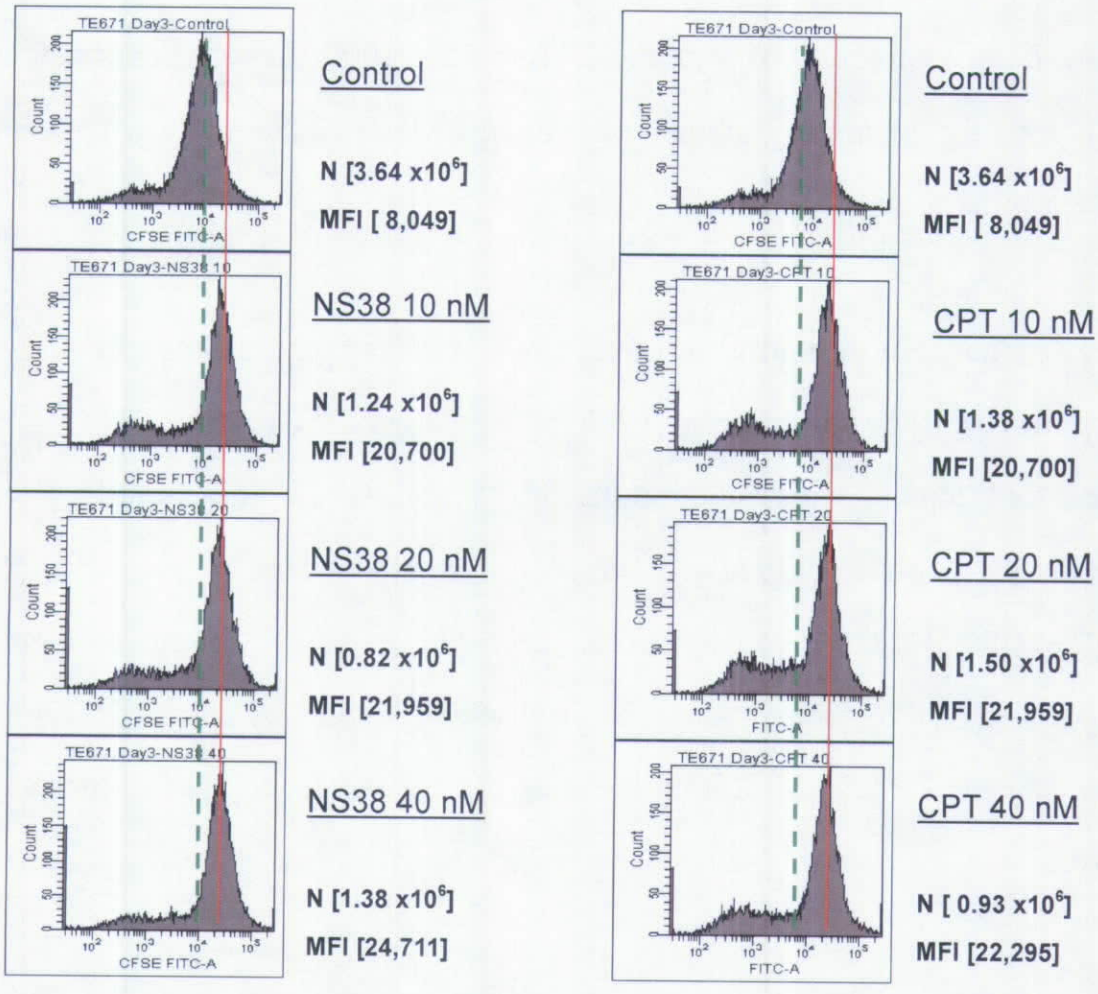
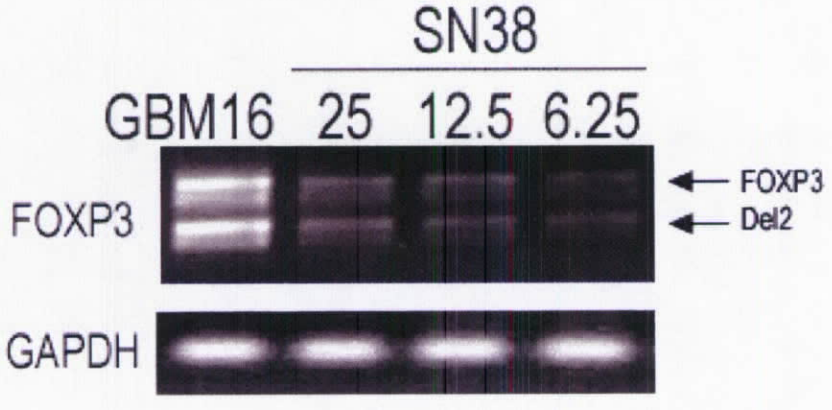


Figure 9 - FACS analysis showing inhibition of the in vitro growth of GBM#16 tumor cells by topoisomerase-1 inhibitor NS38 and CPT. While geometric decrease in the fluorescence (i.e. cell division) with concomitant increase cell numbers was observed in the control untreated, CFSE-labeled GBM tumor cells treated with 10 nM to 40 nM of the two drugs for 3 days showed no such sign of cell division.

Figure 10 – RT-PCR analysis demonstrating inhibited expression of Fox-p3 RNA expression in MT-2 leukemic cells { an experimental model of human Treg lymphocytes} treated with TGF-b (TG) and therapeutic doses of SN38 (SN), doxorubicin (DC), CPT and VP16 for 3 days.0

[A]



[B]

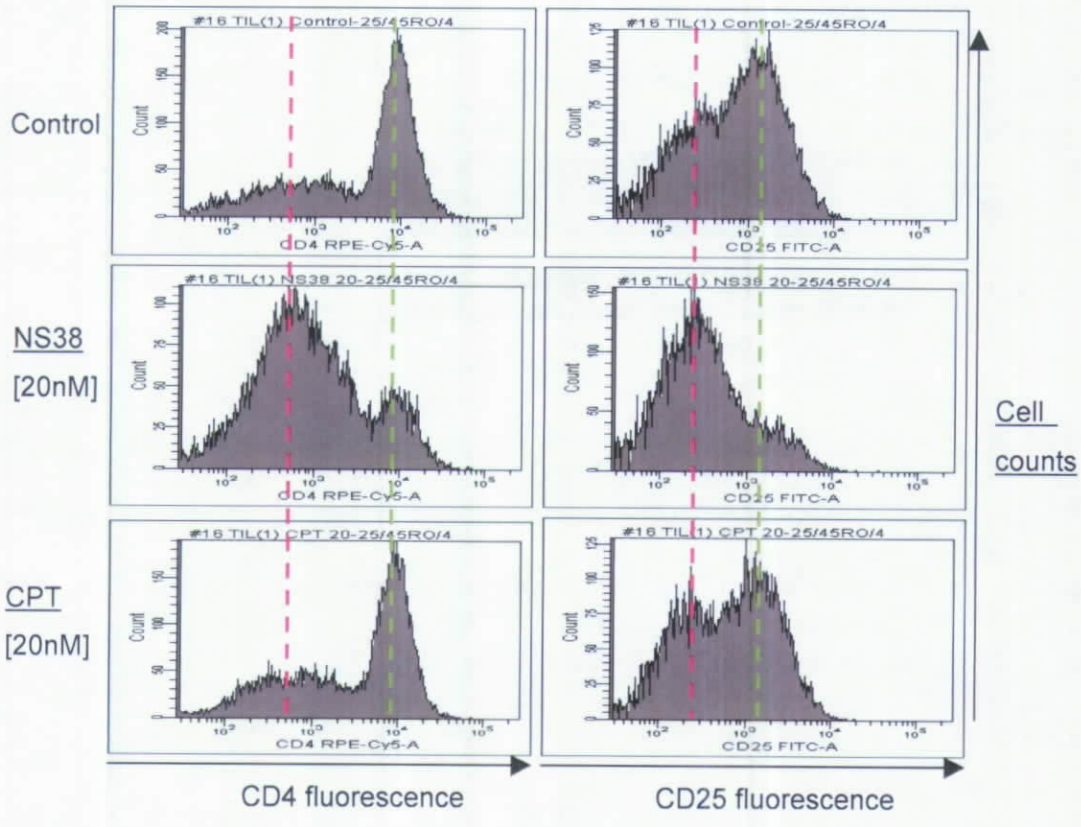


Figure 11 – Effects of topoisomerase-I inhibitors, NS38 and CPT, on the Fco-p3 RNA content and CD4 and CD25 phenotypes of Treg tumor infiltrating lymphocytes isolated from GBM#16 patient before vaccine therapy.

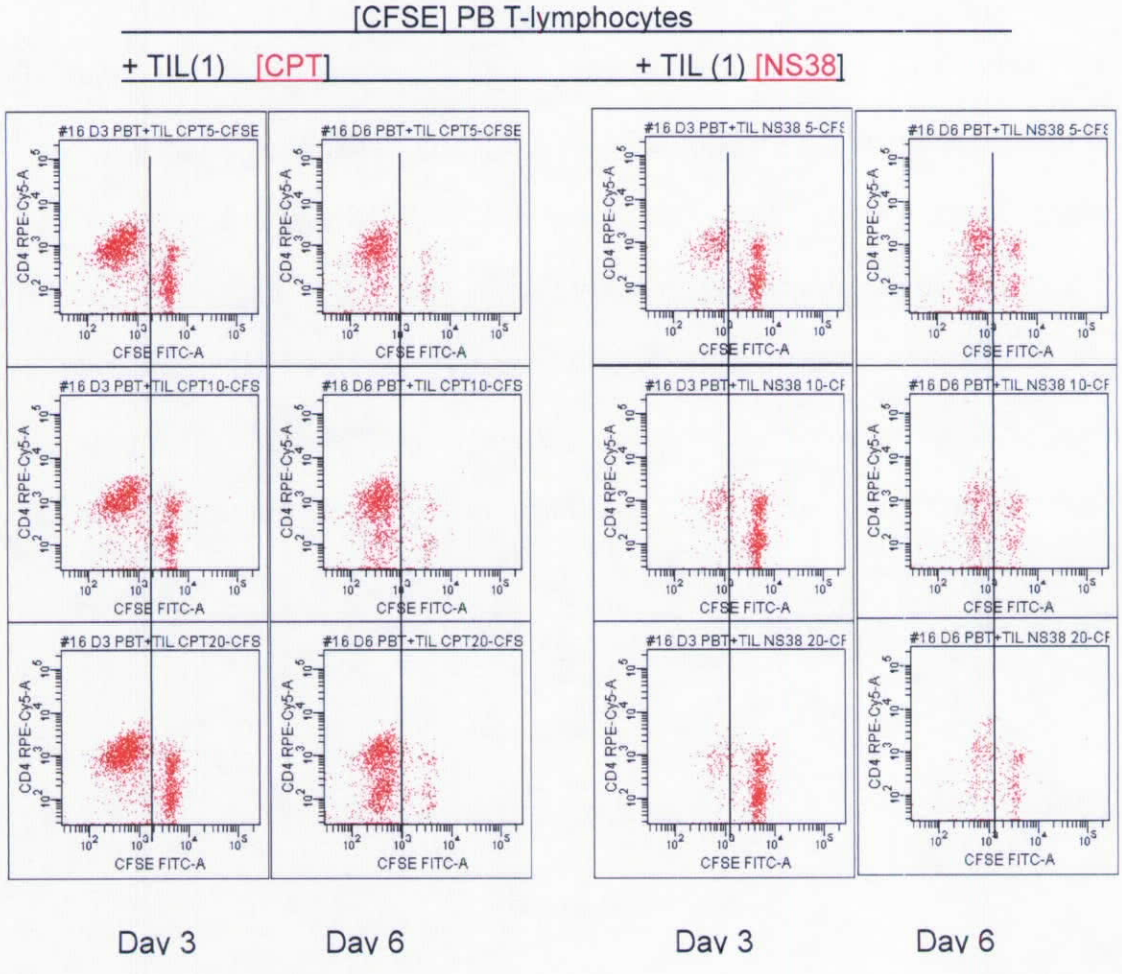


Figure 12 – GBM#16 TIL(1) Treg cells were treated with 5 nM, 10 nM and 20 nM (3 rows from top to bottom row) of CPT (left panel) and NS38 (right panel) for 46 hours, washed, then co-cultured with CFSE-labled autologous PB T lymphocytes for 3 days and 6 days and harvested for multi-color FACS analysis to measure the decrease of cell-associated CFSE fluorescence. The result showed that CPT abrogate the suppressor activities (i.e. not inhibiting the geometric dilution of CFSE fluorescence) without affecting the viability and cell growth of Treg TILs, whereas NS38 was cytotoxic to the TIL Treg with only slight immuno-suppressive effect on autologous PB T lymphocytes (i.e. still inhibiting the CFSE fluorescence dilution),.

9. **Inhibition of cyclo-oxygense-2 in GBM tumor cells may modify the immuno-sppressor properties of GBM tumor cells.** We found that a COX-2 inhibitor, celecoxib, at therapeutic doses of 15-30 uM was not cytotoxic to GBM tumor cells. However, our preliminary results indicated that celecoxib treatment could decrease the the immuno-suppresive activities of GBM tumor cells after ionizing radiation exposure which is known to activate the Akt-2 pathway and hence may indirectly increase the expression of cyco-oxygenase-2 in tumor cells (data not shown).

DISCUSSION

In this translational medicine research project, we have been able to address the two key challenging questions regarding the poor efficacy of autologous dendritic cell-based tumor vaccine adjuvant therapy for post-surgical residual glioblastoma multiforme. As we have found that TILs from GBM tumors of patients prior to vaccination comprised mainly of CD4(+)CD25(+)CD45RO(+)Fox-p3 RA(+) T regulatory cells that are immuno-suppressive, namely inhibiting the growth and functions of other T lymphocyte subsets, especially the tumor-specific immune CD8(+) cytotoxic T lymphocytes or CTLs. We have found that the mature high IL-12 producing DCs could stimulate the growth of CD8(+)CTLs. This is consistent with our finding that TILs comprised mainly Treg cells before DC vaccinationbut shifted to mainly CD8(+) CTLS. Thus, CD4(+)CD25(+) Treg are likely the culprit of un-satisfactory response to DC-based tumor vaccine. And indeed, tumor cells of GBM do produce certain factors to stimulate the growth of CD4(+)CD25(+) Treg cells, On the other hand, mature dendritic cell produce high amounts of interleukin-12 to stimulate the growth of tumor-specific CTLs.

With specific aim #2, we were able to address mainly two issues, namely whether various T cell subsets, in particular the immuno-suppressor Treg lymphocytes and tumor-specific immune CTLs, are similar or differ in their susceptibility to damages by ionizing radiation and to chemotherapeutic agent and whether or not the recurrent GBM tumor cells survived from radiotherapy and/or chemotherapy are susceptible to immune CTL attack. With the limited time for the experimental study, we have managed to obtain some significant

results, some of which are preliminary and remain to be confirmed by repeated experiments to show their reproducibility. Despite these limitations, we have reached some conclusion regarding the future clinical trial. Our results suggest that dendritic cell-tumor vaccine therapy should be initiated as soon as possible after surgical tumor resection and should wait until the completion of radiotherapy in the newly onset GBM. Radiotherapy is likely to stimulate the Treg lymphocytes and also to enhance the immuno-suppressive function of GBM tumor cells. The results of our phase-1 clinical trial now clearly show that new GBM patients have waited to complete radiotherapy for DC-tumor vaccine therapy show worse prognosis (i.e. shorter survival rate and period) than recurrent GBM patients who have started the vaccination right a way. Thus, for the phase-2 clinical trial on, post-surgical DC-tumor vaccine therapy should be instituted prior or simultaneously with radiotherapy in new cases of GBM.

We will continue to explore two aspects. One is the chemo-modulation of Treg lymphocyte functions. Our results with topoisomease-I inhibiting drugs CPT and NS38 are highly significant in terms of clinical implication. Studies along this line would be important. The other aspect is the "GBM cancer stem cells". Despite the use of relatively crude detection method of rhodamine-123 uptake and expulsion, our results regarding the radiation-resistance of GBM cancer stem cells are worthy of further exploration. Even more encouraging is the observation that GBM "cancer stem cells" remain as susceptible to immune CTL cytotoxic attack as the parent GBM tumor cells. This means that immunotherapy might be of value in the dismal recurrent cases of GBM after exhausting the use of radiotherapy and chemotherapy.

CONCLUSION

The major implications of the results accomplished by this translational medicine research project are that our dendritic cell maturation procedures should be enforced, if not improved, to ensure the high production of interleukin-12 , that the dendritic cell-tumor vaccine adjuvant therapy for GBM patients should start right after surgical resection and before or simultaneously with post-surgical radiotherapy and that we should continue to search for chemotherapeutic agents (such as CPT?) that can temporarily inhibit Treg cells for simultaneous application with cancer immunotherapy. In addition we should continue to improve our procedures to culture GBM tumor cells and characterize GBM "cancer stem line" cells, to expand the in vitro growth of Treg immunosuppressor T lymphocytes and tumor-specific immune CTLs and discover the chemotherapeutic drugs that can modulate of these important regulatory and immune lymphocytes for eventual clinical application to treat human diseases.

REFERENCES (pending completion)

九十五年度計畫重要研究成果及對本署之具體建議

（本資料須另附乙份於成果報告中）

計畫名稱：樹突細胞腫瘤疫苗治療：臨床試驗後之轉譯醫學研究

主 持 人：楊文光　　計畫編號：DOH94-TD-B-111-TM007

1. 本計畫之新發現或新發明

1）樹突細胞腫瘤疫苗製造的新技術和新配方(擬申請專利)

2）人類膠質胚原腫瘤細胞之新種株

3）人類 T 免疫控制細胞(Treg lymphocyte)之新種株

4）人類抗膠質胚原腫瘤之特殊免疫殺傷淋巴細胞

5）建立可發現免疫調整藥物之細胞技術平台

2. 本計畫對民眾具教育宣導之成果

目前僅限於參與臨床試驗受治療之惡性腦瘤病人及其家屬之宣導，以及計畫主持人在學校及學術機構的演講。

3. 本計畫對醫藥衛生政策之具體建議

希望根據本計畫的研究成果所策劃和改進之癌症免疫治療新方法能夠獲德衛生署很快的核准，進行臨床試驗，以嘉惠罹患此惡性腦瘤之病人。

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