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$NaHCO_3$ enhances the antitumor activities of cytokine-induced killer cells against hepatocellular carcinoma HepG2 cells

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Abstract. The extracellular pH is lower inside solid tumors than in normal tissue. The acidic environment inhibits the cytotoxicity of lymphocytes *in vitro* and promotes tumor cell invasion. In the present study, both *in vitro* and *in vivo* experiments were conducted to investigate how $NaHCO_3$ would affect the antitumor activities of cytokine-induced killer (CIK) cells against hepatocellular carcinoma (HCC) cells. For the *in vitro* experiments, HepG2 cells were cultured at pH 6.5 and 7.4 in the presence of CIK cells or CIK cell-conditioned medium (CM_{CIK}). For the *in vivo* experiments, nude mice were xenografted with HepG2-luc cells and divided into four groups: i) CIK cells injection plus $NaHCO_3$ feeding; ii) CIK cells injection plus drinking water feeding; iii) normal saline injection plus $NaHCO_3$ feeding; and iv) normal saline injection plus drinking water feeding. The results indicated that the viability and growth rate of HepG2 cells were remarkably suppressed when co-cultured with CIK cells or CM_{CIK} at pH 7.4 compared with those of HepG2 cells cultured under the same conditions but at pH 6.5. In the xenograft study, a marked synergistic antitumor effect of the combined therapy was observed. $NaHCO_3$ feeding augmented the infiltration of cluster of differentiation 3-positive T lymphocytes into the tumor mass. Taken together, these data strongly suggest that the antitumor activities of CIK cells against HepG2 cells were negatively affected by the acidic environment inside the tumors, and neutralizing the pH (for example, via $NaHCO_3$ administration), could therefore reduce or eliminate this influence. In addition, it should be recommended that oncologists routinely prescribe soda water to their patients, particularly during CIK cell therapy.

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Introduction

Hepatocellular carcinoma (HCC), the most frequent primary tumor of the liver, is one of the leading causes of cancer-associated mortality worldwide (1). Regarding treatment options for HCC, immunotherapy remains an important adjuvant treatment, despite advances in surgery, chemotherapy and radiotherapy (2). Cytokine-induced killer (CIK) cell therapy is currently one of the most commonly used immunotherapies (3). In order to improve the efficacy of CIK cell therapy, numerous strategies, including gene transfection (4), stimulation with certain growth factors (5), pretreatment with antitumor drugs (6), or combination with genetically modified dendritic cells (7), have been attempted in practice. However, a number of clinical trials of CIK cell therapies failed to demonstrate any noticeable improvement over other therapeutic regimens, neither in terms of HCC control rates nor long-term survival rates (8), suggesting that other factors, such as the environment where CIK cells are functioning, should also be considered for immunotherapies.

It has been demonstrated that the high metabolic demand of rapidly proliferating cancer cells, in conjunction with a shift toward glycolytic metabolism reflecting both tumor hypoxia and oncogene-induced changes in gene expression, leads to an often greatly increased production and extrusion of acid by cancer cells (9,10). Therefore, the extracellular pH (pHe) of malignant solid tumors is usually acidic, ranging from 6.5 to 6.9, whereas the pHe of normal tissues is significantly more alkaline, ranging from 7.2 to 7.5 (11). This disturbance of the acid-base balance is able to remodel various physiological functions, and results in solid tumors becoming more invasive and metastatic (12). In addition, it may also induce undesired resistance to immunotherapy (13). By contrast, inhibition of the acid extrusion of tumor cells may alleviate their resistance to immunotherapy (14). However, none of these speculations has ever been confirmed for CIK cell immunotherapy *in vitro*

or *in vivo*. In the present study, the antitumor activities of CIK cells against HepG2 cells were evaluated both *in vitro* and *in vivo* under environments with acidic and alkaline pH.

Materials and methods

Cell culture. HepG2 cells (ATCC, Manassas, VA, USA) or HepG2-luc cells (HepG2 cells stably transfected with a firefly luciferase gene), were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in an incubator at 37˚C with humidified atmosphere and 5% CO_2 in air. Cells were adapted in acidic (pH 6.5) or alkaline (pH 7.4) environments for three passages prior to be used for experiments. The pH values of the medium were accordingly adjusted with lactic acid, $NaHCO_3$ and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Preparation of CIK cells. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by standard Ficoll separation, as previously described (5). Human peripheral blood samples were obtained with full informed consent from patients with HCC. In total, six samples were collected between January 2014 and July 2014 at the Department of Gastroenterology of Renmin Hospital, Hubei University of Medicine (Shiyan, China). The isolated cells were resuspended in RPMI-1640 medium supplemented with 1,000 U/ml interferon (IFN)-γ (R&D Systems, Inc., Minneapolis, MN, USA) and incubated at 37˚C for 24 h. Then, recombinant human interleukin (IL)-2 protein (cat. no. 202-IL-050; R&D Systems, Inc.) and mouse anti-cluster of differentiation (CD)3 monoclonal antibody (cat. no. MAB100; dilution, 1:1,000; R&D Systems, Inc.) were added at 500 U/ml and 50 ng/ml, respectively. Subsequently, the cells were refreshed with RPMI-1640 medium supplemented with IL-2 (500 U/ml) every other day for 10 days prior to being subjected to flow cytometry analysis.

Flow cytometry. A set of conjugated monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ, USA), including anti-CD3-fluorescein isothiocyanate (FITC; cat. no. 561806; dilution, 1:20) as a T-cell marker, anti-CD4-phycoerythrin (PE; cat. no. 565999; dilution, 1:20) as a helper T-cell marker, anti-CD8-PE (cat. no. 561950; dilution, 1:20) as a cytotoxic T-cell marker and anti-CD56-PE (cat. no. 561903; dilution, 1:20) as a natural killer (NK) cell-marker, were used to define the phenotypes of CIK cells. In total, $1x10^6$ CIK cells were harvested and washed once with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Haimen, China), and resuspended in 100 μl PBS/BSA. The cells were then incubated with the above conjugated monoclonal antibodies separately for 20 min at 4ºC, washed twice with PBS and resuspended in 400 μl PBS. Flow cytometric analysis was performed with a BD FACSCalibur™ flow cytometer (BD Biosciences), and the data were analyzed using the WinMDI software, version 2.9 (The Scripps Research Institute, La Jolla, CA, USA). The dead cells and debris were gated out.

HepG2 cell apoptosis was analyzed using the annexin V/propidium iodide (PI) double staining method. HepG2 cells were plated at a density of $3x10^5$ cells/well in a BD Falcon® 12-well plate (BD Biosciences) and cultured in CIK cell-conditioned medium (CM_{CIK}), HepG2 cell-conditioned medium ($CM_{control}$) or a 1:1 mixture of the two conditioned media (thus, the percentages of CM_{CIK} in the above media were 100, 0 and 50%, respectively). The pH of the media was adjusted to 6.5 or 7.4, correspondingly. After 48 h of incubation, the cells were collected, washed and resuspended in PBS. Then, annexin V-FITC and PI (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were added to the cells, which were incubated at 4˚C for 20 min prior to being subjected to flow cytometer analysis. All the experiments were performed in triplicate.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT colorimetric assay was used to determine the cytotoxic activity of CIK cells against HepG2 cells *in vitro* (15). The CIK cells [effector (E) cells] and the HepG2 cells [target (T) cells] were co-cultured in a 96-well plate with medium at pH 6.5 or 7.4. The ratios of CIK and HepG2 cells (E/T ratio) were set as 10:1, 20:1, 40:1 and 80:1. In addition, E cells or T cells alone were used as controls. The MTT assay was performed 20 h later to evaluate the cell viability, and the optical density (OD) values were measured at 570 nm. The assays were conducted in triplicate. The cytotoxic activity was calculated as follows: Cytotoxic activity (%) = $[1-(OD_{E+T}-OD_E)/OD_T] \times 100\%$.

Fluorescence assay of cytotoxic effects of CIK cells on HepG2-luc cells. HepG2-luc cells were co-cultured with CIK cells or in CM_{CIK} at pH 6.5 or 7.4 respectively. The viability of HepG2-luc cells was assessed fluorescently using the IVIS® Spectrum *in vivo* imaging system (Caliper Life Sciences; PerkinElmer, Inc., Waltham, MA, USA). In co-culture, HepG2-luc cells were plated at a density of 8,000 cells/well into a BD Falcon® 96-well plate (BD Biosciences), and CIK cells were then added to the 96-well plate at E/T ratios of 0:1, 10:1, 20:1, 40:1 and 80:1. In culture with CM_{CIK}, HepG2-luc cells were plated at a density of 1,500 cells/well in a BD Falcon® 96-well plate (BD Biosciences) with medium containing 0, 25, 50, 75 and 100% CM_{CIK}. The assays were performed in triplicate. After 20 h of co-culture or 48 h of culture in CM_{CIK}, 0.15 mg/ml D-luciferin was added into each well, and the luminescence activity (LA) was measured after additional 5-min incubation. Based on the LA values, the cytotoxicities of CIK cells were calculated according to the following formula: CIK cytotoxicity (%) = $[(LA_{0:1}-LA_{x:1})/LA_{0:1}] \times 100\%$, where $LA_{0:1}$ is the LA of HepG2-luc cells cultured in the absence of CIK cells, and $LA_{x:1}$ is a variable that refers to the LA of HepG2-luc cells co-cultured with 10, 20, 40 or 80-fold CIK cells. The cytotoxicities present in the CM_{CIK} were calculated according to the following formula: CM_{CIK} cytotoxicity (%) = $[(LA_0-LA_y)/LA_0] \times 100\%$, where LA is the LA of the HepG2-luc cells, LA_0 is the LA of HepG2-luc cells cultured in the absence of CM_{CIK}, and LA_y is a variable that represents the LA of HepG2-luc cells cultured with 25, 50, 75 and 100% CM_{CIK}.

Xenograft assay. A total of 20 female nude mice (6-8 weeks-old) were purchased from the Animal Experiment Center of Wuhan University (Wuhan, China). All mice were maintained in the Animal Experiment Center of the Hubei University of Medicine (Shiyan, China) at a controlled

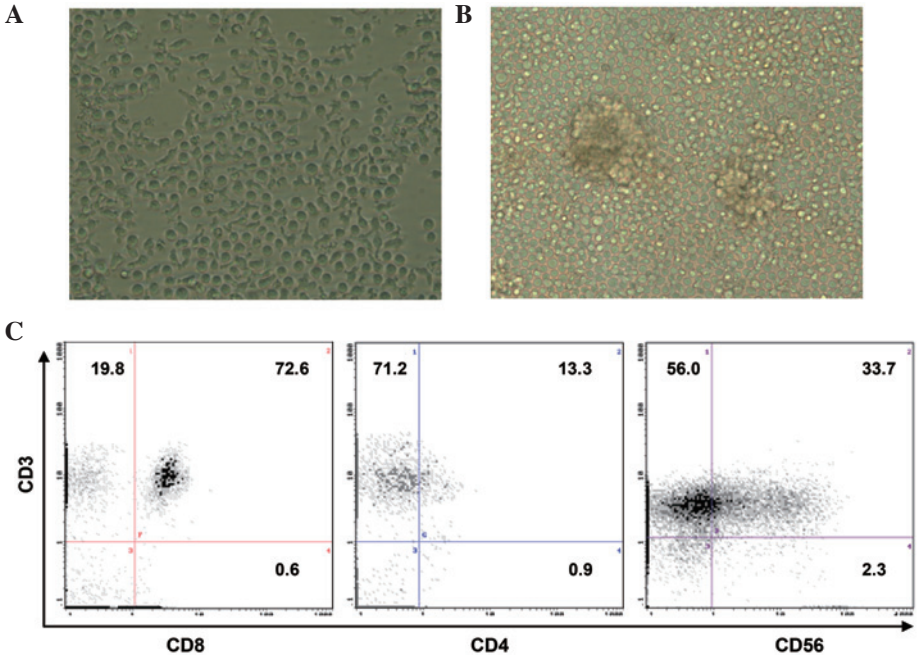

Figure 1. Characterization of CIK cells. (A) Morphology of CIK cells under a phase-contrast microscope after 10 days of induction (magnification, x200). (B) A cluster of CIK cells were observed under a phase-contrast microscope (magnification, x200). (C) Flow cytometry analysis revealed that the percentages of $CD8^+$, $CD4^+$ and $CD3^+$ $CD56^+$ cells were 73, 14 and 34%, respectively. CIK, cytokine-induced killer; CD, cluster of differentiation.

temperature and humidity, under a 12-h light/dark cycle, with *ad libitum* access to sterile food and water. The present study was performed with the approval of the Hubei University of Medicine ethical committee, and all the experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In order to eradicate residual NK cells, the nude mice were administered 200 cGy of whole body irradiation and subcutaneously injected with $1x10^7$ HepG2-luc cells. Subsequently, the mice were randomly divided into four groups according to the different treatments received: i) CIK cells injection plus oral administration of $NaHCO_3$; ii) CIK cells injection plus drinking water feeding; iii) normal saline injection plus oral administration of $NaHCO_3$; and iv) normal saline injection plus drinking water feeding (which served as control). CIK cells ($1.5x10^7$ cells/injection) or an equivalent volume of normal saline were intravenously injected in the mice through their tail veins on days 0, 7, 14, 21 and 28, while $NaHCO_3$ (200 mmol/l) or drinking water feeding were conducted on a daily basis. The LA of the tumors was monitored and measured with the IVIS® Spectrum *in vivo* imaging system on days 0, 10, 20 and 30. The mice were sacrificed on day 30, and the tumor weights were measured.

Immunohistochemical analysis. The grafted tumor masses were fixed in 10% formalin, embedded in paraffin and sectioned with a thickness of 3 μm for immunohistochemical studies. Rabbit anti-human CD3 polyclonal antibody (cat. no. ab828; dilution, 1:50; Abcam, Cambridge, MA, USA) and mouse anti-human perforin monoclonal antibody (cat. no. ab47225; dilution, 1:50; Abcam) were used for immunostaining. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ab6721; dilution, 1:1,000; Abcam) and goat anti-mouse (cat. no. ab6789; dilution, 1:1,000; Abcam) secondary antibodies were used for detection of the primary antibodies. All procedures were conducted according to the manufacturer's protocol. Images of the sections were acquired using an Olympus BX-60 microscope (Olympus Corporation, Tokyo, Japan) to determine the $CD3^+$ and $perforin^+$ cell densities. The number of positive cells was blindly counted in 10 randomly selected independent fields (0.16 mm^2 at x400 magnification) for each section by two independent observers.

Statistical analysis. Differences between groups were compared using analysis of variance, and the least significant difference test was used for multiple mean comparisons. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was conducted using SPSS software v13.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of CIK cells. The CIK cells induced with the method described above appeared regular, round, transparent and variable in size when observed under a microscope. They exhibited the ability of growth in suspension, and multiple characteristic cell clusters were formed (Fig. 1A and B). After 14 days, the absolute number of human PBMCs cultured in the presence of IL-2 and anti-CD3 antibody increased by >250-fold, from 5 million PBMCs to 1,300 million CIK cells. When the phenotypes of the cultured cell population were examined by fluorescence-activated cell sorting analysis, the cell population was observed to be composed of 92% $CD3^+$, 34% $CD3^+$ $CD56^+$, 14% $CD4^+$ and 73% $CD8^+$ cells (Fig. 1C).

The cytotoxic activity of CIK cells is significantly compromised under an acidic environment. Both the MTT assay

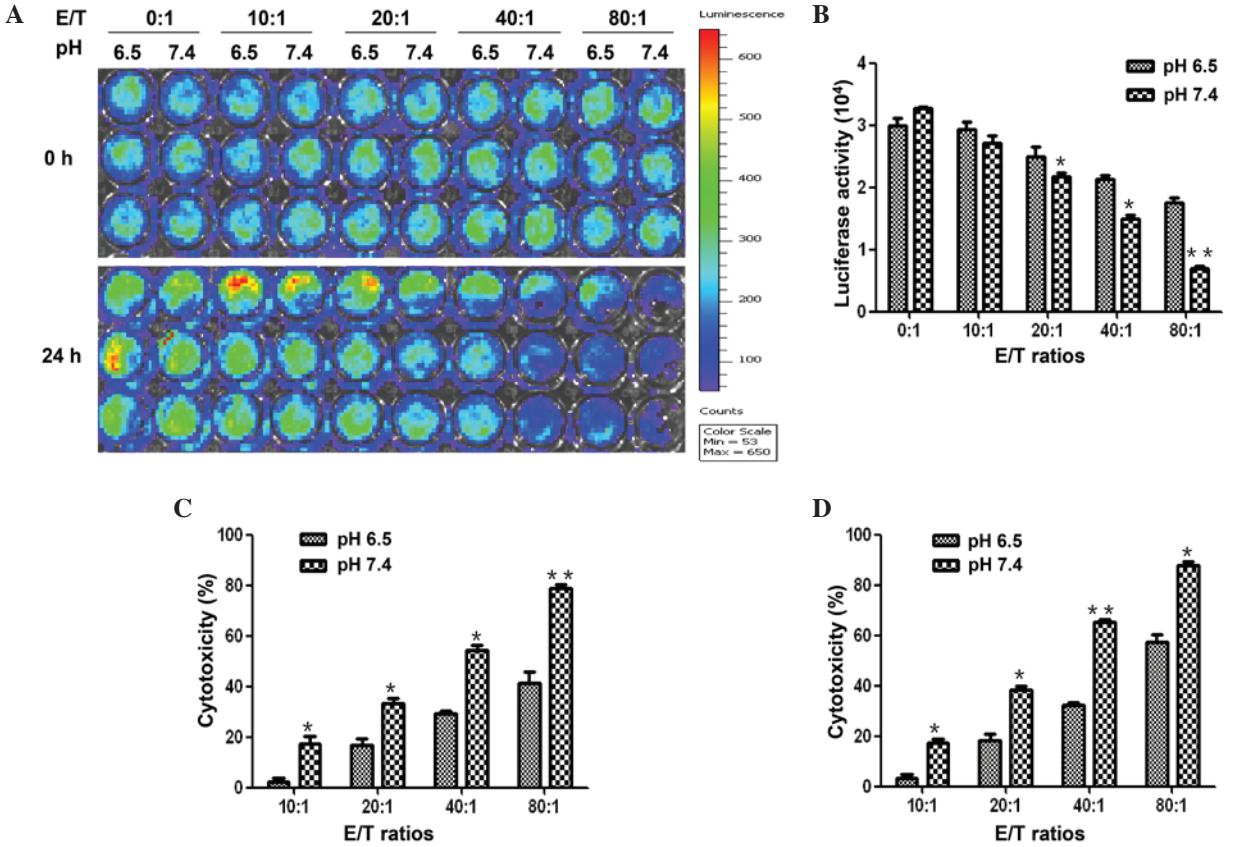

Figure 2. Cytotoxicity of cytokine-induced killer cells against HepG2-luc cells under alkaline and acidic environments. (A-C) Fluorescence assay. (A) Fluorescence images were captured at 0 and 20 h. (B) The fluorescence activities measured after 20 h of co-culture decreased in an alkaline environment, while the E/T ratios (C) and the percentages of dead HepG2-luc cells increased. These results were highly consistent with the (D) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay results. All the experiments were performed in triplicate. Error bars indicate the mean ± standard deviation. *P<0.05, **P<0.01 in *t* test. E, effector cells; T, target cells; luc, luciferase.

and the fluorescence assay demonstrated that CIK cells had a significantly higher cytotoxic activity against HepG2-luc cells in the medium with pH 7.4 than in the medium with pH 6.5. In the fluorescence assay, HepG2-luc cells were co-cultured with CIK cells at an E/T ratio of 0:1 10:1, 20:1, 40:1 or 80:1 at pH 6.5 or pH 7.4. The fluorescence intensities were significantly lower with pH 7.4 than with pH 6.5 for each paired cultures (P<0.05; Fig. 2A and B). The cytotoxic activity calculated based on the fluorescence intensity showed that the cytotoxicity of CIK cells were significantly higher with pH 7.4 than with pH 6.5 (P<0.05; Fig. 2C).

In the MTT assay, 19, 38, 60 and 80% of HepG2-luc cells died when co-cultured with CIK cells at an E/T ratio of 10:1, 20:1, 40:1 and 80:1, respectively, in the medium with pH 7.4. However, only 4, 18, 30 and 45% of HepG2 cells died, respectively, at the same E/T ratios with pH 6.5 (P<0.05; Fig. 2D). Highly consistent with the results of CIK cells in fluorescence assay, the CM_{CIK} displayed stronger cytotoxicity activities in pH 7.4 condition than in pH 6.5 condition. The fluorescence intensities of HepG2-luc cells cultured in medium containing 50, 75 and 100% CM_{CIK} were significantly lower with pH 7.4 than with pH 6.5 (P<0.05; Fig. 3A and B). The cytotoxicity of CM_{CIK} in pH 7.4 condition was stronger than that in the pH 6.5 condition (Fig. 3C).

In addition, the annexin V/PI double staining method was used to determine the percentage of cells that exhibited apoptosis and necrosis (Fig. 4). At a CM ratio of 50%, the CM_{CIK}-induced apoptosis and necrosis in pH 6.5 and pH 7.4 medium were 15 and 26%, respectively. Furthermore, at a CM_{CIK} ratio of 100%, the percentage of apoptosis and necrosis in pH-6.5 and pH-7.4 medium were 25 and 45%, respectively. These results indicated that the acidic environment inhibits the antitumor activity of CIK cells.

The activity of CIK cells against HCC is enhanced by $NaHCO_3$ feeding in nude mice. In order to further investigate whether $NaHCO_3$ treatment would enhance the antitumor activity of CIK therapy *in vivo*, HepG2-luc carcinoma-bearing nude mice were grouped and treated as above described. Nude mice were selected due to their lack of cellular immunity (16). The results revealed that there was no difference between the $NaHCO_3$ feeding group and the control group in terms of luminescence intensities from the tumors. The luminescence intensities of the tumors in the CIK group were significantly reduced compared with those in the control group, while those in the CIK plus $NaHCO_3$ feeding group were also significantly reduced compared with those in the CIK group (Fig. 5A and B). The tumor masses were isolated on day 30 (Fig. 5C) after grafted from each group, and the average weights of the tumors were consistent with the luminescence intensity analysis (Fig. 5D).

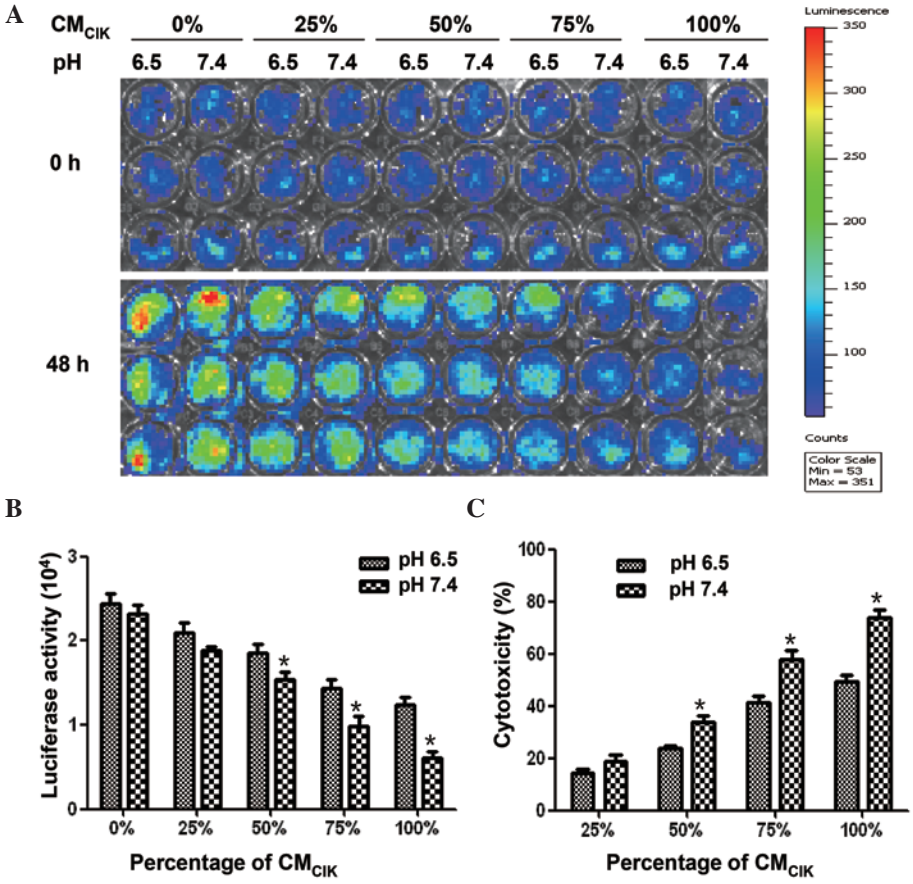

Figure 3. Cytotoxicity of CM_{CIK} against HepG2-luc cells under alkaline and acidic environments. HepG2-luc cells were cultured with different ratios of CM_{CIK} at pH 6.5 and 7.4. (A) Fluorescence images were captured at 0 and 48 h. (B) Luciferase activities were measured at 48 h. (C) The percentages of dead HepG2-luc cells were calculated based on their luminescence activity at 48 h. The experiment was performed in triplicate. Error bars indicate the mean ±standard deviation. *P<0.05 in *t* test. CM_{CIK}, CIK cell-conditioned medium; CIK, cytokine-induced killer; luc, luciferase.

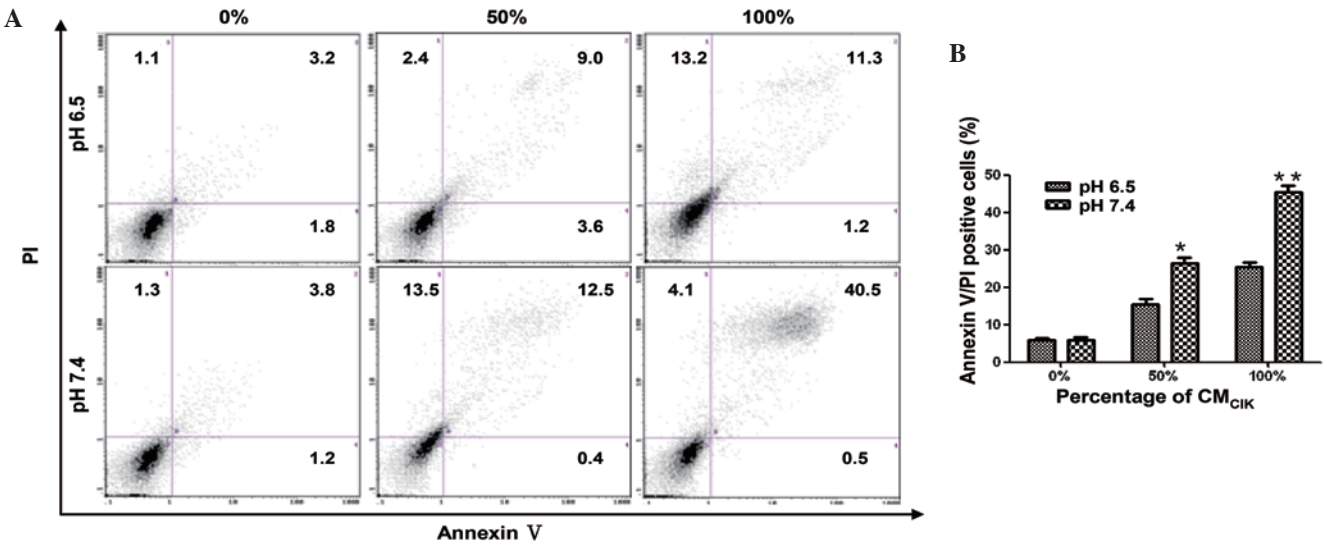

Figure 4. Apoptosis and necrosis analysis of HepG2 cells cultured with CM_{CIK} by annexin V/PI staining. Increased apoptosis and necrosis in an alkaline environment were observed when the proportions of CM_{CIK} were 50 and 100%. (A) Flow cytometry assay. (B) Histogram comparing the percentages of apoptosis and necrosis of HepG2 cells at pH 6.5 and 7.4. The experiment was performed in triplicate. Error bars indicate the mean ± standard deviation. *P<0.05, **P<0.01 in *t* test. CM_{CIK}, CIK cell-conditioned medium; CIK, cytokine-induced killer; PI, propidium iodide.

$NaHCO_3$ enhances the infiltration of CIK cells within the tumor tissue. Since $CD3^+$ T lymphocytes are the main components of CIK cells and perforin is important in the antitumor effects of CIK cells (17), an immunohistochemical study was further performed on the excised tumor tissue with anti-CD3 and anti-perforin antibodies. The results

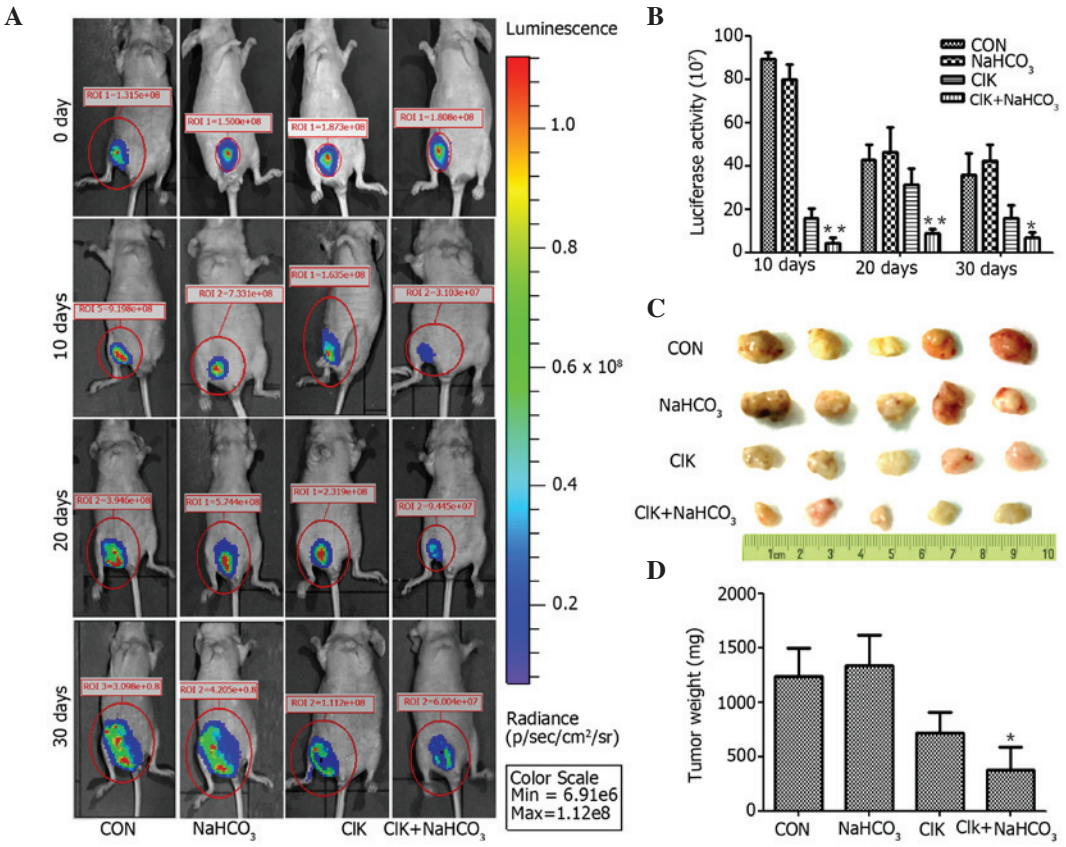

Figure 5. Xenograft experiments. (A) *In vivo* fluorescence images of HepG2-luc-derived tumors of nude mice from four different experimental groups. (B) Histogram revealing that the fluorescence activities were reduced in the CIK+$NaHCO_3$ group. Each experimental group was composed of 5 mice. Error bars indicate the mean ±SD. *P<0.05, **P<0.01 in *t* test. (C) Size of the tumor mass from the mice in the four different experimental groups. (D) Histogram revealing that the average weight of the tumor mass was significantly reduced in the CIK+$NaHCO_3$ group. Error bars indicate the mean ± SD. *P<0.05 in *t* test. ROI, regions of interest; CIK, cytokine-induced killer; CON, control; luc, luciferase; SD, standard deviation.

revealed that there was significantly more accumulation of $CD3^+$ T lymphocytes and perforin in tumor tissue treated with CIK cells plus $NaHCO_3$ compared with tumor tissue treated with CIK cells alone (Fig. 6). There were almost no accumulation of $CD3^+$ T lymphocytes or perforin in the tumor tissue from the other two groups. This result could explain why tumor growth was remarkably suppressed in the CIK+$NaHCO_3$ group.

Discussion

The acidic pH of the extracellular environment has a direct influence on a broad range of immunological functions (18). Previous studies on polymorphonuclear leukocytes demonstrate that this acidic pH mainly causes inhibition of chemotaxis, respiratory activity and bactericidal capacity (19,20). Evidence of impaired lymphocyte cytotoxicity and proliferation at acidic pH is also beginning to emerge (21). In addition, clinical acidosis is accompanied by immunodeficiency (22). Furthermore, other evidence has suggested that acidic microenvironments may play a role during neoplastic growth and invasion (23).

CIK cells are *ex vivo*-expanded T lymphocytes that share phenotypic and functional properties with both NK and T cells (24). In view of the conclusions of previous studies, it is reasonable for us to infer that acidic microenvironments of solid tumors will affect the antitumor activity of CIK cells. Based on the mechanism underlying the antitumor effects of CIK cells, including the fact that CIK cells bind to T cells, form cellular conjugates with tumor cells and secrete a large number of cytotoxic cytoplasmic granules that are cytolytic to T cells (25), and the fact that CIK cells secrete a number of cytokines (including IFN-γ, IL-2 and tumor necrosis factor) that attack tumor cells (26), the present study compared the antitumor activities of CIK cells or CM_{CIK} against HCC at pH 6.5 and 7.4. The results revealed that the antitumor activities of both CIK cells and CM_{CIK} against HepG2 cells were significantly compromised under an acidic environment, with 35 and 25% reduction in cytotoxicity and tumor inhibition, respectively. These results strongly suggested that neutralization of the acidic environment in the tumor tissue should be equally important as the CIK cell therapy itself. CIK cells would not be fully active unless the acidic environment in tumor tissue is neutralized.

Previous experimental and mathematical models have successfully demonstrated that it is possible to upregulate the pHe of tumors with little effect on systemic pH by chronic administration of sodium bicarbonate (27,28). In addition, recent studies documented that oral administration of HCO_3^- could actually inhibit tumor metastasis in prostate, breast and colon cancer cell line-derived tumors (29-31). This apparently occurred by reducing the extracellular acidity of the

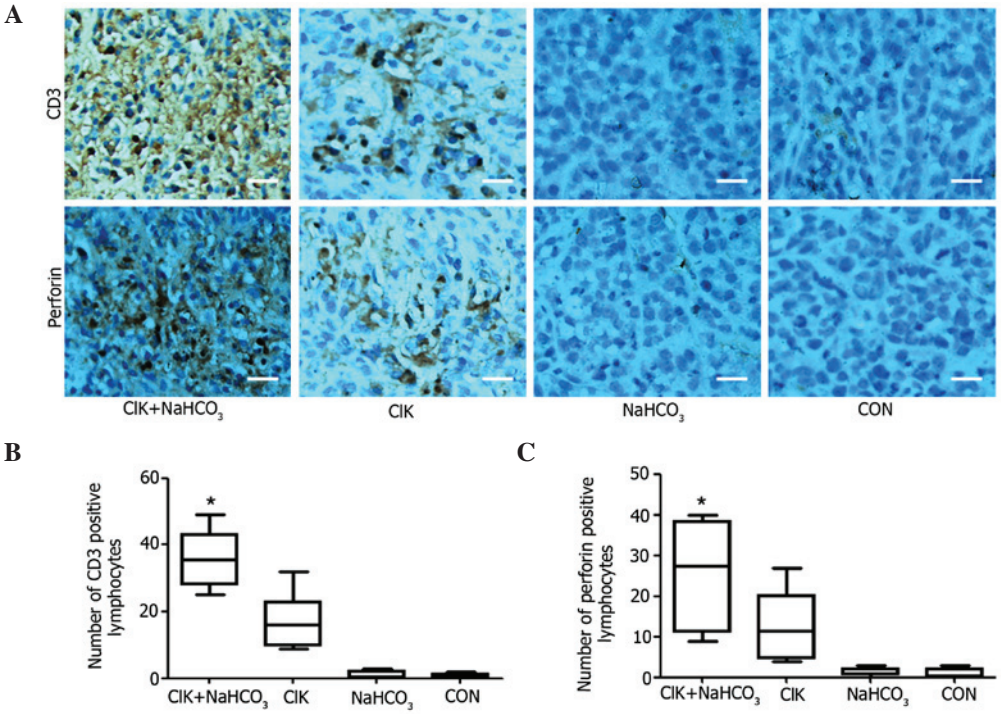

Figure 6. Immunohistochemical study. Sections were prepared from the tumors isolated from the mice in the four different treatment groups on day 30 after HepG2-luc cells were injected into mice. (A) Immunostaining with anti-CD3 and anti-perforin antibodies revealed that there was more infiltration of T lymphocytes in the CIK+$NaHCO_3$ group than in the CIK only group. A total of 10 individual microscopic fields (0.16 mm^2; magnification, x400; scale bar, 25 μm) around the apoptotic areas were randomly selected to count the positive cells for (B) CD3 and (C) perforin, and the average numbers were summarized in a histogram (boxes, 75% percentile; lines, median; bars, standard deviation). *P<0.05 in *t* test. CIK, cytokine-induced killer; CON, control; CD, cluster of differentiation.

acid-extruding tumors, in the absence of changes in the pH of the blood or normal tissues (29-31). Previous findings suggested that certain enzymes such as cathepsins and matrix metalloproteases, which are involved in tumor invasion, were inhibited by alkalinization (32). In the present study, oral administration of $NaHCO_3$ was combined with CIK cell therapy in HCC-bearing nude mice to investigate whether $NaHCO_3$ could enhance the antitumor activity of CIK cells against HCC. The present data revealed a remarkable synergy between oral administration of $NaHCO_3$ and CIK cell therapy in treating grafted HepG2-luc carcinomas in nude mice, suggesting that oral $NaHCO_3$ administration may be sufficient to reduce the acidity of the tumor microenvironment, eventually enhancing the efficacy of CIK cell therapy. These findings and the *in vitro* results could explain one of the reasons why tumor cells thrive in the acidic environment, that is, due to the fact that the immune system is compromised in the acidic environment, thus facilitating the survival of tumor cells. Notably, oral administration of $NaHCO_3$ alone failed to inhibit the growth of the tumor in nude mice. This result was consistent with a previous study (28), in which $NaHCO_3$ administration was observed to have no effect on the growth of primary tumors in severe combined immunodeficiency mice. Thus, although $NaHCO_3$ inhibits spontaneous metastases by attenuating the function of certain enzymes, without the immune system, $NaHCO_3$ is unable to inhibit the primary tumor growth. However, this is not the true situation of the patients with tumors in the clinic, since the majority of these patients are immunologically intact (33). Therefore, even without receiving any immunotherapy such as CIK cell therapy, it would be beneficial for these patients to ingest alkaline food or drinks, since it may aid the T lymphocytes to fight against the tumor cells in their bodies.

The present immunohistochemical studies on the tumor tissue from each group revealed a significantly augmented number of $CD3^+$ T lymphocytes infiltrating into the tumor mass when $NaHCO_3$ feeding was combined with CIK cell therapy, compared with CIK cell therapy alone. In addition, the perforin secreted by T lymphocytes, which is important in killing T cells, was also abundantly detected by immunohistochemistry. These results suggest that the acidic environment appeared to prevent CIK cells from migrating into the tumor tissue, which may partially explain why immune cells such as CIK cells could not function well in an acidic environment. However, there may be other mechanisms involved, such as the acidic environment inhibiting the cytokine secretion of CIK cells, thus compromising their binding ability to the T cells. Further studies to explore the exact molecular mechanisms are warranted.

In summary, both the *in vitro* and *in vivo* experiments of the present study demonstrated that the antitumor activities of CIK cells against HepG2 cells were significantly enhanced under alkaline or relatively less acidic environments, although the molecular mechanism involved require to be further investigated. The present results have provided novel insights for oncologists to treat their cancer patients, particularly when immunotherapy is considered.

Acknowledgements

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