Enhancing Hyper- Hypothermia Integration Therapy (HIT) Antitumor Effect with Toll-Like Receptor 7 Agonist

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Abstract—Integration of hyperthermia and hyperthermia (cryosurgery), so called “HIT”, on cancerous tissue may increase tumor destruction compared to single treatment. Imiquimod, a toll-like receptor agonist, shows high efficacy in boosting naïve and adaptive immune response against cancer. In this study, we test whether combined HIT with imiquimod can establish a systemic antitumor effect against colorectal cancer in a mouse model. Tumor on mouse flank was established by injection of tumor cells subcutaneously. These tumors were treated with HIT (heat and freeze) with/without topical imiquimod. After 16 days treatments, brain tumors in these mice were established by direct cells inoculation into their brains. Clinical scores and survival of mice were monitored by investigator who blinded to the study. Tumors grew in situ were inhibited by HIT. Mice received combination therapy of HIT and topical imiquimod survived longer than other mice. The results suggest this regiment may have potential to treat metastatic tumor in clinic.

Keywords—Colorectal cancer, metastasis, hyperthermia, cryosurgery, toll-like receptor

I. INTRODUCTION

ALIGNANT tumor is the leading cause of death in recent 7 years in Taiwan. Colorectal cancer is the third common cancers among all cancers. Traditional treatments of colorectal cancer are surgical resection, chemotherapy, radiation and target therapy. The studies of cancer therapies by physical means such as hyperthermia and/or cryosurgery are limited.

Hyperthermia, a procedure that increases the temperature of tumor-loaded tissue to 40-43°C is usually combined with chemotherapy and/or radiation [1]. Cryosurgery (or hypothermia) with liquid nitrogen to destruct cancer cells is an easy and safe method with minimal scarring [2]. However, tumor larger than 4 cm responses poor to cryosurgery [3]. Combination of hyperthermia and cryosurgery may increase tumor cell death [4]. We call this therapy as hyper-hypothermia integration therapy (HIT).

Imiquimod, a member of low molecular weight imidazoquinolamines [5], is currently used to treat warts as an immunomodulator [6]. It also used to boost host immune responses against different cancers by enhancing innate and adaptive immune response via binding to toll-like receptor 7 (TLR-7) [6-8].

In this study, we intent to increase systemic antitumor effects of HIT with TLR7 agonist in a brain metastasis animal model.

II. MATERIALS AND METHODS

Cells. The murine colon cancer cells (CT-26) is a gift from Professor HY Lei, Department of Microbiology and Immunology of the University. Cells were cultured in RPMI medium supplement with 10% FBS and 1% streptomycin penicillin, at 37°Cin a humidified incubator under 5% CO2.

Animal. BALB/c mice aged 6-8 weeks were purchased from National Chung Kung University Laboratory Animal Center. The CT-26 cells (10⁶ cells per 50μl) were injected subcutaneously into the right flank of mice. Tumor volume was measured by digital caliper and calculated with the formula: (L x W²)/2, which L is the maximum diameter and W is the smallest diameter of tumor.

Hyper- hypothermia integrative therapy (HIT). Tumor overlying skin temperature of the mice was kept at 45°C for 10 minutes by placing a thermoelectric cooler on the skin. It was then freezed immediately with liquid nitrogen spray for 20 seconds and allowed to thaw spontaneously. The second spray was done repeatedly after thaw. A thermocouple was connected to a computer to monitor the skin temperature continuously.

Topical immune modulator. A thin layer (around 60 mg/cm²) of imiquimod cream (5% w/w, Aldara, 3M, USA) was applied evenly on the tumor and 3mm adjacent normal skin after HIT and occluded overnight (around 18 hours) with a semipermeable dressing (TegadermTM, 3M, USA). The dressing was removed 24 hours later, the topical medication was repeated every other day for 16 days.

Brain tumor establishment and rechallenge. Metastatic brain tumor model was established by injection of CT-26 cells (50000/10μl) into cerebral cortex. Cells were injected through the thin bone between right eye and ear link line.
Outcome evaluation. Tumor size, brain tumor blood flow, body weight, activity and survival of mouse were evaluated. Damages of tumor were examined pathologically. All parameters were assessed by another investigator who blinded to the study.

Statistics. One Way Analysis of Variance (ANOVA) was performed to determine whether there were significant differences between the different treatments. Bonferroni t-test was applied for multiple pairwise comparisons among individual groups. The Kaplan-Meier method was used for mouse survival after cancer cell inoculations. Data were analyzed using SigmaStat™ (Systat Software Inc. CA, USA) version 3.11. A P value less than 0.05 was considered significant. Data were calculated from 2-3 separated independent experiments.

III. RESULTS

HIT and imiquimod inhibit colon cancer growth in mice.
Mice were divided into 4 groups: 1) control (n=3), 2) received topical imiquimod (n=3), 3) treated with HIT (n=5), and 4) HIT and topical imiquimod (n=6). Fig. 1A shows tumor growth on day 17 after CT-26 cells inoculation in mice. Tumors in mice treated with topical imiquimod showed a trend of slightly growth inhibition (not significant, P > 0.05). On the other hand, tumors in HIT and HIT-imiquimod groups revealed significant inhibition of tumor growth (P < 0.01 and P < 0.001, respectively, compared with the control, Bonferroni t-test). Histopathologically, HIT and imiquimod caused whole layer tumor necrosis and vascular damages (data not shown).

Mortality of mice with brain tumor correlates with tumor cells loading
Metastatic brain tumor model was established by injection of CT-26 cells into cerebral cortex. Cells were injected through the thin bone between right eye and ear (Fig. 2A). For examining the relevance of tumor size and mortality, mice were injected with various numbers of CT-26 cells and a modified clinical score evaluation chart was used [9]. Clinical score evaluation chart was composed of 4 main sections including levels of hair ruffle, mobility, rotation behavior and body weight and there are several subsections in mobility and rotation behavior sections. The highest score is 10 while 0 means death of the mice.

Fig. 2B shows the clinical scores of the mice after intracranial injection of different amounts of tumor cells. The more the cells were inoculated, the higher mortality in the mice.

HIT-imiquimod inhibits tumor growth in brain and prolongs survival.
To investigate whether HIT or HIT-imiquimod elicits systemic immune responses against tumor, CT-26 cells were injected into the brain of mouse. Control mice were mice with established flank tumor without treatments before brain tumor establishment. Naïve mice were health mice with brain tumor cells injection. After cancer cells were injected into brain, mice showed extrados, swollen head, blindness, and decreased mobility. On day 20, all mice injected with PBS survived (score 10) while all naïve mice died (score 0) (Fig. 3). Mice in HIT and HIT-imiquimod had higher clinical scores. Fig. 4 shows 80% of mice in HIT-imiquimod group survive on day 16 after intracerebral tumor cell injections. In control group, only 30% mice survived on day 10 after cell injections. Topical imiquimod provided partial immunity against tumor cells in brain.

Fig. 1. Integrate hyper- hypothermia (HIT) and imiquimod inhibits tumor growth. The tumor size on day 17 in control mice (n=3), mice received HIT (n=5), imiquimod (n=3) and HIT-imiquimod (n=6), respectively (A). Changes in tumor volume on the right flank during treatments (B) (** P < 0.01; *** P < 0.001).

Fig. 2. (A) Metastatic brain tumor model was established by injection of CT-26 cells into cerebral cortex. (B) Inject different CT-26 cells concentration into brain and estimate the clinical scores. (0: PBS injection)
In our study, we found imiquimod boost systemic immune and topical imiquimod eradicated metastatic melanoma in lung photoimmunotherapy which combines photodynamic therapy surface of dendritic cells. Recently, in situ pro-inflammatory cytokines through Toll-like receptors on the carcinoma. Imiquimod stimulates the production of antitumor properties. It has been reported to be effective in the treatment of various cutaneous neoplasms including actinic keratosis, basal cell carcinoma and superficial squamous cell carcinoma. Imiquimod stimulates the production of pro-inflammatory cytokines through Toll-like receptors on the surface of dendritic cells. Recently, in situ photoinmunotherapy which combines photodynamic therapy and topical imiquimod eradicated metastatic melanoma in lung [11]. In our study, we found imiquimod boost systemic immune response against brain tumor. It may explain by the ability of enhancing the production of cytokines such as interferon-alpha, interleukin (IL)-6, and tumor necrosis factor (TNF)-[alpha] from dendritic cells by imiquimod [12]. Tumor cell destruction by HIT may generate strong tumor antigens in the way similar to whole tumor lysate generated by cancer vaccine [13]. Indeed, tumor lysates generated by different methods such as UVB, freeze/thaw, photodynamic therapy have different strength to elicit immune responses against tumor [14].

V. CONCLUSION

Our study showed HIT association with topical imiquimod enhanced antitumor effects either locally or in distant metastasis brain tumor model. This regimen may have potential in treating metastatic cancer in clinic.

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