

In vivo distribution and antitumor effect of infused immune cells in a gastric cancer model

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Abstract. Adoptive cellular transfer has been employed for cancer immunotherapy, including patients with gastric cancer. However, little is known about the distribution of effector cells after their injection via different pathways. In this study, we used human gastric cancer cells (BGC823) tagged with enhanced green fluorescent protein (EGFP) to establish a subcutaneous gastric cancer model in nude mice. Cytokine-induced killer (CIK) cells and cytotoxic T lymphocytes (CTLs) were generated from human peripheral blood and labeled with red fluorescent PKH26. A portion of CIK cells was armed with CEA/CD3-bispecific single-chain antibody. When CIK cells were injected into nude mice with established subcutaneous gastric cancer via peritumoral (p.t.), intravenous (i.v.) and intraperitoneal (i.p.) infusion respectively, the distribution of cells was observed using a live fluorescence imaging system. We found that only a very small number of CIK cells could travel to the tumor site after i.p. or i.v. infusion, and they inhibited subcutaneous tumor growth *in vivo* only immediately following injection. In contrast, p.t. injection resulted in a significantly higher accumulation of CIK cells at the tumor site for 48 hours and mediated the greatest tumor inhibition compared with the other two injection methods. In addition, we compared the antitumor activity of CIK, CEA/CD3-bispecific Ab-CIK and CTL cells *in vitro* and *in vivo* after p.t. injection. Among the three types of immune cells, CTLs demonstrated the strongest antitumor activity both *in vitro* and *in vivo*. CEA/CD3-bispecific single chain antibody could effec-

tively link T lymphocytes and tumor cells expressing CEA, and resulted in significantly higher accumulation of CIK cells at the tumor site compared with the parental CIK cells. This study indicates that peritumoral injection of immune effector cells by minimally invasive surgical procedures represents an effective delivery method of adoptive cellular immunotherapy. Tumor-specific immune cells, such as CTLs, are a better choice of effector cells than CIKs in cellular immunotherapy. Furthermore, CD3⁺ immune cells armed with the CEA/CD3-bispecific single chain antibody could more effectively travel to and accumulate at the site of tumors expressing CEA, such as gastric cancer.

Introduction

Gastric cancer is the fourth most common cancer in the world (1). Standard methods of treatment, e.g. surgery, chemotherapy and radiotherapy have shown mixed success for early-stage patients. However, the prognosis of patients with advanced-stage gastric cancer is still extremely poor. Currently, transfusion of lymphocytes or adoptive cellular immunotherapy (ACI) have been used for the treatment of cancer both in animal studies (2-4) and clinic trials (5-7). ACI has also been used to treat gastric cancer with encouraging results, indicating that ACI can improve the prognosis of some patients, especially those who cannot tolerate surgery (8).

Cytokine-induced killer cells (CIKs) are commonly used as immune effector cells to treat cancer (9-11). The major component of the heterogeneous CIK cells, NKT cells, expresses both T cell marker CD3 and NK cell marker CD56 and exhibits non-major histocompatibility complex (MHC)-restricted cytotoxicity. This represents an effective mechanism in the treatment of cancer. Infusion of CIK cells is typically through an intravenous pathway; however, little is known regarding the number of cells that actually arrive at the tumor site with this method and how long these cells could survive *in vivo*.

Apart from CIK cells, many other immune cells such as lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TILs) and antigen-specific cytotoxic T lymphocytes (CTLs) have been used in adoptive therapy (5). CTLs

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play a central role in antitumor immunity (12,13) and have demonstrated therapeutic efficacy of cancer immunotherapy both *in vivo* and *in vitro*. However, the *in vivo* distribution of these cells following various methods of injection is also unclear.

In this study, we infused PKH26-labeled human CIK cells or CTLs into nude mice with established EGFP-positive human gastric cancer through different pathways and sequentially examined the tissue distribution of CIK cells and CTLs using live fluorescence imaging. This study intended to identify the effective ACI cell type as well as the effective route of immune cell delivery to the tumor.

Materials and methods

Mice. Four-week-old female BALB/c nude (nu/nu) mice were acquired from the Animal Center of the Academy of Military Medical Sciences (Beijing, China). All animals were maintained in a pathogen-free environment and all animal protocols followed the experimental procedures of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Cell line. The poorly differentiated human gastric adenocarcinoma cell line, BGC823, was purchased from the Chinese Academy of Medical Sciences (Beijing, China). These cells are kept in our laboratory at the Institute of General Surgery, General Hospital of PLA (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA).

DNA transfection and isolation of stable EGFP-expressing cells. BGC823 tumor cells were transfected with the pEGFP-C1 plasmid (Clontech, Mountain View, CA, USA) using the Xfect™ transfection reagent (Clontech). The neomycin resistance gene (neoR) in pEGFP-C1 plasmid allows stably transfected tumor cells to be selected using G418. After transfection, the cells were passaged at a ratio of 1:5 in selective medium that contained 200 µg/ml geneticin (G418; Sigma). The level of G418 was increased to 2,000 µg/ml in a stepwise manner (increased every day by 100 µg/ml). Cell clones expressing high levels of EGFP were isolated by limit dilution in 96-well plates. The EGFP-expressing clones were then amplified and transferred by conventional culture methods. This resulted in the identification of a cell line with bright EGFP fluorescence, designated as BGC823-EGFP that was used in this study.

Tumor challenge. Nude mice were challenged with 1x10⁷ BGC823-EGFP cells, subcutaneously. Whole-body images were taken using the IVIS-200 Imaging System (Xenogen, Alameda, CA, USA) on Day 10.

Dendritic cell isolation and culture. Peripheral blood mononuclear cells (PBMCs) from healthy donor were cultured with Cellix-901 medium (Beijing XinMingLiTai Bio-technique, Co., Ltd., Beijing, China) for 2 h. Adherent cells were collected and further cultured in Cellix-901 medium with 1,000 IU/ml rhIL-4 (Cell Genix) and 1,000 IU/ml rhGM-CSF for 7 days to generate a dendritic cell (DC)-enriched cell population. DCs were then pulsed with 3 µl/ml *Pseudomonas aeruginosa* and

20 µg/ml BGC823 cell lysate followed by incubation for 12 h before use for the next step. To prepare BGC823 cell lysate, BGC823 cells were digested with trypsin, washed in normal saline and centrifuged at 450 x g for 5 min. The supernatant was then discarded and cells were resuspended in sterile water, and were frozen and thawed repeatedly 3 times. Lysate was obtained after centrifugation and filtration.

CIK, CTL and CIK-CEA/CD3-bscAb cell isolation and culture. Using a blood cell separator (Institute of Biomedical Engineering, Chinese Academy of Medical Sciences), 2-4x10⁹ PBMCs from healthy donors were obtained. To prepare CIK, cell concentration was adjusted to 2x10⁶ cells/ml in fresh, serum-free Cellix-601 medium (Beijing XinMingLiTai Bio-technique, Co., Ltd.) with 2,000 U/ml rhIFN-γ and incubated at 37°C in a humidified atmosphere of 50 ml/l CO₂. After 24 h, anti-CD3 (50 ng/ml) and rhIL-2 (1,000 U/ml) were added. On Days 0, 4, 7 and 10 of culture, cell densities were determined and phenotypes were identified by flow cytometric analysis (FACS) (Becton-Dickinson, USA). To make CTL cells, PBMCs were incubated in tissue culture flasks with the Ag-pulsed DCs. To generate CIK-CEA/CD3-bscAb cells, CEA/CD3-bispecific single chain antibody (Beijing ABT Genetic Engineering Technology, Co., Ltd., Beijing, China) was added into the medium to bind CIK cells to obtain CIK-CEA/CD3-bscAb cells.

Immune cell labeling and in vitro assays. Immune cells were labeled with red fluorescent PKH26 (Sigma-Aldrich, Co., LLC.) following the kit instructions. CIK-, CIK-CEA/CD3-bscAb- and CTL-mediated cell cytotoxicity was evaluated using the lactate dehydrogenase (LDH) release assay according to the manufacturer's protocol. Cytotoxicity was assessed as $[(A_{\text{sample}} - A_{\text{spontaneous}}) / (A_{\text{maximum}} - A_{\text{spontaneous}})] \times 100\%$, (A, absorbance).

Tracing immune cells in vivo and evaluating the inhibition of tumor growth. Seven days after BGC823-EGFP cells were implanted into the nude mice, the tumor-bearing animals were separated into 3 groups. All mice were injected with 1x10⁷ labeled CIKs, intraperitoneally (i.p., Group 1) ; intravenously (i.v., Group 2) or peritumorally (p.t., Group 3), respectively. The migration and distribution of the infused CIK cells were then observed using the IVIS-200 Imaging System (Xenogen) at 4, 24, 48 and 96 h after CIK cells injection. The lung, liver, spleen, kidney, stomach and intestine were collected on Days 2 and 8 for histopathological analysis. Tumor size and weight were assessed on Day 35 to evaluate the therapeutic efficacy of transferred CIK cells.

In separate experiments, we set up 3 groups to compare the therapeutic efficacy of CIK, CIK-CEA/CD3-bscAb and CTL cells. Each group of labeled immune cells was injected peritumorally at 1x10⁷ cells/mouse. Migration and distribution of the infused immune cells were then observed at 4, 24, 48, 96 and 144 h after cell infusion.

Statistical analysis. Statistical analyses were performed with the SPSS 17.0 statistical software using the Student's t-test and ANOVA. P-values of <0.05 were considered statistically significant between the experimental groups.

Table I. *In vitro* antitumor effects of CIK cells before and after labeling with PKH26 (mean ± SD, n=3).

Groups	E/T ratio	5 h		10 h	
		LDH	Cytotoxicity, %	LDH	Cytotoxicity, %
CIK	5:1	0.457±0.023	70.51±3.61	0.555±0.024	95.43±4.84
	10:1	0.697±0.081	84.44±4.48	0.894±0.090	100
CIK-PKH26	5:1	0.460±0.017	68.88±3.67	0.555±0.023	93.78±3.06
	10:1	0.701±0.063	83.54±4.98	0.901±0.028	100

LDH, lactate dehydrogenase release assay; CIK, cytokine-induced killer cells.

Table II. Antitumor effect of different immune cells *in vitro* (mean ± SD, %, n=3).

Groups	5 h		10 h	
	5:1 E/T ratio	10:1 E/T ratio	5:1 E/T ratio	10:1 E/T ratio
CIK	70.51±3.61	84.44±4.48	95.43±4.84	100
CIK-CEA/CD3-bscAb	74.09±2.01	88.01±2.88	100 ^c	100
CTL	85.59±5.14 ^{a,b}	97.47±2.20 ^{a,b}	100 ^c	100

^aP<0.05 vs. the CIK group; ^bP<0.05 vs. the CIK-CEA/CD3-bscAb group; ^cP<0.01 vs. the CIK group. CIK, cytokine-induced killer cells; CTL, cytotoxic T lymphocytes.

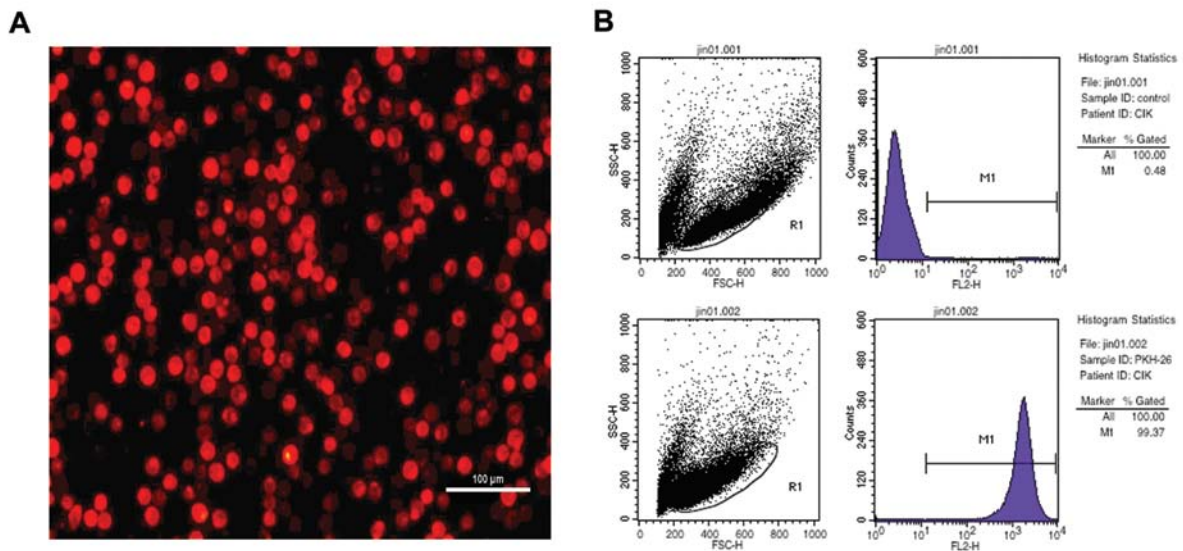


Figure 1. CIK cells after labeling with PKH26. (A) Strong red fluorescence was detected with fluorescence microscopy after labeling; (B) FACS analysis showed that 99.37% of CIK cells were successfully labeled with PKH26.

Results

Antitumor effect of different immune cells in vitro. Firstly, we detected the antitumor effect of labeling on the immune cells. No difference was observed in cell morphology after labeling with PKH26. Strong red fluorescence was detected by fluorescence microscopy (Fig. 1A) and FACS analysis showed that 99.37% of CIK cells were PKH26-positive (Fig. 1B).

There was no significant difference in the antitumor activity between CIK and CIK-PKH26 cells (Table I).

In addition to CIK cells, CIK-CEA/CD3-bscAb cells and CTLs also showed strong antitumor activity against BGC823 cells *in vitro*. At E:T cell ratios of 5:1 and 10:1 and at 5 and 10 h, the BGC823 cell killing activity was as follows: CTL > CIK-CEA/CD3-bscAb > CIK cells (Table II).

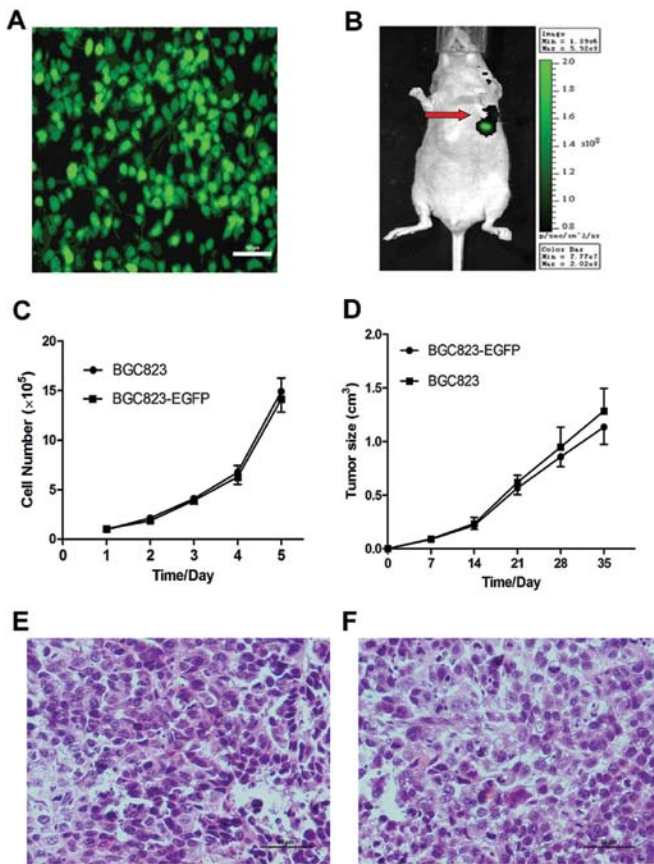


Figure 2. The human gastric cancer cell line BGC823 was transduced with the pEGFP-C1 plasmid using the Xfect transfection reagent, resulting in a high-level of EGFP expression. (A) Strong green fluorescence was detected with fluorescence microscopy; (B) whole-body image of the nude mouse injected with BGC823-EGFP tumor cells subcutaneously. Picture was taken by IVIS-200 Imaging System and arrow points to the tumor. (C) No difference in *in vitro* growth between BGC823 and BGC823-EGFP was observed over 5 days; (D) no difference in *in vivo* growth between BGC823 and BGC823-EGFP was observed over 5 weeks; (E) H&E slide of BGC823 subcutaneous tumor (magnification, x200); (F) H&E slide of BGC823-EGFP subcutaneous tumor (magnification, x200).

Establishment of human gastric cancer (BGC823) in nude mice subcutaneously. Fluorescence imaging was employed to monitor the growth of human gastric cancer in nude mice. First of all, we successfully transfected BGC823 cells with EGFP (BGC823-EGFP cells) and selected them with G418 (Fig. 2A). We then injected parental BGC823 and BGC823-EGFP cells subcutaneously into the nude mice, respectively. The BGC823-EGFP subcutaneous tumor was visible with the IVIS-200 Imaging System within 10 days after tumor cell implantation (Fig. 2B).

EGFP transfection was found to have no effect on BGC823 cell morphology and cell proliferation *in vitro* (Fig. 2C) or tumor growth *in vivo* (Fig. 2D). In addition, no difference was observed in the histopathological analysis between BGC823 and BGC823-EGFP tumors (Fig. 2E and F).

Distribution and antitumor effect of CIK cells infused via three different pathways. When CIK cells were i.p. injected (Fig. 3A), red fluorescent PKH26-labeled CIK cells tended to gather first in the abdominal cavity. At 24 h post-injection, cells began to spread, and a small amount of accumulation could be observed

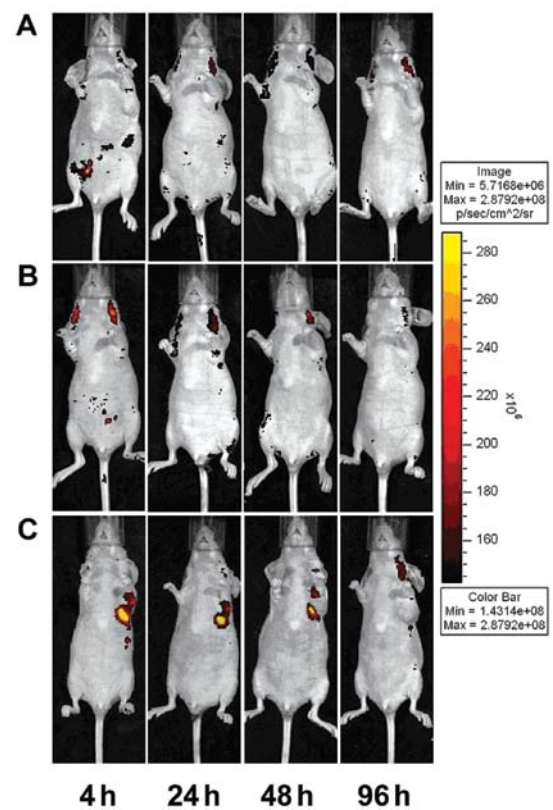


Figure 3. The distribution of CIK cells after infusion into the tumor-bearing nude mice; (A) i.p., (B) i.v. and (C) peritumoral injection groups.

in the tumor area before gradually dissipating. On Day 5 post-injection, the red fluorescence was nearly gone. In the i.v. group (Fig. 3B), CIK-PKH26 cells dispersed rapidly in the body, and a small amount of accumulation could be observed in the tumor area 24 h post-injection. On Day 5, the red fluorescence vanished. In contrast, in the peritumoral injection group (Fig. 3C), CIK-PKH26 cells gathered around the tumor after injection and dissipated very slowly. On Day 5, we could still detect a small amount of red fluorescence around the tumor.

On Day 2 post-injection via p.t. injection, labeled CIK cells were found to infiltrate the tumor bed in high numbers, although this infiltration was mainly localized around the tumor (Fig. 4A), while in the i.p. (Fig. 4B) and i.v. groups (Fig. 4C), the red fluorescent spots were much fewer. In the i.p. group, the labeled CIK cells were detected infiltrating multiple organs. The spleen was the most enriched in CIK cells (Fig. 4D), followed by the liver (Fig. 4E), kidney (Fig. 4F), intestine (Fig. 4G), stomach (Fig. 4H) and lung (Fig. 4I). On Day 8 post-injection, we found only a small quantity of red fluorescent spots in the liver and spleen in the i.p. and i.v. groups; however, in the peritumoral injection group, some labeled CIK cells were still found infiltrating the tumor bed (Fig. 4J), liver (Fig. 4K) and spleen (Fig. 4L).

The infusion of CIK cells via each of the three pathways, particularly peritumorally, was found to inhibit subcutaneous tumor growth in nude mice. Three weeks after CIK transfusion, tumors of the peritumoral injection group were significantly smaller than the normal saline (NS)-treated control group. Although not statistically significant, tumors of the i.p. and i.v. groups were also smaller. Four weeks after transfusion,

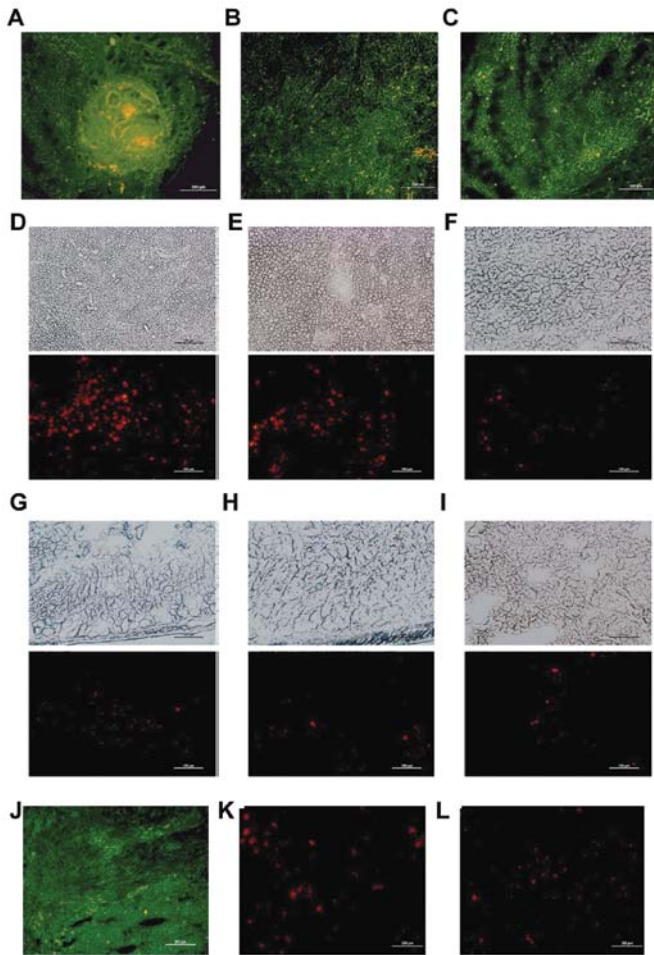


Figure 4. Frozen sections of tumors. (A) Peritumoral injection, (B) i.v. and (C) i.p. groups on Day 2. (D) Spleen, (E) liver, (F) kidney, (G) intestines, (H) stomach and (I) lung on Day 2. (J) Tumor, (K) liver and (L) spleen on Day 8.

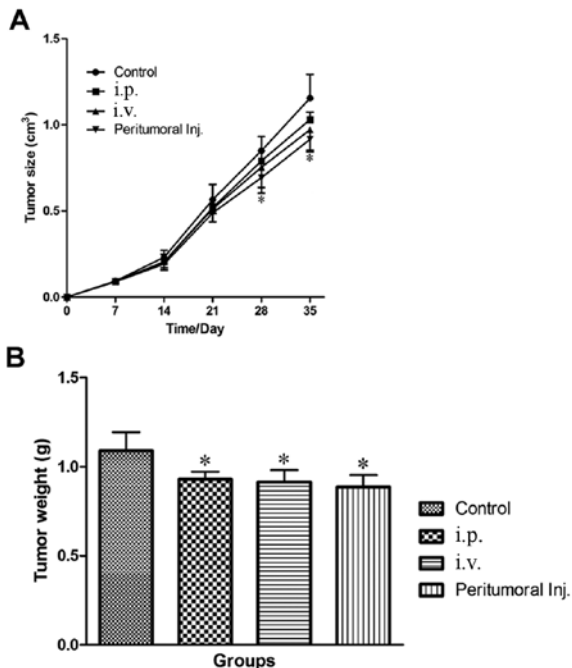


Figure 5. Tumor size and weight after the infusion of CIK cells via different pathways as indicated. Statistical significance was determined using the Student's t-test vs. the NS-treated control group (n=4, *P<0.05).

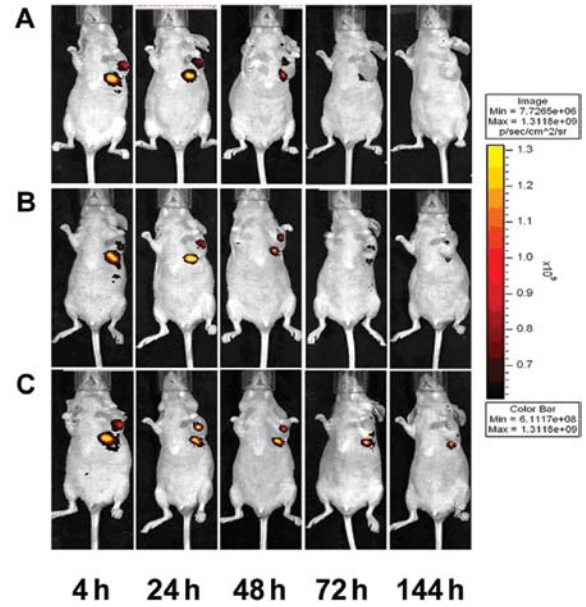


Figure 6. The distribution of different immune cells after peritumoral injection. (A) CIK, (B) CTL and (C) CIK-CEA/CD3-bscAb groups.

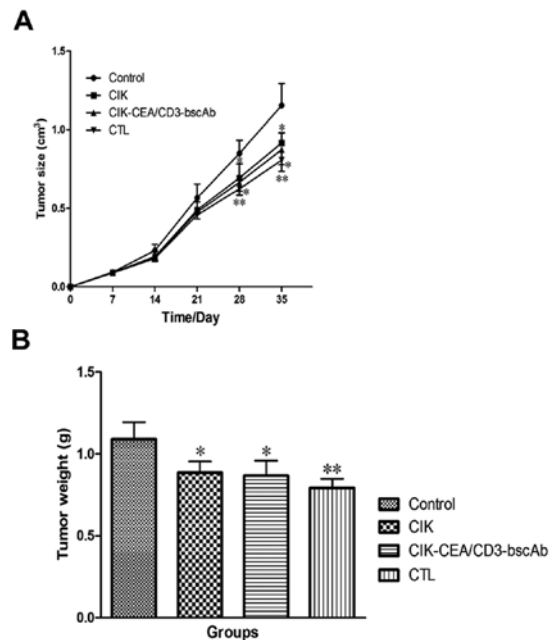


Figure 7. Tumor size and weight after the infusion of 3 different immune cells via peritumoral injection. Statistical significance was determined using the Student's t-test vs. the NS-treated control group (n=4, *P<0.05, **P<0.01).

tumor weight in the peritumoral, i.v. and i.p. injection groups was significantly lighter than the NS-treated control groups (Fig. 5).

Distribution and antitumor effect of different immune cells infused via peritumoral injection. Following peritumoral injection, all of the immune cells were gathered around the tumor. CIK cells were nearly gone 5 days post-injection (Fig. 6A), followed by the dispersal of CTL cells within 7 days (Fig. 6B). However, CIK-CEA/CD3-bscAb cells remained crowded around the tumor 7 days post-injection (Fig. 6C).

Peritumoral injection of three immune cell types, particularly CTLs, was found to inhibit subcutaneous tumor growth in nude mice (Fig. 7). Control tumor weight was 1.09 ± 0.10 g 35 days after implantation. CTL, CIK-CEA/CD3-bscAb and CIK cells injected peritumorally 7 days after tumor implantation reduced tumor weight by 27.29 ± 5.05 , 20.41 ± 8.35 and $18.58 \pm 6.01\%$, respectively.

Discussion

Whole-body imaging has proven to be a useful technology for the study of the dynamics of metastatic cancer. Green fluorescent protein (GFP) expression in cancer cells can help externally to image and follow the natural course or impediment of tumor progression and metastasis (14). Tumor motility, progression and metastasis can be visualized at the single-cell level *in vivo* with GFP (15). EGFP is the enhanced GFP, which is made for a heightened brightness (16). In this study, we have successfully established the stable EGFP-expressing human gastric adenocarcinoma BGC823 cells (BGC823-EGFP). These cells form subcutaneous tumors in nude mice. BGC823-EGFP cells have revealed identical biological characteristics as parental BGC823 cells, and can therefore be used to investigate tumor growth and metastasis (15,17,18).

CIK cells are polyclonal T effector cells generated when cultured under conditions of cytokine stimulation (19). The main effector cells of the heterogeneous CIK cell family express NK and T cell markers CD56 and CD3, respectively, and are referred to as NK-like T (NKT) cells. These cells possess non-MHC-restricted antitumor activity which means they do not require prior specific sensitization to induce the recognition of target cells (9,20,21). Over the years, CIK cells have been used for their antitumor activity against a variety of tumor targets (10,11,22). In this study, we found that CIK cells killed 100% of BGC823 cells within 10 h at the E:T cell ratio of 10:1 *in vitro*. CIK cells also exhibited antitumor activity *in vivo*. In our gastric cancer nude mouse model, infused CIKs inhibited subcutaneous tumor growth. Importantly, we observed that CIK cells infused via different pathways demonstrated different distribution and tumor inhibitory effect.

CIK cells are usually infused intravenously in adoptive cellular immunotherapy (ACI). Hazelrigg *et al* (23) found that the immune cells first arrived at the lungs after intravenous transfusion. In 2-6 h, accumulation in the lungs reached a peak and dissipated, with gradual accumulation in the liver, kidney and spleen. The overall cell distribution tended to stabilize within 24 h. Skitzki *et al* (24) reported that CIK cells could widely immigrate into most organs after intravenous transfusion; distribution was related to blood supply and immune properties of the organs as well as the order in which cells reached each organ. Furthermore, the *in vivo* cytotoxic activity of adoptively transferred immune cells is mainly observed in the initial peak period post-transfusion. These studies suggest that immune cells can effectively reach most organs and tumor tissue while maintaining their cytotoxic abilities. For leukemia, lymphoma and other non-solid tumors, CIK cells can spread throughout the body, including the bone marrow, via intravenous transfusion to kill tumor cells (25,26). However, for solid tumors, such as gastric cancer, the ability of CIKs to target and accumulate in tumor tissue remains to be fully defined.

In our study, we found that, after intravenous transfusion, CIK cells dissipated with the blood circulation and could indeed arrive at the tumor tissue. In comparison, intraperitoneally infused CIK cells first gathered in the abdominal cavity, then distribution followed the same course as the intravenous group by 24 h. In contrast, peritumoral injection resulted in the maintenance of CIK cells in the tumor tissue for the maximum amount of time examined. In this case migration of infused CIK cells to other organs, such as the liver and spleen, was evident but not to the degree of the i.v. or i.p. groups. In terms of tumor growth, peritumoral injection of CIK cells showed improved inhibition. Together, these results indicate that immune cells used for cancer adoptive immunotherapy, if injected directly into the tumor area, may be able to achieve the maximum antitumor effect.

In order to potentiate the antitumor activity of CIK cells, many immunological manipulations have been developed. For example stimulating factors such as Bacille Calmette-Guerin (27) and virus vaccine (28) were added in cell culture to improve cell proliferation. Interleukin-2 (IL-2) genes were transfected into CIK cells to enhance their IL-2 production and potentiate their cytotoxicity (29). DCs were engineered to present tumor antigens to CIK cells with the hopes of enhancing specific recognition of tumor cells and their subsequent killing (30,31). Furthermore, bispecific antibodies for CD3 on T and/or NKT cells and surface antigen on the target cells were used to promote the engagement of CIK cells with target cells (32,33). We have previously found that the gastric adenocarcinoma BGC823 cells express surface carcinoembryonic antigen (CEA). We therefore used CEA/CD3-bispecific single chain antibody to coat CIK cells. CEA/CD3-bscAb can pull together T lymphocytes and CEA-expressing tumor cells (34). In this study, CIK-CEA/CD3-bscAb cells showed stronger antitumor activity than CIK cells against BGC823 cells *in vitro*. Use of CEA/CD3-bscAb to arm CIK cells may represent a potential approach to enhance the antitumor activity of CIK cells.

CTL cells are a subpopulation of T cells with specific cytotoxicity. CD8⁺ CTLs are the most numerous members of the CTL subgroup. After priming by antigen presented by antigen presenting cells, CTLs can recognize and kill corresponding target cells (35,36). Adoptive transfer of antitumor CTLs has already been employed in clinical trials and has shown to be effective in adoptive immunotherapy of ovarian cancer, melanoma, breast cancer and renal carcinoma (12,37-40). In this study, CTL cells were derived from PBMCs and stimulated by DCs loaded with BGC823 tumor cell lysate. We found that the antitumor activity against gastric cancer of the CTLs was much stronger than CIK cells not stimulated with Ag-loaded DCs.

In summary, our results indicate that immune cells such as CIKs and CTLs have strong and direct cytotoxic effects on gastric cancer cells, which suggests that immunotherapy using these immune cells, may become a novel treatment strategy for patients with gastric cancer following surgery, radiotherapy and chemotherapy.

Acknowledgements

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