Effect of cytokine-induced killer cells combined with dendritic cells on the survival rate and expression of 14-3-3ζ and p-Bad proteins in Lewis lung cancer cell lines

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Abstract. In the present study, the function and mechanism of cytokine-induced killer cells (CIK) combined with dendritic cells (DC-CIK) were examined in Lewis lung cancer (LLC) cells. Co-culture of CIK dendritic cells (DC) in vitro was used to investigate their proliferation and the antitumor effects on LLC cells. DC and CIK cells were collected from healthy human peripheral blood mononuclear cells and co-cultured as an experimental group, while LLC cells were cultured alone as a control group. Cell morphology was observed by an inverted microscope and an MTT assay was utilized to detect the proliferation of LLC cells. Expression of 14-3-3ζ and p-Bad were measured by western blot analysis. Compared with the control group, treatment of LLC cells with DC-CIK resulted in decreased cell adherence, reduced cell proliferation and abnormal morphological changes. Additionally, DC-CIK treatment of LLC cells resulted in the decreased expression of 14-3-3ζ and p-Bad protein in LLC cells, which may provide important information pertaining to the possible mechanism of DC-CIK-induced antitumor activity against LLC cells. The present study provides a theoretical and experimental basis for the clinical treatment of DC-CIK cell co-culture.

Introduction

An estimated 1.8 million novel lung cancer cases occurred in 2012, accounting for ~13% of total cancer diagnoses worldwide (1). Sundar et al (2) demonstrated that there are two main types of lung cancer, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), with >80% of lung cancer cases being NSCLC in 2014. The combination of the traditional methods of surgery, chemotherapy and radiotherapy with immunotherapy is a novel modality for anti-cancer therapies to reduce the mortality of patients with cancer. However, there are still unacceptably high rates of relapse and mortality in patients with early-stage, surgically-resectable lung cancer (3). Currently, chemotherapy is the standard treatment for advanced stage and metastatic NSCLC (4). However, chemotherapy is associated with a decline in sensitivity over time and often has a toxicity profile that reduces the overall quality of life of the patients, without significantly improving prognosis (5). Despite numerous advances in treatment modalities, the treatment and mechanism for NSCLC progression remains unclear.

Dendritic cells (DCs) are the most effective antigen-presenting cells and have been applied in cellular immunotherapy research worldwide (6). Since the first DC vaccine for prostate cancer was approved by the FDA, DC-based immunotherapy has become an increasingly promising novel therapeutic option. Cytokine-induced killer cells (CIK) are well known for their antitumor activity (7). In recent years, there has been an upsurge of interest in unraveling the roles of combined DC-CIK therapy on NSCLC, with numerous results indicating that DC/CIK immunotherapy combined with other treatments has a good clinical efficacy and prospects for the treatment of NSCLC (8-11). However, the mechanism by which DC-CIK cells can specifically kill NSCLC remains unclear. Therefore, the present study used DCs to induce CIK cells specifically targeted to NSCLC.

The family of 14-3-3 proteins serve key roles in integrating cellular survival signaling. 14-3-3ζ is a member of a family highly conserved proteins that control key aspects of cellular function, including proliferation, apoptosis, and cell survival (12). Experimental and clinical results from previous studies have suggested that 14-3-3 proteins represent an addiction for numerous cancers and consequently are an attractive target for anti-cancer therapeutics (13,14). The protein has been identified as a putative oncoprotein in several cancers, including NSCLC, liver cancer, head and neck squamous cell carcinoma, and is a potential target for developing a prognostic biomarker and therapeutics that may enhance the antitumor activity of cisplatin for the treatment of NSCLC (15).
An increasing amount of evidence has indicated that dysregulation of apoptosis contributes to the development of human cancers (16). Bad, a proapoptotic Bcl-2 family protein regulates the intrinsic apoptosis pathway. Bad is also regulated by phosphorylation, which leads to its sequestration by 14-3-3 scaffold proteins (17). Phosphorylated (p)-Bad dissociates from Bcl-2 and is sequestered in the cytosol to promote cell survival (18).

In the present study the antitumor effects of DC-CIK cells in Lewis lung cancer (LLC) cell lines were evaluated and the underlying mechanism of these effects was investigated. The DC-CIK cells effects on cytotoxic potentiation and apoptosis were investigated and the cytotoxic effects were evaluated using an MTT assay and apoptosis morphology observation. Expression of 14-3-3ζ and p-Bad were measured by western blot analysis.

Materials and methods

Culture of CIK. Peripheral blood mononuclear cells (PBMCs) were collected from healthy blood donors (2 males, 2 females; median age, 39 years; age range, 28-50 years) with no clinical symptoms of any disease. A total of 10 ml of blood was collected from each donor in evacuated tubes containing 0.1 mg/ml heparin. Mononuclear cells (MNC) were isolated by lymphocyte separating medium (Wuhan Huamei Biotech Co., Ltd., Wuhan, China) and washed by normal saline (centrifugation, 1,341.48 x g for 15 min). Mononuclear cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and recombinant human (rh) interferon (IFN)-γ (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) 1,000 U/ml, CD3 monoclonal antibody (sc-20047; 1:4,000, final concentration, 50 ng/ml; Santa Cruz Biotechnology, Inc.) and 300 units/ml of recombinant human interleukin-2 (rh IL-2) were added to CIK cells and cultured at 37°C in 5% CO₂ incubator.

Medium was changed every three days for half dose and added with 300 U/ml rh IL-2 to maintain the cell concentration at 1x10⁶/ml. On day 14, CIK cells were harvested by trypsin and washed twice with PBS. Ethical approval for the present study was obtained from the Ethics and Welfare Committee of Jinzhou Medical University (Jinzhou, China). Written informed consent was obtained from all participants.

Culture of DC. PBMC were cultured in serum-free RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) at 37°C in 5% CO₂ for 2 h and washed by serum-free RPMI 1640 culture medium twice. Adherent cells were suspended in RPMI 1640 culture medium which contained rh Granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,000 U/ml), rhIL-4 (500 U/ml), rh tumor necrosis factor (TNF)-α (200 U/ml) for 7 days.

DC co-cultured with CIK. Mature DCs were obtained from the aforementioned culture of DC 7 days after GM-CSF TNF-α induction, then CIK and DCs were co-cultured in RPMI-1640 at 37°C in 5% CO₂ in a proportion of 1:5. Cells were cultured together for 3 days.

Culture of LLC cells. LLC cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone; GE Healthcare Life Sciences) and antibiotic solution (penicillin 100 U/ml and streptomycin 100 µg/ml). (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany). Cells were cultured under standard conditions in a 5% CO₂ humidified incubator at 37°C. LLC cells alone were cultured as control group.

Cell migration assay. The cells were divided into 4 groups: DC-CIK, CIK, PBS and the control group (RPMI-1640 medium with 10% fetal calf serum). RPMI-1640 medium with 10% fetal calf serum was added in the upper transwell insert chamber with the control group. DC-CIK, CIK cells or PBS were seeded at the concentration of 0.6x10⁵ cells/well in the upper transwell insert chamber containing a polycarbonate filter (6.5 mm diameter, 0.4 µm pores; Corning Costar, Corning, NY, USA). LLC cells was added to the lower chamber at 2.0x10⁶ cells/500 µl/well in all groups, and the plates were incubated for 7 days at 37°C in 5% CO₂. Both upper and lower chamber cells were cultured in RPMI-1640 medium with 10% fetal calf serum. Images of the LLC cells in each group were captured under an inverted microscope (Olympus Corporation, Tokyo, Japan) to evaluate the number and morphology of cells for 20 min at room temperature at 3.5 and 7 days (magnification, x200). LLC cells were used as target cells, and the CIK and DC-CIK cells cultured for 7 days were used as effector cell mixed for the cell viability assay and western blot analysis.

Cell viability assay. The cell viability of LLC cells was measured in the 7th day. LLC cells were obtained from the aforementioned cell migration assay were diluted with RPMI-1640 medium containing 10% fetal calf serum at 1x10⁵ cells/ml. There were three parallel wells for DC-CIK, CIK, PBS and the control group. The MTT assay was employed to examine cell viability. Briefly, MTT was added to the culture medium at a final concentration of 0.5 mg/ml and cells were incubated at 37°C for 3 h, the culture medium containing MTT was removed. Dimethyl sulfoxide (100 µl) was then added into each well to dissolve the formed blue formazan. Absorbance (A) was read at 490 nm on a microplate reader. Cell viability was calculated as follows: Cell viability (%) = A experiment group/A control group x100%.

Western blot analysis. LLC cells were lysed with RIPA lysis buffer [50 mM of tris-HCl (pH 7.5), 150 mM of NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM of EDTA, 25 mM of NaF, 2 mM Na3VO4, and 1 mM of PMST] containing 1:100 diluted protease inhibitor cocktail (cat. no. P8340; Sigma-Aldrich; Merck KGaA) on ice. The protein concentrations were measured using a Bradford assay subsequent to centrifugation at 13,000 x g for 15 min at 4°C. Equal amounts of proteins were separated on a 10% gel by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For western blot analysis of 14-3-3ζ, ab87361; 1:1,000; Abcam, Cambridge, MA, USA), p-bad (ab171725; 1:1,000; Abcam) and β-actin (A5441; 1:10,000; Sigma-Aldrich; Merck
KGaA), the primary antibodies used in the experiment were probed and incubated overnight at 4°C, followed by secondary antibody reactions with horseradish peroxidase-cinjugated goat anti-mouse IgG (ab205719; 1:5,000; Abcam) for 1.5 h. The detection was evaluated by the 3-bromo-4-chloro-5-indolyl phosphate and nitro blue tetrazolium reaction (Ameresco, Inc., Framingham, MA, USA).

**Statistical analysis.** All statistical analyses were performed using SPSS Statistics 17.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. One-way analysis of variance was used followed by Fisher’s Least Significant Difference test for homogeneous data and followed by Dunnett’s T3 for the heteroscedastic data. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LLC proliferation.** Adherent LLC cells that exhibited a round or semi-shuttle shape growth were examined under an inverted microscope (magnification, x200) at 24-48 h after they were passaged (Fig. 1A). Proliferation began at the first three days following culturing. The cells of passaged 2 exhibited a shuttle or fibroblast shape, with the cells being fully extended (magnification, x400; Fig. 1B).

**Morphology of DCs and CIK cells.** Microscopic observation revealed that adherent DCs were visible at the bottom of the plate. On day 7, the cells exhibited an irregular, fusiform or stellate shape that is characteristic of mature DCs. The DCs were thriving, with evident thick and long dendritic protrusions (Fig. 2A). CIK cells were spherical, uniform and transparent, cells were increased in size and small colonies had formed. On day 12, the number of colonies had markedly increased, exhibiting cellular proliferation (Fig. 2B).

**Morphology of LLC cells following co-culture with DC-CIK.** LLC cellular morphology did not change significantly in all groups at 3 day of co-culture. Transparent LLC cells with a uniform size in the CIK group were superior to the DC-CIK group. LLC cells exhibited inflation, cytoplasmic contraction and an increase in intercellular space in the DC-CIK group at 5 day of cell co-culture (Fig. 3A). LLC cells shrank in size and the intercellular space continued to increase in the CIK cells group 7 day (data not shown). A proportion of LLC cells exhibited cell death in the DC-CIK group. Cell morphology change was evident, and cell size became smaller and further away from the surrounding cells. Connections between cells also disappeared (Fig. 3B). These images demonstrate that DC-CIKs induced morphological changes in LLC cells.

**DC-CIK inhibited LLC cell viability.** In order to examine the cytotoxic effects of DC-CIK on LLC cells, cell viability was examined by an MTT assay after LLC cells were incubated with PBS, CIK or DC-CIK for 7 days. The DC-CIK group was statistically different from the other groups. The results demonstrated that DC-CIK inhibited the proliferation of LLC cells (P<0.01; Fig. 4).

**Effect of DC-CIK on expression levels of 14-3-3ζ and p-Bad.** To illustrate the mechanism of DC-CIK induced apoptosis, the involvement of 14-3-3ζ and p-Bad were investigated by western blot analysis. As depicted in Fig. 5, compared with the control group, the protein level of 14-3-3ζ and p-Bad was slightly decreased in the DC-CIK group. The results demonstrate that DC-CIK reduced the expression of 14-3-3ζ and p-Bad protein in LLC cells.

**Discussion**

NSCLC is the leading cause of cancer-associated mortality in the USA (15). To date, surgery, radiotherapy and chemotherapy are still the principal therapeutic regimens; however, radiotherapy and chemotherapy exhibit heavy toxic side effects, which are detrimental to the survival rate of patients (19).
In the present study, DC-CIK treatment clearly reduced cell viability on LLC cells by downregulating 14-3-3ζ and p-Bad. DC/CIK cell therapy may be an effective treatment strategy. Similar results have been published by a previous study regarding liver cancer cells (24).

14-3-3 proteins bind to a number of functionally diverse signaling proteins including protein kinases and protein phosphatases, and are involved in important cellular processes such as signal transduction, cell cycle control, and apoptosis (15,25). Ectopic expression of 14-3-3 has been discovered in various malignancies, including lung cancer, liver cancer and head and neck squamous cell carcinoma. Enhanced expression of 14-3-3 proteins have been detected in human cancers including lung cancer (26), which correlates with more aggressive tumors and a poor prognosis (17). Downregulation of 14-3-3ζ in head and neck cancer cells and lung cancer cells renders cells more sensitive to chemotherapy (27,28). The over-expression 14-3-3ζ in NSCLC tissues is associated with the severity of disease and similarly targeted knock-down of 14-3-3ζ using RNAi in A549 cells, and also increased the sensitivity of cells to cisplatin (29). These findings suggest that 14-3-3ζ serves an important role in promoting tumor aggressiveness. As 14-3-3ζ expression is closely associated with NSCLC disease, the present study examined DC-CIK for anti-cancer effects on LLC cells. It was determined that DC-CIK reduced the expression of 14-3-3ζ protein. It is supported by the study of Lin et al (30).

The mitochondria-dependent (type II) apoptotic pathway begins with the apoptosis-regulating protein Bcl-2 family, including the anti-apoptotic proteins Bcl-2 and Bcl-xL in addition to the pro-apoptotic proteins Bad and Bax (31). Previous studies have demonstrated that the effect of apoptosis and repressed cell viability may be due to the decreased levels of p-Bad (29,32). Previous report indicated that the expression of p-Bad was increased in colorectal cancer cells, and suggested that the increased expression of the protein in malignant colorectal epithelial cells compared with the normal mucosal epithelial cells may possibly alter the regulation of cell death during colorectal tumorigenesis (33). However the association between p-Bad and cancers remains controversial. A previous study reported this beavercin-induced apoptosis in human NSCLC A549 cells was also accompanied by the upregulation of p-Bad (30). P-Bad expression was detected well in normal gastric mucosal epithelial cells, whereas it was detected in only 51% (31 of the 60) of the cancers. The decreased expression of p-Bad in malignant gastric epithelial cells compared with normal mucosal epithelial cells suggested that loss of p-Bad expression may serve a role in gastric tumorigenesis (34). In the present study, the results demonstrated that LLC cells treated with DC-CIK resulted in decreased cell adherence and abnormal morphological changes which are characteristics of apoptosis and reduced cell proliferation. In addition, DC-CIK treatment resulted in decreased expression of p-Bad protein in LLC cells. Depending on the nature of its target proteins, 14-3-3 binding impacts multiple signaling pathways that determine cell fate and organ development. For example, 14-3-3 associations control Raf signaling fidelity and neutralizes Bad-mediated apoptosis (35). Therefore, further study is required to examine the effects of p-Bad and the association between 14-3-3ζ and p-Bad in LLC cells.
Taken together, the results of the present study indicate that DC-CIK induced cell apoptosis in LLC cells, which was associated with the downregulation of 14-3-3ζ and p-Bad. Whether the effects were directly associated with the modulation of autophagy, requires further study. The data presented provides evidence that DC-CIK may have the potential to serve as a promising adjuvant in the combination therapy for the treatment of NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and XL conducted the conception and design of the present study. YH and FL performed the experiments.

Ethics approval and consent to participate

Ethical approval for the present study was obtained from the Ethics and Welfare Committee of Jinzhou Medical University (Jinzhou, China). Written informed consent was obtained from participants.

Consent for publication

Written informed consent was obtained from participants.

Competing interests

The authors declare that they have no competing interests.

References


