# Specific Antitumor Effects of Tumor Vaccine Produced by Electrofusion between Osteosarcoma Cell and Dendritic Cell in Rats

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**Dendritic cells (DCs) are potent antigen-presenting cells capable of inducing primary T-cell responses. Several immunotherapy treatment strategies involve manipulation of DCs, both *in vivo* and *ex vivo,* to promote the immunogenic presentation of tumor-associated antigens. In this study, an electrofusion protocol was developed to induce fusion between osteosarcoma cells and allogeneic bone marrow-derived DCs. Preimmunization with irradiated electrofusion products was found to provide partial or complete protection from tumor challenge in the UMR106 tumor model. Vaccinated survivors developed long immunological memory. The therapeutic potential of this type of approach was suggested by the ability of UMR106-DC electrofusion products which could induce tumor rejection in a substantial percentage (60%) of hosts bearing pre-established tumor cells. These results tended to indicate that treatment with electrofused tumor cells and allogeneic DCs might be capable of inducing a potent antitumor response and could conceivably be applied to a wide range of cancer indications for which tumor-associated antigens have not been identified. *Cellular & Molecular Immunology*. 2004;1(6):454-460.**

**Key Words:** DC, osteosarcoma, tumor, vaccine, immunotherapy

## Introduction

Although the immune system is there to protect the host from infection, what damps the popular imagination is its potential to recognise and destroy cancer. The immune system can generate potent cytotoxicity (e.g., transplant rejection), but this mechanism can not be fully harnessed for therapeutic benefit in patients with cancer. As it is known that an ever-increasing array of tumour antigens is in existence, the challenge lies in generating a sufficiently potent response towards the tumour antigens. Dendritic cells (DCs) are the best professional antigen-presenting cells (APCs) and they have been used extensively in this context because they can increase the surface expression of major histocompatibility complex (MHC) antigens of class I and class II, and co-stimulatory molecules (required for efficient presentation of peptides and stimulation of T cells) (1) and can synthesize a variety of immunologically important cytokines such as IL-1, TNF-α and IL-12. Results from numerous studies have indicated that DCs pulsed with defined tumor-associated peptides (2, 3), tumor lysate (4, 5), apoptotic or necrotic tumor cells (6, 7), just like DCs genetically modified to express tumor-associated antigens using viral vectors (8, 9) or tumor RNA (10-12), are all capable of eliciting a tumor-specific cytotoxic T-cell response. To further develop effective DC-based strategies, fusion of DCs with tumor cells is particularly attractive. It offers the theoretical advantage of ensuring a broad and continuous source of unaltered tumor antigens as well as superior antigen presenting functionality of DCs. Several studies have shown that fusion cells were functionally active in stimulating both $CD4^+$ and $CD8^+$ T cells and eradicating established tumor metastases (13-15). The fusion hybrids combine the antigens from the tumor cells with the antigen-presenting and co-stimulatory properties of DCs, allowing for effective presentation of the full complement of potential antigens within the tumor, both known and unknown. However, the traditional fusion method using polyethylene glycol (PEG) is often plagued by its too widely ranging efficiencies, toxicity, poor reproducibility, and varying susceptibilities among individual tumor cell partners. We have recently described an alternative means of generating DC-tumor cell hybrids by exposing cells to electric fields. The success of fusion has

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*Abbreviations:* DC, dendritic cell; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorting; IL, interleukin; TNF, tumor necrosis factor; MHC, major histocompatibility complex.

unequivocally been verified by a number of analyses including FACS, cytospin, confocal immunofluorescence, and DNA content. The efficiency of electrofusion is usually tens to hundreds times higher than that of the chemical methods (16, 17).

Osteosarcomas are the prominent primary bone cancers in humans, excluding hemopoietic malignancies. They mainly affect children and adolescents and are usually highly aggressive and eventually lethal. The current grading and staging systems of osteosarcoma are based on a combination of clinicopathologic features, namely, the histological appearance of the tumor and its local and/or disseminated spread. However, an unequivocal discrimination of osteosarcomas into categories with distinct clinical behavior and prognosis has not yet been achieved, largely due to the protean morphologic presentations of these tumors. Combinations of adjuvant chemotherapy and surgery have resulted in improved survival rates (18-22). In an attempt to individualize the therapeutic interventions offered to osteosarcoma patients, immunotherapy might make a contribution to the prevention and cure. Although clinical trials have already been conducted on several malignant tumors, there is actually little or no information available on the performance and nature of the response elicited by electrofused DC-tumor cell in animal osteosarcoma models. In this study, an electrofusion protocol was developed and optimized and the activity of the electrofusion products generated was tested in tumor models possessing different growth and immunogenic properties. The level of efficacy and longevity of the immune response induced by vaccination were investigated.

## Materials and Methods

*Rats and cell line*

Male SD and Wistar rats, 4 to 6 weeks old, were purchased from the laboratory animal research centre of Fourth Military Medical University. The rats were maintained in microisolator cages under specific pathogen-free conditions. The UMR106 osteosarcoma cell line syngeneic to SD rats was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cell line was cultured in RPMI-1640, 10% FBS (Sigma) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) confirmed to be mycoplasma-free by routine testing.

*Dendritic cells*

Bone marrow-derived DCs were generated as described previously (23) with modifications. Briefly, bone marrow was flushed from femurs and tibias and cells were depleted of erythrocytes with lysis buffer. The cells were washed and cultured overnight in culture medium RPMI-1640, consisting of 10% FBS. Non-adherent cells were harvested, resuspended in complete medium containing 1,000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) plus 500 U/ml recombinant rat interleukin 4 (IL-4) and 1,000 U/ml recombinant rat tumor necrosis factor-α (TNF-α) (all cytokines from R&D Systems), and cultured in 6-well plates at $2 \times 10^6$ cells/well. Fresh cytokines and medium were added on day 3. Non-adherent and loosely adherent cell clusters of proliferating DCs were harvested on day 12, and resuspended in complete medium containing fresh cytokines. Cells were washed with PBS and incubated with mouse anti-rat Abs directed against OX62, MHC class I, MHC class II, CD80 and CD86 (all prime antibodies from Serotec Systems) for 1 h on ice. After washed with PBS, the cells were incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated goat anti-mouse IgG (PharMingen) for 30 min. Samples were then washed, fixed with 2% paraformaldehyde, and analyzed by FACScan (Becton Dickinson).

*Preparation of electrofusion vaccine*

DCs and tumor cells were mixed at a 5:1 ratio and suspended in 0.3 M glucose solution containing 0.1 mM $Ca(CH_3COO)_2$, 0.5 mM $Mg(CH_3COO)_2$, and 0.3% bovine serum albumin The pH of the fusion medium was adjusted to 7.2-7.4 with L-histidine (all chemicals were from Sigma Systems). After centrifugation, the cells were resuspended in the same fusion medium without bovine serum albumin. Routinely, 0.5 ml of cell suspension containing $6 \times 10^6$ cells were processed using a specially designed electroporation cuvette, precoated on one side with paraffin wax (50 μl per cuvette). For electrofusion, a pulse generator (model ECM 2001, BTX Instrument, Genetronics, San Diego, CA) was used. Electrofusion involves two independent but consecutive steps. The first reaction is to bring cells in close contact by dielectrophoresis, which can be accomplished by exposing cells to an alternating electric field of relatively low strength (24). Cell fusion can then be triggered by applying a single square wave pulse to induce reversible cell membrane breakdown in the zone of membrane contact. For the current study, the optimal conditions for maximum electrofusion efficiency without substantial cell death (not lower than 70% viability by Trypan Blue staining) were found to consist of two consecutive rounds of an alignment pulse of 50 V for 5 s followed by a fusion pulse of 250 V. The entire process was repeated a second time to maximize fusion efficiency. The fusion mixture was allowed to stand for 5 min before suspending in complete medium and then incubated at 37°C overnight. The nonadherent cells consisted of mainly DCs, and the adherent cells consisted of mainly fusion hybrids and tumor cells. The electrofusion products were purified by monoclonal antibody OX62 (a DC marker not expressed on tumor cells) sticking to the magnetic beads (Miltenyi Biotec) and then irradiated with 200 Gy with an Biological X-ray irradiator to ensure inactivation of the tumor cells and DCs. Samples from several cuvettes were pooled (before irradiation) when required to achieve the number of cells needed for the study. The irradiated pooled sample was then diluted in 0.3 M glucose to the appropriate concentration for immunization.

*Immunization with electrofusion vaccine and tumor challenge*

Groups of 10 rats were immunized with doses of $1 \times 10^6$ electrofused cells. Freshly irradiated electrofusion products were diluted to the appropriate concentration in 0.3 M glucose and delivered intradermally with a 27-gauge needle in a total volume of 0.2 ml into the SD rat's groin. Injection into the skin resulted in the formation of a raised

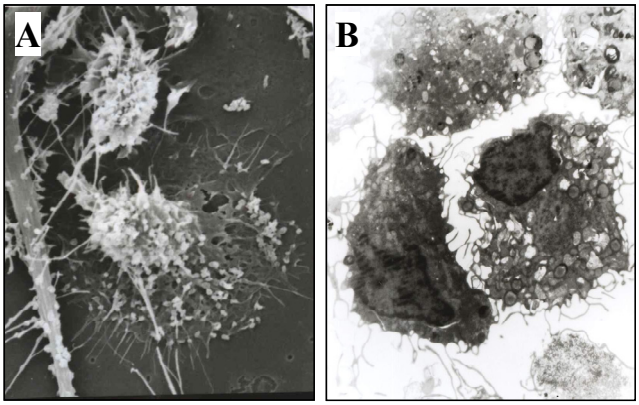


**Figure 1.** (A) DCs under scan electron microscope. DCs on day 10 displayed typical morphology with elongated dendritic processes under scan electron microscope (× 3,000). (B) DCs under transmission electron microscope. DCs on day 10 displayed typical morphology with elongated dendritic processes under transmission electron microscope (× 4,000).

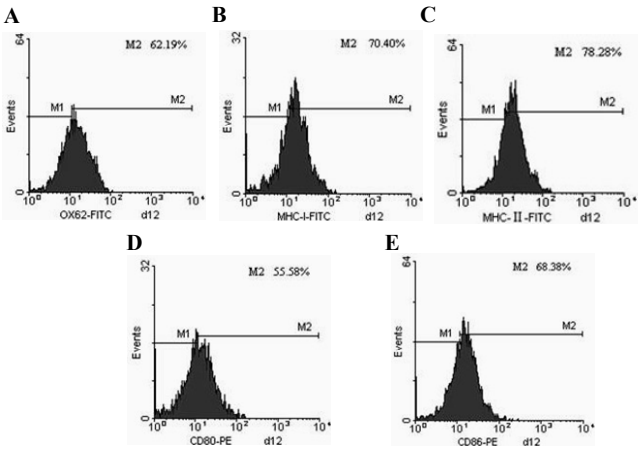


**Figure 2.** Dendritic cells were analyzed by flow cytometry. DCs were stained with a FITC-labeled antibody against OX62 (A), MHC I (B) and MHC II (C), or with a PE-labeled antibody against CD80 (D), CD86 (E).

"bubble" indicating an intradermal location, although some of the material might have been absorbed into the subcutaneous layer. In the pretreatment model, the rats were immunized twice, once on day 0 and again on day 14. The electrofusion products were prepared freshly each time. A lethal subcutaneous tumor challenge was carried out 1 week after the last immunization (on day 21). For the tumor challenge, vaccinated SD rats were injected with $1 \times 10^7$ UMR106 cells. In the therapeutic model, tumor cells were injected on day 0 and vaccination with electrofusion products was done on days 3, 7 and 14.

## Results

*Identification of dendritic cells*

After culture and induction, DCs displayed typical morphology with elongated dendritic processes under both inversion microscope and electron microscope, respectively (Figure 1). DCs expressed high level surface antigens, including 62.19% for OX62, 70.40% for MHC class I, 78.28% for MHC class II, 55.58% for CD80, 68.38% for CD86 (Figure 2).

*Characterization of UMR106 cells fused with dendritic cells*

Bone marrow-derived DCs were mixed with tumor cells at a 5:1 ratio and put into an electroporation cuvette. The mixture was submitted to an alignment pulse to promote cell-cell contact, and then fusion pulse was applied to induce cell membrane fusion (Figure 3). Delivery of the alignment and fusion pulses were then repeated a second time to maximize the fusion. Then the fusion products were purified by monoclonal antibody OX62 sticking to the magnetic beads. Unfortunately, there were no perfect antibodies against rat osteosarcoma-associated antigens to label the UMR106 cells, the fusion products could not be stained for expression of both a tumor marker and a DC marker. We could only affirm the existence of electrofusion products in virtue of the high expression of OX62 on adherent cells (OX62 could not be expressed on adherent osteosarcoma cells, while DCs would never be adherent), and the improvement of MHC class I expression. FACS analysis of a purified fusion products obtained with UMR106 tumor cells and DCs was shown in Figure 4.

*Immunization against tumor challenge*

The ability of irradiated, electrofused tumor-DC products to induce an antitumor response and provide protection against tumor challenge was evaluated in UMR106 tumor models. In this study, rats were immunized intradermally with $1 \times 10^6$ electrofused cells on days 0 and 14. The intradermal route of immunization was chosen because it has been reported that mature DCs injected by this route can traffic to the draining lymph nodes (24). One week after the second immunization (day 21), the rats were challenged with a lethal dose of tumor cells, and then were monitored for tumor growth and survival over time. The results were shown in Figure 5. About 70% of the rats

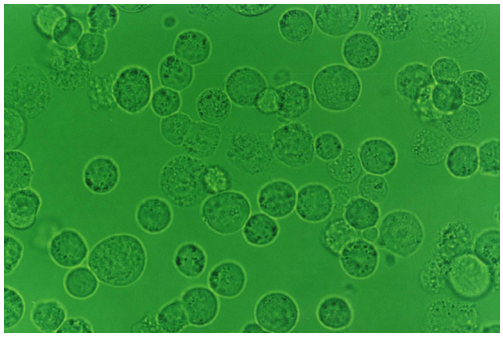

**Figure 3.** Electrofusion products under inversion microscope (× 400). The mixture was subjected to an alignment pulse to promote cell to cell contact and then to a fusion pulse to cause cell membrane fusion. The entire process was repeated a second time to maximize fusion efficiency. The character of these electrofusion products was detected 30 min after the fusion pulse was administered.

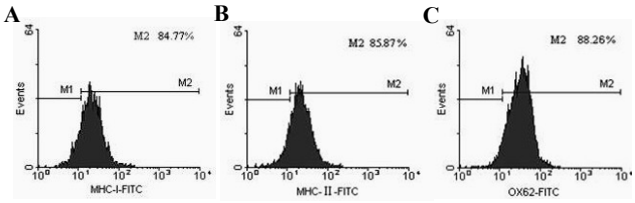


**Figure 4.** Electrofusion products were analyzed by flow cytometry. (A) DCs stained with a FITC-labeled antibody against MHC I (84.77%); (B) DCs stained with a FITC-labeled antibody against MHC II (85.87%); (C) DCs stained with a FITC-labeled antibody against OX62 (88.26%).

immunized with $1 \times 10^6$ electrofused cells were typically able to reject tumor challenge and remained tumor-free. In contrast to the results obtained with the fusion products, immunization with tumor cells that underwent the electrofusion process alone, DCs that underwent electrofusion alone, or a mixture of these two populations failed to provide the same level of antitumor protection. The difference indicated that optimal antitumor efficacy did in fact require the presence of both the tumor and the DC component during the electrofusion process and neither could be solely attributed to immunization with inactivated tumor cells.

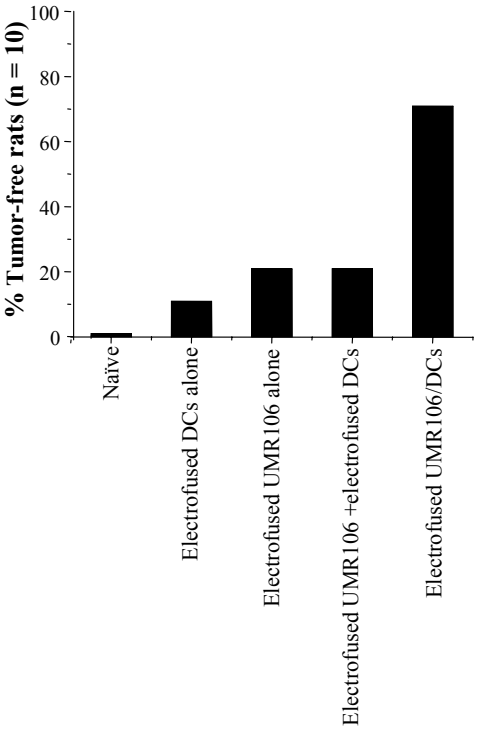


**Figure 5.** Induction of antitumor protection by immunization with tumor cell-allogeneic DC fusion vaccine. In the UMR106 model, SD rats were separately immunized with DCs that underwent the electrofusion process alone ($1 \times 10^6$), UMR106 cells that underwent the electrofusion process alone ($1 \times 10^6$), a mixture of the above two ($1 \times 10^6$), UMR106 cells electrofused with Wistar DCs (dose of $1 \times 10^6$), on days 0 and 14, and then challenged *s.c.* with $1 \times 10^7$ UMR106 cells on day 21. Results are shown by the percentage of tumor-free rats on day 70 after tumor injection. Naïve unvaccinated rats were used as a negative control in this study.

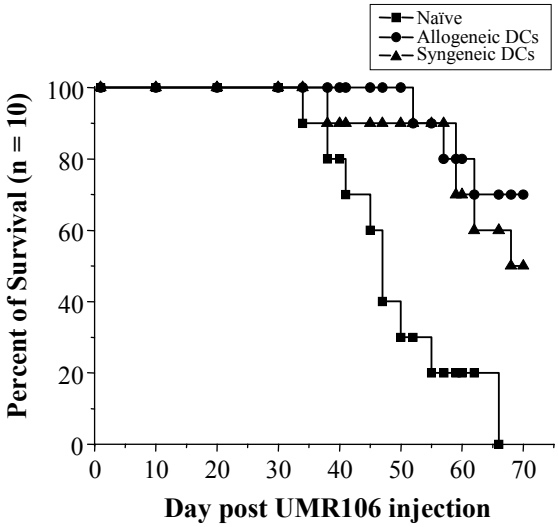


**Figure 6.** Activity of electrofusion products generated with syngeneic versus allogeneic DCs. A comparison of electrofusion products generated with allogeneic versus syngeneic DCs was conducted in the UMR106 models. SD rats were immunized with the irradiated electrofusion products of UMR106 tumor cells and syngeneic SD DCs or allogeneic Wistar DCs ($1 \times 10^6$) on days 0 and 14, and then were challenged *s.c.* with $1 \times 10^7$ UMR106 cells on day 21. Naïve unvaccinated rats were used as a negative control in this study. Results are shown by the percentage of the surviving rats over time after tumor challenge.

*Comparison between syngeneic and allogeneic dendritic cells as fusion partner*

In this study, allogeneic DCs were selected as a fusion partner (for example, Wistar DCs fused to SD UMR106 tumor cells) with the aim to stimulate high frequency alloreactive $CD4^+$ T cells and provide a potent source of help for the development of tumor-specific CTLs. The use of tumor-allogeneic DC fusion products would be expected to have the advantage of simultaneously provision of antigen presentation, co-stimulation, and allogeneic help in the same local microenvironment. To confirm the validity of this approach, we conducted a side-by-side comparison of the antitumor response elicited by fusion products generated with syngeneic versus allogeneic DCs. In the UMR106 tumor model, immunization with fusion products generated with tumor cells and allogeneic DCs (from Wistar rats) seemd to be superior to the products generated with syngeneic DCs as assessed by the percentage of rats capable of rejecting tumor challenge, though the difference was not very obvious (Figure 6).

*Longevity of the antitumor response*

As described earlier, immunization with DC-tumor cell fusion products was found able to elicit protective activity against tumor challenge (Figures 5 and 6). To determine whether the immunization could result in a long-term immunological memory, rats surviving an UMR106 tumor challenge after the injection with UMR106-allogeneic DC

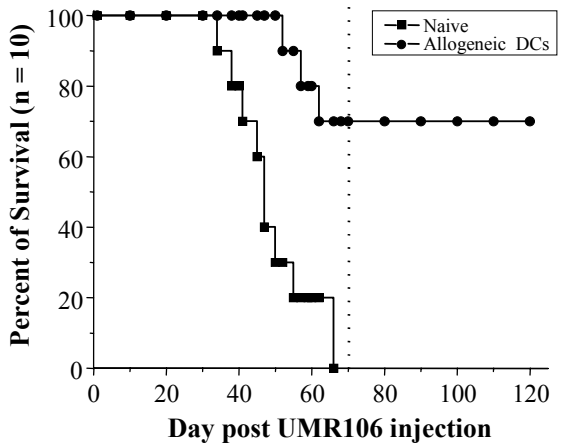


**Figure 7.** Longevity of the immune response elicited by electrofusion vaccine. SD rats were immunized with the irradiated electrofusion products of UMR106 cells and allogeneic Wistar DCs ($1 \times 10^6$) on days 0 and 14 and were then challenged *s.c.* with $1 \times 10^7$ UMR106 cells on day 21. The vaccinated rats that survived the challenge (70%) received a second *s.c.* injection of UMR106 cells, 7 weeks after the first challenge. All the immunized rats survived. Naïve unvaccinated rats served as negative control.

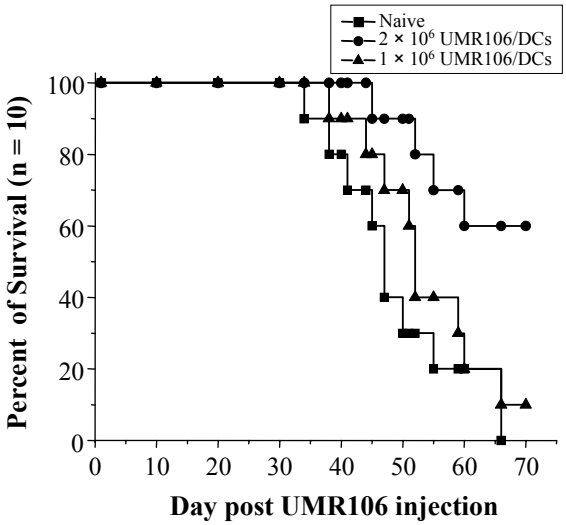


**Figure 8.** Active treatment of rats bearing UMR106 tumor cells with electrofusion vaccine. SD rats were injected *s.c.* with $1 \times 10^7$ UMR106 cells on day 0 and were then treated with the irradiated electrofusion products of UMR106 tumor cells and allogeneic Wistar DCs at a dose of $1 \times 10^6$ or $2 \times 10^6$ on days 3, 7, and 14. Naïve unvaccinated rats were used as negative control. Results are shown by the percentage of the surviving rats over time after tumor challenge.

fusion products, were rechallenged with a second lethal dose of tumor cells 70 days later. A parallel group of age-matched naïve rats injected with UMR106 cells gradually succumbed to tumor growth. In contrast, all of the fusion product-immunized rats were able to reject this secondary tumor challenge, indicating that they had developed a long-term memory response (Figure 7).

*Therapeutic activity of tumor cell-DC fusion products*

To determine whether the tumor cell-DC fusion vaccines could be of any use in a clinically relevant setting, the therapeutic activity of UMR106-allogeneic DC fusion products were tested in rats bearing pre-established UMR106 tumor cells. The rats were injected subcutaneously with $1 \times 10^7$ UMR106 cells on day 0 and were then treated with fusion vaccine at a dose of $1 \times 10^6$ or $2 \times 10^6$ electrofused cells on days 3, 7 and 14. As shown in Figure 8, the level of tumor rejection obtained was found decreased as compared with that observed in a preimmunization setting (Figure 5). Little or no antitumor protection was observed in the rats treated with $1 \times 10^6$ electrofused cells, a dose sufficient to achieve substantial tumor protection in pretreatment studies. However, substantial antitumor protection (60% long-term survivors) was obtained with a higher dose of the vaccine ($2 \times 10^6$ electrofused cells). These results seemed to suggest that active treatment of tumor growth with tumor cell-DC electrofusion vaccines could provide a therapeutic benefit.

## Discussion

The availability of an animal model to study osteosarcoma has opened several new paths for investigation. The murine and human diseases are similar in terms of pathology and, more importantly, in the interaction between the immune system and the tumor. Both human and murine osteosarcoma have low levels of MHC class I expression, which can be upregulated by treatment with cytokines, and both are capable of eliciting some level of cellular and humoral immune reactivity. So far, the efficacy of DC-based immunotherapy for osteosarcoma has not yet been well documented. Our current work aimed to establish a rat osteosarcoma model in healthy SD rats with a normal immune system and to explore whether DC-based immunotherapy could treat osteosarcoma effectively *in vivo*. The results indicated that immunization with the fusion products generated by the electrofusion of allogeneic DCs and osteosarcoma cells was capable of inducing an immune response leading to tumor rejection in both pretreatment and therapeutic settings.

In immunotherapy, DC-based vaccine affords a promising new approach for the clinical response of cancers and has become an issue of the highest interest. Fused DC-tumor cells present to $CD4^+$ T-helper cells a high level T cell co-stimulatory and MHC molecules, both of which are absent in most tumor cells. This engagement results in the upregulation of cell surface markers on T-helper cells and the secretion of various cytokines. The $CD4^+$ T cell therefore provides 'help' by generating potent CTLs which are the principal effectors of specific antitumour immune responses (25).

Electrical cell fusion is an essential step in some of the most innovative methods in modern biology, such as the production of monoclonal antibodies, the cloning of mammals, and vaccination against cancer. Compared with the chemically induced cell fusion *via* polyethylene glycol (PEG), electrical cell fusion is a method with higher efficiency. Technically, electrofusion is truly something interdisciplinary. To achieve success in it requires an appreciation and familiarity with both physics and

electronics which is something rare among immunologists. Although simple in overall concept, the mechanism of electrofusion is still not fully understood. As a result, improvement of the methodology remains largely on an empirical basis.

One important advantage of immunization with an electrofusion product is the potential to induce an immune response against all possible tumor antigens, known or unknown. Several *in vitro* and *in vivo* applications have been explored for the use of electrofused DC-tumor cell hybrids as APCs (26, 27). The immunotherapeutic potential of these hybrid cells also has been confirmed in human clinical vaccination trials (13, 28, 29). Incidentally, before immunized with the rats, the fusion products of the tumor cell and the dendritic cell should be irradiated appropriately, which was to improve the therapeutical safety. After irradiation the cell survives but cannot divide so that no risks of losing control will be worried about.

In this study, the intradermal route was chosen, because there were some special advantages in it. The optimal number of DCs largely depends on the route and effectiveness of DCs' migration. Subcutaneous (30), intradermal (31), intravenous (32), and intranodal (33) approaches to the delivery of DCs have been respectively evaluated clinically. The intranodal approach bypasses the requirement for vaccine-loaded DCs to migrate into the lymphoid tissue and simply relies on their capacity to express effective T-cell stimulative capacity. The intravenous route results in the dispersal of DCs into lung, liver, spleen, and bone marrow, but not into the lymph nodes or tumour sites (34). In contrast, studies using intradermal injection of bone marrow-derived DCs have demonstrated direct migration of DCs into the draining lymph nodes. Another DC vaccination study also has shown that $CD4^+$ T cells are primed to a xenoantigen via various routes, but Th1 responses developed only after intradermal DC administration (35). And it could be speculated that the intradermal route was something predominant for this project.

UMR106 is a badly aggressive, poorly immunogenic osteosarcoma cell line, which has strong carcinogenic capability (100%) and a high lung metastasis frequency (not lower than 70%) according to our previous research (results not shown). However, in this study, preimmunization with the irradiated electrofusion products obtained an exciting result, that is, 70% of the treated rats lived on. Another experiment demonstrated that all of the rats that survived the primary tumor challenge were typically able to reject the second tumor challenge and remained tumor-free, indicating that they had developed a long-term memory response.

The test to compare syngeneic dendritic cells with allogeneic ones as fusion partner showed that allogeneic vaccines were in fact just more potent and provided a little higher levels of tumor protection in the UMR106 tumor model, which were not obvious statistically, though. Nevertheless, we felt satisfied with the outcome, because the option of using allogeneic DCs as a fusion partner seemed to project a practical advantage, for in a clinical setting, allogeneic DCs can be generated conveniently from stored peripheral mononuclear cells from normal healthy volunteers from the general population.

In summary, this study demonstrated it was feasible to generate a large number of DC-tumor cell hybrid cells by the electrofusion technique. Compared with other methods, electrofusion could be reproducible and the fusion rate tended to be high. Allogeneic DCs fused with osteosarcoma cells were capable of inducing a potent antitumor response and could be employed to treat the malignant bone tumor effectively. This approach could conceivably be applied to a wide range of cancer indications for which tumor-associated antigens have not been identified.

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