Effectiveness evaluation of dendritic cell immunotherapy for osteosarcoma on survival rate and *in vitro* immune response

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ABSTRACT. The aim of this study was to investigate the effects of dendritic cell (DC) therapy in osteosarcoma. Bone marrow DCs from Wistar (allograft group) and Sprague Dawley (SD) (homograft group) rats were electrically fused with the SD-derived osteosarcoma cell line UMR106 to generate a DC-osteosarcoma fusion (DOF) tumor vaccine, which was co-incubated with SD T lymphocytes to stimulate T cell proliferation. CD8+ and CD4+ cell percentages were measured by flow cytometry; tumor-cytotoxic effects of cytotoxic T lymphocytes (CTLs) were measured by the MTT assay. Active immunotherapy was applied to SD osteosarcoma model rats via subcutaneous injection of the tumor vaccine. Significant potentiation of T lymphocyte proliferation was observed in both groups. In the homograft group, the CD8+/CD4+ ratio was elevated to 78.2 from 55.1% after stimulation ($P < 0.05$) whereas the CD4+ cell percentage was reduced from 61.3 to 21.2% ($P < 0.05$). Similarly, in the allograft group the CD8+ and CD4+ cell percentages significantly increased (33.8 to 69.6%) or decreased (61.3 to 28.1%)

after stimulation, respectively ($P < 0.05$). The preferential homograft group response was not significant ($P > 0.05$). Induced UMR106-specific CTLs showed a significantly higher tumor-cytotoxic effect after stimulation ($P < 0.05$). After DOF active immunotherapy, tumor bodies displayed atrophy or disappearance, leading to higher survival times and rates (60 and 70% in the allograft and homograft groups) ($P < 0.05$). This study demonstrated that osteosarcoma immunotherapy using a DC-fused tumor vaccine can effectively stimulate T lymphocyte proliferation and induce the tumor-cytotoxic activity of CTLs.

Key words: Dendritic cells; Immunotherapy; Cytotoxic T lymphocytes; Osteosarcoma

INTRODUCTION

Osteosarcoma is a commonly occurring malignant bone tumor in children and adolescents, characterized by high malignancy, high metastatic rate, and fast progression, all of which lead to a high mortality rate (Dudenhöffer-Pfeifer et al., 2013; Andersen, 2014; Choo et al., 2014; Dawoodji et al., 2014). The pathogenesis of osteosarcoma, however, remains unclear.

Dendritic cells (DCs) are widely accepted as strong antigen presenting cells with critical roles in the activation, regulation, and maintenance of the cellular immune response. Accordingly, increasing interest has been displayed in the potential role of DCs in tumor biotherapy (Leviyang, 2013; Kim et al., 2014; Li et al., 2014a). Recently, tumor immunotherapy based on DCs has gained credence for clinical practice, as DCs have been demonstrated to protect animals from immune attack in addition to the induction of a specific proliferation response (Li et al., 2014b). In clinical application, DCs-derived vaccines have elicited satisfactory effects in treating breast, colorectal, and lung cancers (Patch et al., 2013; Pedersen et al., 2014).

Tumor vaccines are produced from tumor cells originating from their own host or from other individuals of the same species that have undergone *in vitro* culture and processing and have been back-transfused to the host to stimulate an anti-tumor immune response, and therefore have a value for application in tumor treatment (Quatromoni et al., 2013). Recently, DCs-derived vaccines have become widely used in tumor treatment. These are generated through a conjugation between DCs and tumor antigens for the purpose of activating the specific anti-tumor immune response. In addition, the potentiation of immune cell activity by co-incubation of DCs with other immune cells facilitates tumor toxicity (Shedlock et al., 2013; Simor et al., 2013; Shi et al., 2014).

This study evaluated the efficacy of cytotoxic T lymphocytes (CTLs) and the animal survival rate following immunotherapy for osteosarcoma using DCs *in vitro* and *in vivo*, respectively, in an attempt to provide supporting evidence for the clinical application of DC immunotherapy against osteosarcoma.

MATERIAL AND METHODS

Experimental animals and cell lines

Rats were used for all experiments, and all procedures were approved by the Zhuhai

Campus of Zunyi Medical College, Guangdong, China. Male Wistar and Sprague Dawley (SD) rats aged between 4-6 weeks, with average weight of 224.3 ± 2.4 g were utilized in this study. All rats were provided by the Animal Center of Zhuhai Campus of Zunyi Medical College and were kept in an SPF-grade facility. The osteosarcoma cell line UMR106 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was maintained at the Zhuhai Campus of Zunyi Medical College.

Cell culture

Wistar rats were sacrificed by decapitation and laid flat on a pre-sterilized operation table. Femurs from both left and right hind limbs were removed, and rinsed in 200 µL 1X phosphate-buffered saline (PBS). Cells were collected by centrifugation at 1200 rpm for 3 min in lymphocyte separation buffer (TaKaRa, Shiga, Japan). After the supernatants were discarded, fresh culture medium (Beyotime, Shanghai, China) was added and cells were incubated at 37°C with 5% CO_2 for 24 h. DC morphology was observed under an inverted microscope, and cell markers CD44 were detected using flow cytometry as described below.

UMR106 osteosarcoma cells were cultured with RPMI-1640 medium (HyClone, UT, USA) containing 10% fetal bovine serum. Cells displaying a monolayer growth pattern were collected, mixed with DCs at a 1:5 ratio, and fused using a cell fusion apparatus (Lonza, Amagazaki, Japan) to obtain allograft DC-osteosarcoma fusion (DOF) tumor vaccine cells.

DOF cell screening and assessment

DOF cells were collected after a 24-h incubation and were separated in an anti-rat OX62 antibody-labeled magnetic MiniMACS separation column. Positive cells were collected and utilized to make a DOF cell suspension (1 x 10^5 /mL), which was rinsed twice in PBS and stained for 0.5 h using an FITC-labeled anti-rat CD44 antibody (1:500; Santa Cruz, CA, USA), followed by washing in PBS. Cells were then labeled using a PE-conjugated anti-rat OX62 antibody (1:1000; AbD Serotec, Kidlington, UK) for 0.5 h, rinsed in PBS, and double-fluorescent labeled cells were detected using flow cytometry (Beckman, CA, USA).

Culture of CTLs, proliferation assay, and subtype analysis

CTLs were prepared and cultured using the same methods as those used for DCs. In the proliferation assay, DOF cells were used as the stimulating cells while unfused DCs and UMR106 cells were employed as controls. After radioactive sterilization under 30 Gy ^{60}Co, cells were inoculated onto 96-well cell culture plates, into which 0, 1 x 10^3, 5 x 10^3, and 2 x 10^4 cells were added along with 1 x 10^5 CTLs to a final volume of 200 µL in each well. In each treatment group, three control wells were completed in parallel. After continuous incubation for 60 h, cell proliferation was determined as described in the MTT kit instruction manual. The cellular absorption value (A value) at 490 nm was measured using an enzyme immunoassay analyzer, and the average values were calculated. The stimulation index (SI) was calculated using the formula: SI = A value in stimulated cells / A value in unstimulated cells. Flow cytometry was used to detect the CD8+/CD4+ ratio of CTLs in SD rats before and after DOF stimulation (see below) and to analyze their subtypes.

Cytotoxicity assay

To culture the cells prior to assessment, the T cell concentration was adjusted to 1 x 10^5 /mL and cells were inoculated onto 24-well cell culture plates, with 1 mL cell suspension added into each well. Equal concentrations of stimulatory DOF cells and unfused DCs were added into each well on 24-well plates with a 500-μL total volume. After 1-week continuous incubation, the cytotoxicity of the collected CTLs was measured by the MTT method using DOF-stimulating T cells (DOF-T) as effector cells and untreated DC T cells (DC-T) or pure T cells (T) as control groups. In brief, cells were inoculated onto 96-well plates at 2 x 10^6/mL concentration with 200 μL per well. Target UMR106 cells were added according to the respective effector/target cell ratio (5:1, 10:1, 20:1, and 40:1) in triplicate along with blank control wells. After a 48-h incubation, freshly prepared MTT solution was added to each well. The kill rate was calculated using the formula: killing rate (%) = [1 - (A values in experimental cells - A values in effector cells) / (A values of target cells - A values of blank controls)] x 100%.

Active immunotherapy on animals

To generate an osteosarcoma animal model, we administered osteosarcoma cells via subcutaneous injection into the right hind limbs of SD rats aged between 4 and 6 weeks with average body weights of 221.5 ± 1.8 g. All animals were then randomly divided into saline control, DOF treatment, inactivated tumor cell treatment, and DC treatment groups (N = 10 per group). DOFs were collected, screened, and irradiated under 30 Gy ^{60}Co to produce the tumor vaccine. After being washed with PBS, 2 x 10^5 cells (in 0.2 mL) were intradermally injected into the groins of subject rats at day 4, 8, and 16 after the injection of osteosarcoma cells. Animal conditions were observed daily to plot the respective 2-month survival curve for each group.

Data analysis

The SPSS 13.0 software package (SPSS, Chicago, IL, USA) was utilized to process all collected data, which were tested for analysis of variance (ANOVA). The T cell proliferation effects and CTL cytotoxic activities were compared using the Student *t*-test while the efficacy of DOF active immunotherapy was determined using Kaplan-Meier survival analysis. $P < 0.05$ was defined as statistical significance.

RESULTS

Culture and characterization of DCs

The growth pattern of DCs was observed under an inverted microscope. At 72 h following plating, 90% were round-shaped and adherent. Beginning from day 4, there were increasing numbers of cells in suspension, with increased size and edges that had become rough instead of smooth. After 1 week, most cells were in suspension with further enlarged volumes and obvious burr-shaped processes (Figure 1).

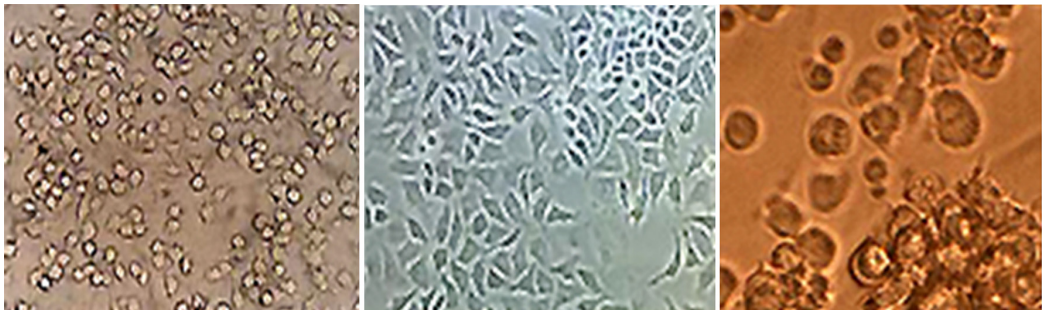

Figure 1. Cultured DC morphology (200X). DC, dendritic cell.

Assessment of DOFs

Following initial screening, DOFs were assayed by flow cytometry with FITC-labeled CD44 and PE-labeled-OX62 (Figure 2). The percentages of double-positive cells were 49.8 and 52.6% in the allograft and homograft groups, respectively, with no significant between-group difference ($P > 0.05$).

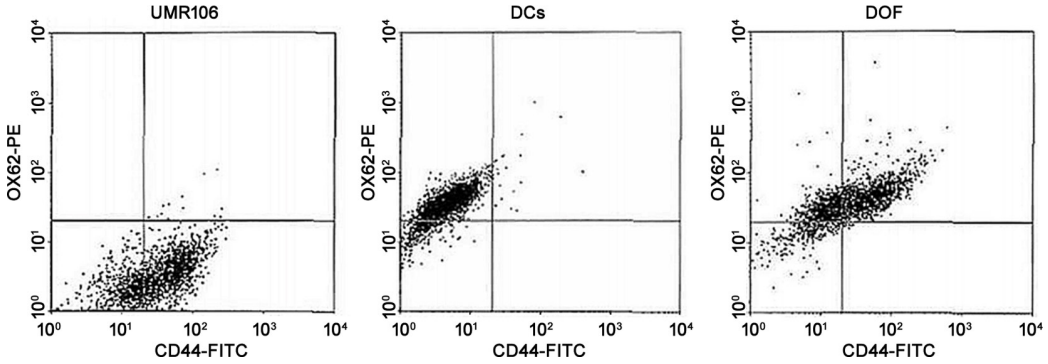

Figure 2. Flow cytometry results of all cell lines.

Stimulatory assay of T cell proliferation

The SIs in all groups are listed in Table 1. DOFs had significantly enhanced stimulatory effects on T cell proliferation compared to untreated DCs and UMR106 cells ($P < 0.05$). Such stimulation effects were positively correlated with the reactive cell ratio ($r = 0.678$, $P < 0.05$). The DOF-induced stimulatory effect in the homograft group was higher than that in the allograft group but there was no significant difference between groups ($P > 0.05$). The number of CD8+ cells was significantly increased to 80% after DOF stimulation, while the percentage of CD4+ cells decreased to 18.3% with significant between-group difference ($P < 0.05$).

Assay of CTL efficacy and survival rates

The results of CTL cytotoxic effects are summarized in Figure 3. Cells in the DOF-T group had significantly higher cytotoxicity against UMR106 cells compared to those in the

DC-T or T cell groups ($P < 0.05$). There was no significant difference in the killing effects between the DC-T and T cell groups ($P > 0.05$). The CTL effect was lower in the allograft group compared to the homograft group, but this difference was not significant ($P > 0.05$). The kill effect statistics are shown in Figure 3. After active immunotherapy, tumor bodies in both allograft and homograft groups after DOF treatment showed atrophy or even disappearance, leading to longer survival times. The survival rates in both groups were 60 and 70%, respectively, which were statistically significant compared to those of other groups ($P < 0.05$).

Table 1. Assessment of stimulatory effects on T cell proliferation using stimulatory index.

	Allograft group			Homograft group		
Ratio	1:100	1:20	1:5	1:100	1:20	1:5
DOFs	18	22	48	20	26	56
DCs	12.4	14	16	12.4	13.5	20
UMR106s	4	5	7	4	6	5
P value	0.035	0.026	0.012	0.033	0.019	0.011

DCs = dendritic cells; DOFs = DC osteosarcoma fusion cells; UMR106 = Sprague Dawley rat-derived osteosarcoma cell line.

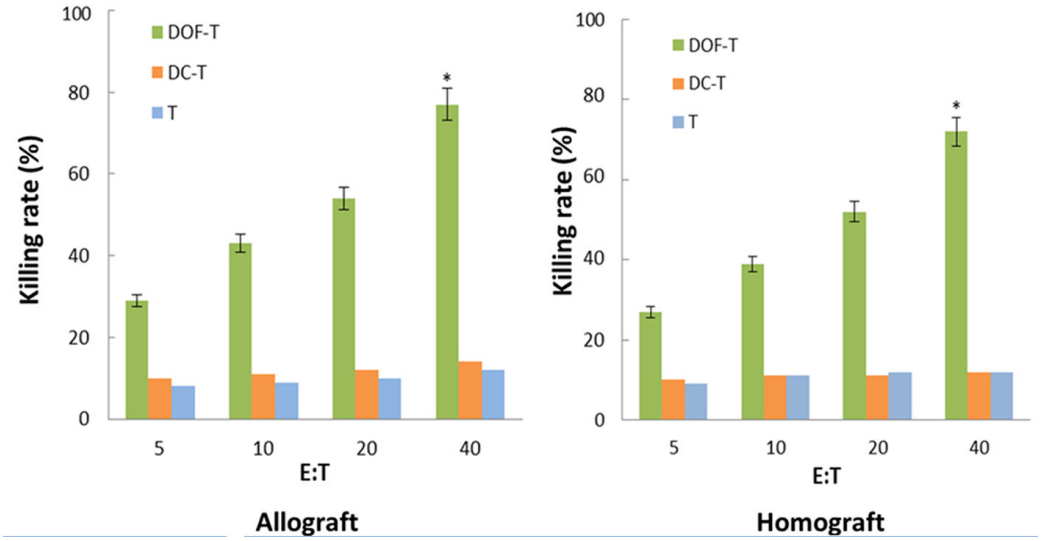

Figure 3. Summary of CTL effects with different effector/target cell ratios (E:T). *$P < 0.05$ compared to the control group.

DISCUSSION

Tumor treatment primarily consists of surgical resection, chemotherapy, radiotherapy, or combined therapy, all of which cause severe impairment as they can result in an abnormal hemogram profile and lower blood cell quality, making it difficult to accept further immunotherapy. Therefore, the application of tumor treatment plans that compromise bodily function

to a lesser degree is now welcomed by patients with tumors, and is the focus of much current tumor research (Sumida et al., 2012; Wang et al., 2013a; Solodeev et al., 2014).

Under normal circumstances, the human immune system can recognize certain mutant tumor cells through the processing and presentation of tumor antigens to T lymphocytes by DCs, provoking the recognition of these tumor cells as abnormal cells, to be killed and cleared by activated T cells. This process allows the human immune system to effectively monitor and prevent tumor pathogenesis inside the body (Wei et al., 2012; Wilde and Schendel, 2012; Wang et al., 2013b; Wu et al., 2014). Currently, the most widely used treatment plan in clinical practice is the DC-cytokine-induced killer (CIK) method, in which DC primarily recognizes antigens and activates the acquired immune system, while CIK can kill tumor cells via their own cytotoxicity and secretion of cytokines, thus generating an effective immune response by their joint effects (Xu et al., 2013; Yu et al., 2013). The combination of these two pathways can effectively inhibit tumor growth and proliferation, in addition to aiding the body in its recovery of immune functions, thereby reducing residual tumor cell numbers as much as possible. As a more advanced and effective method than those previously employed, DC-CIK can significantly improve patient life quality and extend survival time, and has received satisfactory clinical efficacy reviews (Zhang et al., 2012; Zeng et al., 2013; Zanon et al., 2014).

There are two common sources of DCs: from healthy donors and purified from patient peripheral blood. Both sources have their unique advantages. The detailed comparison between these two sources, however, has received little research interest. This study was based on previous research and compared the outcome effects between allograft and homograft treatment plans. Similar CTL effects and animal survival rates were observed in both the allograft and homograft groups following immunotherapy against osteosarcoma ($P > 0.05$), suggesting that either allo- or homografted DCs can yield satisfactory treatment efficacies. However, due to the immune system impairment in tumor patients, we suggest that the allograft stratagem represents the preferred choice for clinical treatment.

Conflicts of interest

The authors declare no conflict of interest.

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