

The effects of Cytokine-induced killer cells in the treatment of cholangiocarcinoma

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1. Introduction / Rationale and background to the study

Cholangiocarcinoma is a cancer that starts in bile duct epithelial cells called cholangiocytes. Its incidence was confined to certain localities in the past; however, this form of cancer is becoming more prevalent worldwide. For instance, the incidence rate of bile duct cancer in England underwent a 16-fold increase between 1971 and 2001 while the rate in the United States rose by 120% between 1976 and 2000.⁽¹⁾ In Thailand, both liver cancer and bile duct cancer are ranked among the top 5 causes of death, killing 28,000 persons in 2004.⁽²⁾

The only curative treatment for cholangiocarcinoma by far is surgery. However, fewer than 30% of cholangiocarcinoma patients can have the tumors removed as the remaining majority of cases are

diagnosed at inoperable advanced or metastatic stages. The five-year survival rates range from 20% to 43% for patients with intrahepatic cholangiocarcinoma and from 20% to 40% for those with hilar cholangiocarcinoma.^(1,3) After tumor resections, most cholangiocarcinoma patients experience recurrence. Neoadjuvant or adjuvant radiation therapy and/or chemotherapy do not seem to significantly lengthen the overall survival time. At present, the first-line chemotherapy treatment for advanced cholangiocarcinoma includes a combination of gemcitabine⁽⁴⁾ and other chemotherapy drugs such as 5-FU or oxaliplatin. Review of literature concerning the single and combined uses of gemcitabine to treat cholangiocarcinoma in clinical trials (mostly phase II trials)⁽⁵⁻⁷⁾ has shown that in gemcitabine monotherapy studies, the objective response rate was 0-36%, the stable disease rate was 13-15%, the time to tumor progression was 2 to 10.7 months, and the overall survival time was 4-14 months. On the other hand, in studies of gemcitabine with other chemotherapy drugs (i.e. 5-FU, mitomycin, oxaliplatin, capecitabine, cisplatin, docetaxel, and irinotecan), the objective response rate was 9.3-64%, the stable disease rate was 9.3-53%, the time to tumor progression was 3-10 months, and the overall survival time was still 4.7-18 months only. It can be concluded that current chemotherapeutic agents do not prove to be very successful for the treatment of advanced or metastatic cholangiocarcinoma. Hence, further studies to develop new drugs for more effective treatment of cholangiocarcinoma are warranted.

There are now reports on cancer treatment by immunotherapy with cytokine-induced killer (CIK) cells. CIK cells are lymphocytes stimulated with anti-CD3 monoclonal antibody, interleukin (IL)-2, IL-1, and interferon gamma (IFN- γ), and the cytotoxic activity of CIK cells are not restricted by major histocompatibility complex (MHC) on target cells. Both in vitro and in vivo studies have indicated that CIK cells exhibit high cytotoxicity on tumor cells. According to scientific findings, CIK cells express CD4 and CD8 markers and most of them express surface CD3 and CD56 molecules as well. Previous studies have discovered that CIK cells are 73 times as cytotoxic as lymphokine activated killer (LAK) cells due to the fact that they are non-major histocompatibility complex restricted, and that these killer cells show efficacy against cancerous cells with high expression of tumor antigens such as those in liver cancer, lung cancer, colon cancer, breast cancer, ovarian cancer, leukemia, melanoma, kidney cancer, and lymphoma.⁽⁸⁻³⁵⁾

To date, a number of clinical trials have been conducted to evaluate the efficacy of CIK cells in the treatment of patients with various types of cancer. In a study involving Hodgkin and non-Hodgkin lymphoma patients, the injections of 1×10^9 - 1×10^{10} CIK cells in these patients were reported to cause no side effects.^(21,31) According to a phase III randomized controlled trial (RCT), CIK cells enhanced immune system and reduced recurrences in patients with hepatocellular carcinoma (see literature review). Another study (an RCT) indicated that concomitant chemotherapy and immunotherapy with CIK cells significantly decreased tumor marker levels (MG7-Ag, CA 72-4, CA 19-9, and CEA) in stage IV gastric cancer patients. These patients were reported to also have stronger immune system, better life quality, and greater initial response to treatment when compared to the gastric cancer patients undergoing chemotherapy alone.⁽¹⁵⁾ A study involving non small cell lung cancer patients also concluded that CIK-cell therapy in combination with chemotherapy was superior to chemotherapy alone⁽³³⁾ in treating this form of cancer.

In a recent study by A. Wongkajornsilp et al., CIK cells have been found to inhibit cholangiocarcinoma cell growth in SCID mice. Nevertheless, no clinical trials have been performed to assess CIK cells in the treatment of bile duct cancer. Thus, this study aims to investigate CIK cells' efficacy in treating cholangiocarcinoma patients with emphasis on drug safety.

Literature review

A phase I study of CIK cell therapy for patients with hepatocellular carcinoma was reported in Shi et al. (2004). In the study, peripheral blood mononuclear cells were collected from 13 HCC patients, and after stimulated with interferon (IFN)- γ , antibody to CD3, and interleukin-2, the cells were injected back into the patients on day 14. It was found that PBMC collected from the patients 10 days before and 10 days after CIK injection changed from 33.5% and 7.7% to 36.6% ($P < 0.05$) and 18.9% ($P < 0.05$) respectively. After the therapy, patients were reported to have improvements in their overall health conditions, with more appetite and better sleep. Tumor size was also reduced in 3 patients. Most of the patients experienced 6-8 hours of fever (37.5-40 °C), which developed 6 hours after CIK cell injection, but no other side effects (e.g. effects on kidney and liver) were observed. The study concluded that CIK cell therapy was safe and effective.⁽⁸⁾

A phase 1 clinical trial by Leemhuis (2005) reported the use of autologous CIK cells in treating 9 Hodgkin and non-Hodgkin lymphoma patients. According to the study's in vitro findings, CIK cells exhibited cytolytic activities against tumor cells (B-cell; OCI-Ly8) at a 40:1 effector-target cell ratio. The patients received $1 \times 10^9 - 1 \times 10^{10}$ CIK cells per injection and no immediate side effects were observed. Partial response was reported in 2 patients and stabilization of disease in other 2 patients. The study concluded that CIK cell treatment had beneficial effects for lymphoma patients at the risk of recurrence following chemotherapy.

Weng et al. (2008) conducted a randomized controlled trial on 85 hepatocellular carcinoma patients who previously received a combined treatment with transarterial chemoembolization and radiofrequency ablation. The patients were divided into 2 groups: CIK group and non-CIK group. It was found that the recurrence rates at month 18 were 15.6% in patients receiving CIK cell injections and 40% ($P < 0.05$) in those receiving no injections. The study indicated that CIK cell therapy enhanced immune system and lowered recurrence in HCC patients.⁽³¹⁾

Jiang et al. (2006) reported a clinical trial involving 57 stage IV gastric cancer patients. The patients were divided into 2 groups, one receiving CIK cell therapy combined with chemotherapy and the other receiving only chemotherapy. In the former group, tumor markers (MG7-Ag, CA 72-4, CA 19-9, and CEA), were significantly reduced, and patients' immune system, life quality, and initial response to treatment were better than those of patients treated by chemotherapy alone.⁽¹⁵⁾

Wu et al. (2008) carried out a randomized controlled trial to investigate the effects of CIK cell therapy when combined with chemotherapy drugs in the treatment of inoperable non small cell lung cancer patients. The patients were divided into 2 groups, with group A receiving docetaxel-cisplatin only and

group B receiving chemotherapy drugs plus CIK cells. It was found that host immune function and quality of life of group B patients were improved to a greater extent than those of the other group, with the disease control rate of 89.7% (compared to 65.5% in group A patients ($P = 0.030$)) and the time to progression at 6.65 months (as opposed to 4.67 months for group A patients). The overall survival rate of group B patients was also significantly higher than that of group A patients ($P = 0.029$). No adverse side effects were observed in patients injected with CIK cells. It was concluded that non small cell lung cancer patients benefited more from chemotherapy plus immunotherapy than from chemotherapy alone.⁽³³⁾

A pilot clinical trial by Oliosio et al. (2009) studied the use of autologous CIK cells in treating patients with refractory lymphoma and patients with metastatic solid tumors. The study aimed to establish whether it is feasible to culture an ample number of autologous CIK cells for patients previously treated with chemotherapy and to explore the side effects of CIK cell therapy in 12 patients (6 with lymphoma, 5 with stage IV renal cancer, and 1 with hepatocellular carcinoma). According to the study findings, injections of CIK cells at the average dose of 28×10^9 cells ($6-61 \times 10^9$ cells) produced minimal side effects. Complete response was reported in 3 patients and stabilization of disease in 2 patients over a median follow-up time of 33 months (9-44 months). The preliminary data suggested that CIK cell therapy was safe and helped enhance patients' immune system by increasing the number of effector immune cells.⁽²⁵⁾

A randomized controlled trial by Hui et al. (2009) was performed in 127 hepatocellular carcinoma patients who had undergone radical tumor resection. The patients were chosen at random and divided into 3 groups. Group 1 (41 patients) received 3 injections of CIK cells; group 2 (43 patients) received 6 injections; and group 3 (43 patients) received no additional treatment with CIK cells. Disease free survival in group 1 and group 2 was found to be significantly better than that of group 3 ($P = 0.001$ and 0.004). The study concluded that CIK cell therapy had the potential for preventing post-operative recurrence in HCC patients.

Wongkajornsilp et al. (2009) studied the effects of CIK cells in treating cholangiocarcinoma cells in SCID mice. It was found that CIK cells' antitumor potency was at their highest when CIK cells were co-cultured with dendritic cells. Tumor infiltrating CIK cells were detected in tumor cells 2 to 14 days after treatment. The study suggested that CIK cell therapy was a promising option for clinical trials of cholangiocarcinoma treatment. Up to now, however, no clinical trials concerning the effects of CIK cells on cholangiocarcinoma patients have been conducted.

2. Study objectives

2.1 Primary objectives

- To evaluate the safety of adjuvant therapy with autologous CIK cells in cholangiocarcinoma patients

- To investigate the numbers of CD3+ CD56+ cells in patients prior to and following the treatment

2.2 Secondary objectives

- To assess the patients' overall survival rate
- To assess the time to tumor progression in the patients (TTP)*
- To study the patients' CEA and CA19-9 levels before and after the treatment

*According to Thailand FDA's guidelines for study endpoints, TTP is defined as a measure of time after a disease is treated until the disease starts to worsen. In this study, TTP is measured from the beginning of treatment to the study endpoints, which include:

- Evidence of disease progression in accordance with RECIST criteria
- Death from any cause
- The clinical trial end date
- The last assessment of tumor size before patients start an alternative cancer treatment
- The scheduled follow-up visit last attended by the subject
- The study termination date as specified by researchers

3. Study design

3.1 Overview of study design and drug administration

This study is a phase II clinical trial of adjuvant immunotherapy with CIK cells following tumor resections (R1 or R0 resection) in early-stage cholangiocarcinoma patients. The trial took place at Rajavithi Hospital.

3.1.1 Clinical visits

Patients interested to participate in the trial were fully informed in writing of the nature and purpose of the proposed treatment procedure, of their rights and responsibilities as the study participants, and of potential benefits and risks of the treatment. Patients had opportunities to ask questions and were given an ample amount of time to make decisions. As consents were obtained prior to patients' next scheduled clinical visits, the consent forms were signed and dated before the study began. All participants received a copy of the clinical study information sheet and a copy of their signed and dated consent form for their reference.

3.1.2 Drug preparation process

A peripheral blood sample of 50cc (3 tbsp.) drawn from each participant was collected into a tube containing heparin to prevent the formation of blood clots and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll. Then, the PBMC cells were cultured in x-vivo medium and supplemented with 1000U/ml IFN-g. On day 1, after 24-hour incubation at 37°C in 5% CO₂ incubator, 100 ng/ml anti-CD3, 1000 U/ml IL-2, and 1000 U/ml IL-1 α were added into the culture. The cells were incubated at 37°C in 5% CO₂ incubator and the culture medium containing 1000 U/ml IL-2 per cell concentration of 3×10^6 cells/ml was replaced every 3 days. After 21 days of incubation, the cells were harvested, washed with normal saline solution for at least 3 times, and then resuspended in 100 ml of clinical-grade normal saline containing 0.2% human serum albumin. After that, the numbers of CD3, CD8, and CD56 cells were counted with the expectation for a 100-fold increase. All cell culture procedures were performed in a sterile laboratory under the supervision of Thailand's National Science and Technology Development Agency (NSTDA). Samples of cell cultures were also sent to Rajavithi Hospital for contamination testing.

After day 26 of cell culture, patients received one-hour intravenous infusions of $1 \times 10^9 - 1 \times 10^{10}$ ⁽¹³⁻¹⁵⁾ autologous CIK cells. The infusions repeated every four weeks for four times (on day 1, 29, 57, and 85). After each infusion, patients were hospitalized for one day, at the expense of research team (sponsored by Rajavithi Hospital), so as to be monitored for any adverse drug reaction. During the hospitalization, patients' temperature and vital signs were checked every 4-6 hours. Every 2 months, CT scan or MRI was performed on patients and the levels of blood CBC, LFT, CEA, CA19-9, CD3, CD8, and CD56 were identified.

The trial is to be terminated under the following circumstances:

1. There is evidence of disease progression.
2. Patients cannot tolerate CIK cell toxicity.
3. Patients die.
4. Patients refuse treatments, do not attend scheduled clinical visits, or withdraw from the clinical study.

3.2 Safety considerations

3.2.1 Side effects after CIK cell infusions

Previous studies show that side effects of CIK cell infusions are not severe, with fever (37.5-39 °C) as the only symptom in most patients. The current study, therefore, gave priority to sterile preparation of CIK cells in the laboratory. All solutions used in the cell preparation were of clinical grade and the preparation took place in a class 10000 cleanroom laboratory under the supervision of NSTDA. Samples of all cell cultures were also sent to the laboratory at Rajavithi Hospital for bacterial contamination testing.

3.2.2 Patient care after CIK cell infusions

After each infusion with CIK cells, patients were hospitalized for one day, at the expense of research team, so as to be monitored for any adverse drug reaction. During the hospitalization, patients' temperature and vital signs were checked every 4-6 hours. Every two months, the levels of patients' blood CBC, LFT, CEA, CA19-9, CD3, CD8, and CD56 were identified and CT-scan or MRI was performed. If fever was observed, patients would be given Paracetamol. And if fever persisted despite 2 days of paracetamol, patients would be given an antibiotic drug and checked for blood toxicity. In case of blood toxicity, additional drugs would be prescribed according to the sensitivity and specificity of the infecting agents.

3.2.3 Number of participants

A total number of 10 patients volunteered to participate in this clinical trial.

4. Study population

4.1 Target population

The target population is patients clinicopathologically diagnosed with operable (R0 or R1 resection) early-stage cholangiocarcinoma.

4.2 Criteria for inclusion and exclusion

Inclusion criteria

To be eligible for participation in this study, patients must:

1. Be 18-60 years of age
2. Have histological confirmation of cholangiocarcinoma
3. Have undergone surgery in which tumor has been completely removed or residual tumor is less than 1 cm in size
4. Have a Karnofsky Performance Status (KPS) score of higher than 70 and a life expectancy of greater than 3 months
5. Have normal functions of the body's vital organs including normal bone marrow function (Neutrophil $> 1.5 \times 10^9/L$; PLT $> 75 \times 10^9/L$; AST,ALT < 5 UNL (Upper Normal Limit); TB < 5 UNL; INR < 1.5)
6. Voluntarily agree to participate in the study and sign the informed consent form

Exclusion criteria

1. Patients received or used concomitant anticancer medications, including interferon and herbal medicines approved by a local organization, during the 12-month period prior to study participation.

2. Patients are pregnant or breastfeeding, or patients have childbearing potential and lack acceptable method of contraception.
 3. Patients have a past medical history of other cancers, except for cured basal cell carcinoma and cured in-situ cervical carcinoma.
 4. Patients are suffering from brain metastases and have cognitive impairment.
 5. Patients are suffering from other serious medical conditions, e.g. infectious diseases, pre-existing cardiovascular diseases including heart failure and stable or unstable angina.
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5. Follow-ups

5.1 Follow-up phase

Patient follow-ups were performed every two months until the patient's death or until the study termination by mutual agreement between the research team and the study participants.

During each follow-up, patients' data pertaining to their one-year survival rate, their overall health, their quality of life, their tumor assessment, and their treatment-related problems were collected.

5.2 Screening examination and eligibility screening form

The study regimen was to be started only after patients had voluntarily signed the informed consent forms.

To evaluate consenting patients' eligibility to participate in this clinical trial, screening examinations were performed and recorded in details in eligibility screening forms by research personnel or assigned staff. Screening tests were documented for all prospective participants irrespective of their eligibility assessment results. All the data, including the patient's name, address, and contact information, were kept confidential.

5.2.1 Screening examination procedures

Tumor assessment (by contrast CT or MRI) was performed within 2 weeks before eligibility determination.

General health assessment was conducted within 7 days before eligibility determination. The assessment involved obtaining patient data on personal profile, height, weight, medical history, previous cancer illness and treatment, and comorbid conditions and treatment as well as performing physical examination including pregnancy test (if applicable), vital sign measurements and general health check-up, KPS assessment, and blood testing (including measurements of albumin, total bilirubin, glucose, potassium, sodium, calcium, and creatinine clearance)

5.3 Clinical assessments

Clinical assessments primarily aimed to determine drug safety and calculate the levels of CD3 and CD56 cells in patients' blood before and after treatment.

Clinical assessments' secondary objective was to evaluate patients' quality of life, time to tumor progression (TPP), treatment response rate, and overall survival (OS).

Drug safety evaluation was based on the incidence rate of adverse drug reactions during the first 48 hours and the first 7 days following CIK cell therapy. Subsequently, the drug safety evaluation continued on a monthly basis for one year. Adverse reactions were assessed with the EORTC QLQ C-30 questionnaire after selection of items by considering the time length for a 10-point drop in score, EORTC QLQ C-30 score, and KPS score. Quality of life assessment was performed along with the tumor response assessment during the therapy and during each follow-up.

In an individual patient, radiological assessments of tumor size/tumor response were performed with the same imaging modalities (with CT scan or MRI and with X-ray) throughout the study. In cases where CT scan was applicable, both IV and oral contrast materials were employed.

5.4 Laboratory assessments

All laboratory tests performed during the study were documented in case report forms (CRF).

- CEA and CA19-9 tests (in parallel to tumor assessments)
- Full blood counts including white blood cell, absolute neutrophil/granulocyte, red blood cell, hemoglobin, hematocrit, and platelet counts
- Specific blood tests including total bilirubin, AST, ALT, alkaline phosphatase, albumin, glucose, potassium, sodium, calcium, and creatinine analyses
- Pregnancy test (if clinically indicated)
- Flow cytometric analyses of peripheral blood CD3, CD8, CD56 cells

5.5 Follow-up phase

The follow-up phase started upon the completion of study treatment and follow-up appointments were scheduled every two months (+/- 1 week). During each follow-up, data (see Table 7: 5.2) were to be recorded based on the following guidelines.

In patients with no tumor progression during the treatment, tumor evaluation with contrast CT scan or MRI was to be performed during each follow-up for the purpose of TPP calculation.

In survival time analysis, if a patient did not attend a follow-up appointment, the patient was to be contacted by phone or mail. Such a contact had to be documented appropriately by research personnel.

Patients' quality of life, vital signs, and KPS score were to be assessed during each follow-up.

6. Study treatment formula

6.1 Dosage and administration

6.1.1 Packaging and labeling

CIK cells used in the study treatment were packed and labeled by research sponsor.

The number of CIK cells used per infusion were calculated, ranging from 109 to 1010 cells/infusion.

7. Treatment schedule

Patients were to receive CIK infusions every 4 weeks for 4 consecutive cycles or until any of the following circumstances:

- Tumor progression was observed
 - Patients could not tolerate CIK cell toxicity
 - Patients died from any cause.
 - Patients withdrew from the study or became lost to follow-up.
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Study results

In vitro results

1. Number of CD56+/CD3+ cells

PBMCs were used to generate CIK cells under 3 culture conditions: the normal culture of CIK cells, the co-culture of CIK cells and dendritic cells (DC), and the co-culture of CIK cells and dendritic cells treated with zoledronate acid (Zol). Analysis of cultured cells revealed that in comparison with the normal CIK culture and the CIK and DC co-culture, the CIK and Zol-DC co-culture yielded a greater number of CD56+/CD3+ cells with statistical significance ($P < 0.001$; ANOVA). (see Table 1 and Diagram 1).

Table 1: The numbers of CD56+/CD3+ cells as the results of CIK culture under the normal condition, the CIK coDC condition, and the CIK coZol-DC condition as calculated from a total of 4.8×10^8 cells.

Cell culture conditions	Experiment 1	Experiment 2	Experiment 3
	CD56+/CD3+ (%)	CD56+/CD3+ (%)	CD56+/CD3+ (%)
CIK	35.9	29.8	15.7
CIK coDC	52.14	32.14	38.2
CIK coZol-DC	77	68	57.8

Diagram 1: The numbers of CD56+/CD3+ cells as the results of CIK culture under the normal condition, the CIK coDC condition, and the CIK coZol-DC condition.

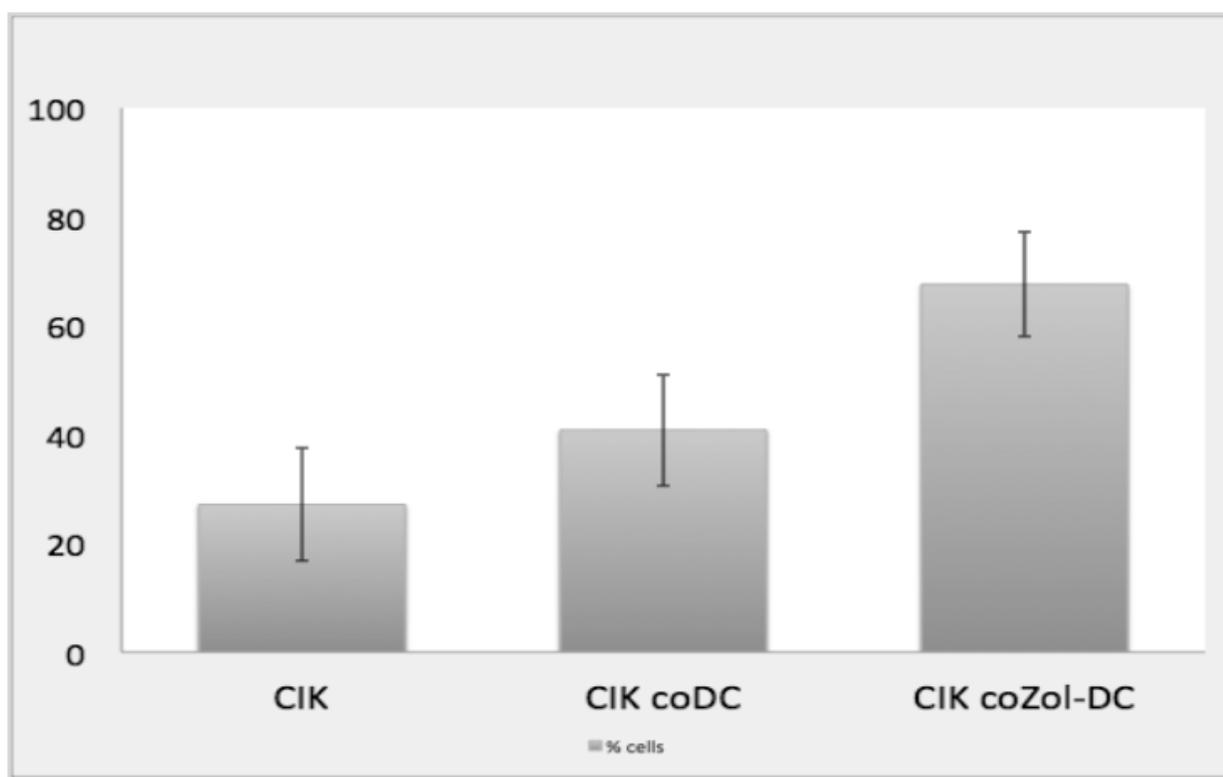


diagram 1

Inhibitory effects of CIK cells on the growth of cholangiocarcinoma cells

15000 cells of three cholangiocarcinoma cell lines, i.e. KKU100, KKUM213, and RMCCA1, were co-cultured with CIK cells for 24 hours under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition. Proliferation of cholangiocarcinoma cell lines was then analyzed using BrDU assay as described in the experiment method section. The analysis revealed that CIK cells under all three culture conditions exhibited inhibition of cholangiocarcinoma cell growth and the antiproliferative

activity of CIK cells depends on the number of CIK cells used in the cell culture. It was also found that CIK cells under the CIK coZol-DC culture condition showed significantly greater antiproliferative potency than those under the other two culture conditions ($P < 0.001$; ANOVA) (see Diagrams 2A-C).

Diagram 2A: The proliferation rates of KKU100 cholangiocarcinoma cell lines when co-cultured with CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition.

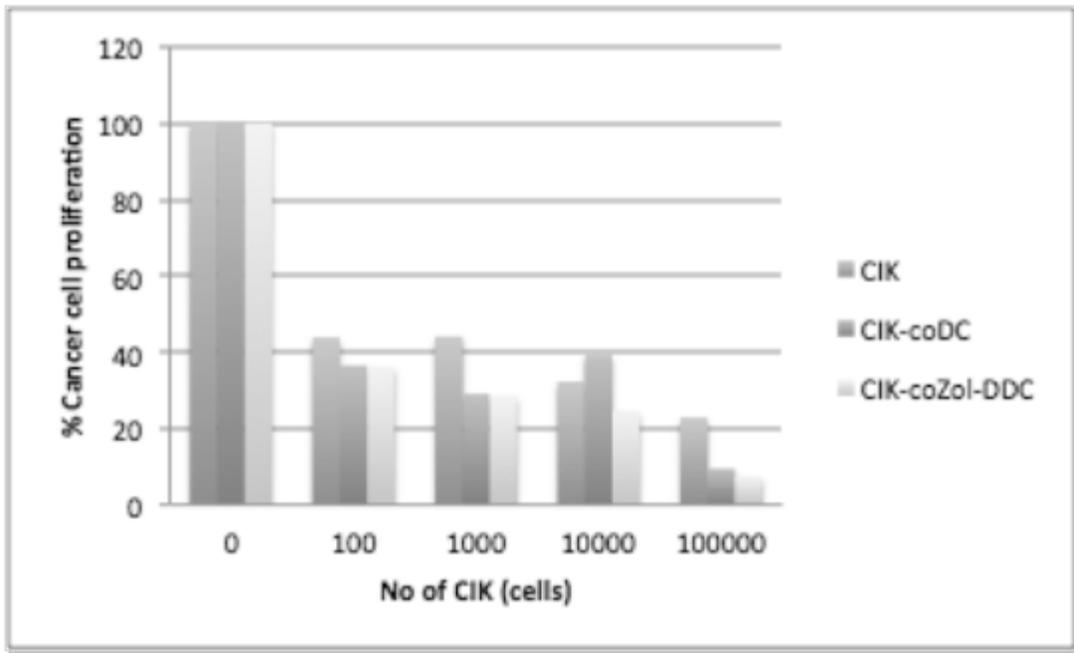


Diagram 2B: The proliferation rates of KKUM213 cholangiocarcinoma cell lines when co-cultured with CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition.

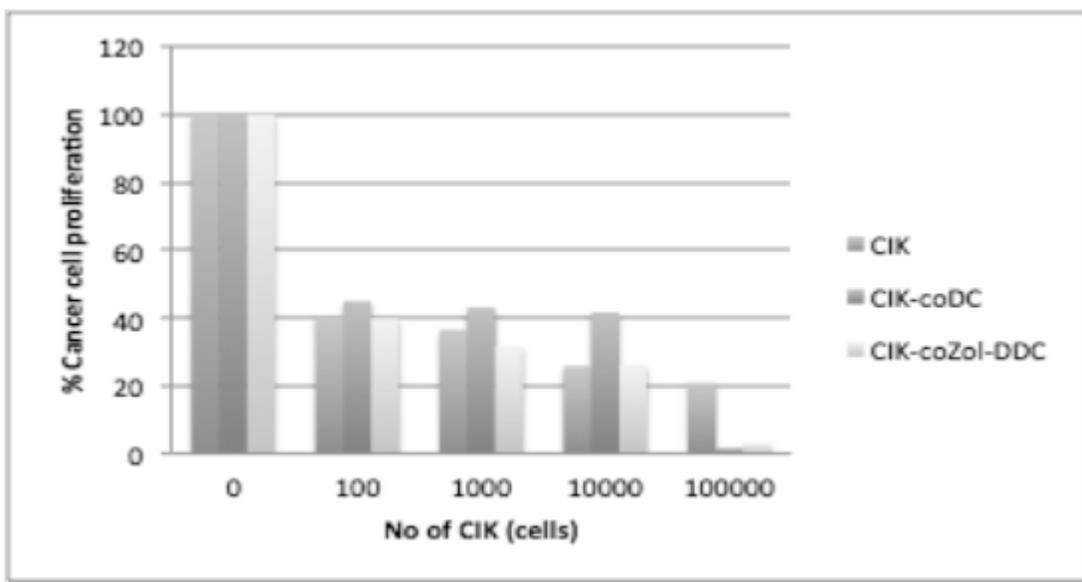
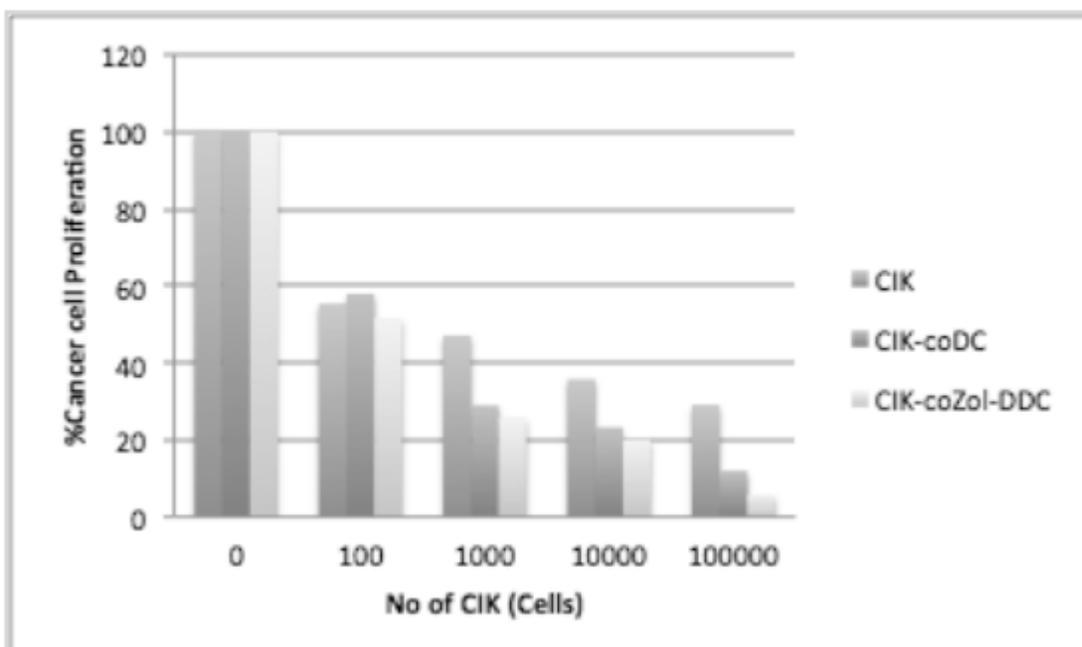


Diagram 2C: The proliferation rates of RMCCA1 cholangiocarcinoma cell lines when co-cultured with CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition



IFN-gamma levels measured in CIK cell culture media

After co-culturing 15000 KKU100, KKUM213, and RMCCA1 cholangiocarcinoma cell lines with CIK cells for 24 hours under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition, ELISA assay was performed to measure IFN-gamma levels in the culture media. The analysis

indicated that IFN-gamma concentration in each culture condition was influenced by the number of CIK cells used in the medium and that the culture medium used in CIK coZol-DC condition yielded the highest concentration of IFN-gamma with statistical significance ($P < 0.001$; ANOVA) (see Diagrams 3A-C).

Diagram 3A: IFN-gamma levels measured in the culture media used in the co-culture of KKU100 cell lines and CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition.

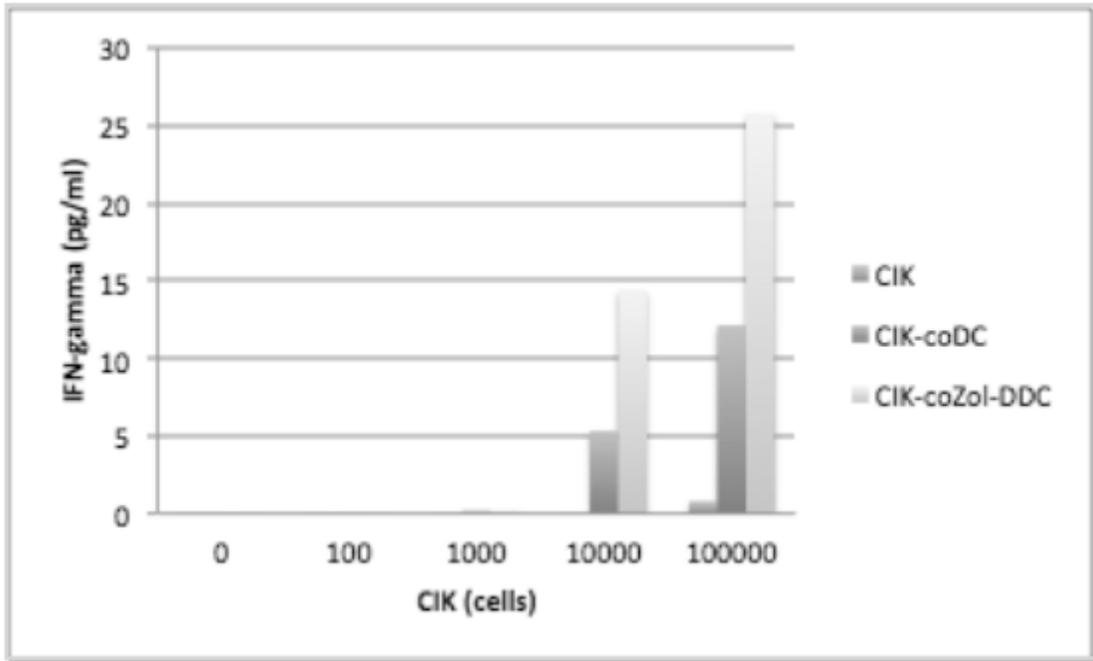


Diagram 3B: IFN-gamma levels measured in the culture media used in the co-culture of KKUM213 cell lines and CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition.

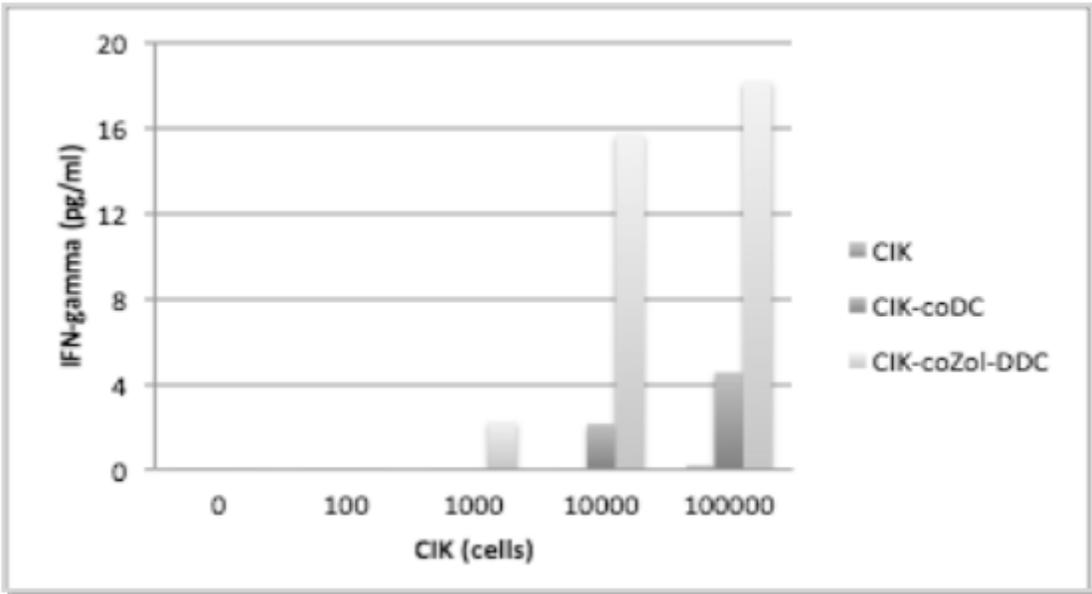
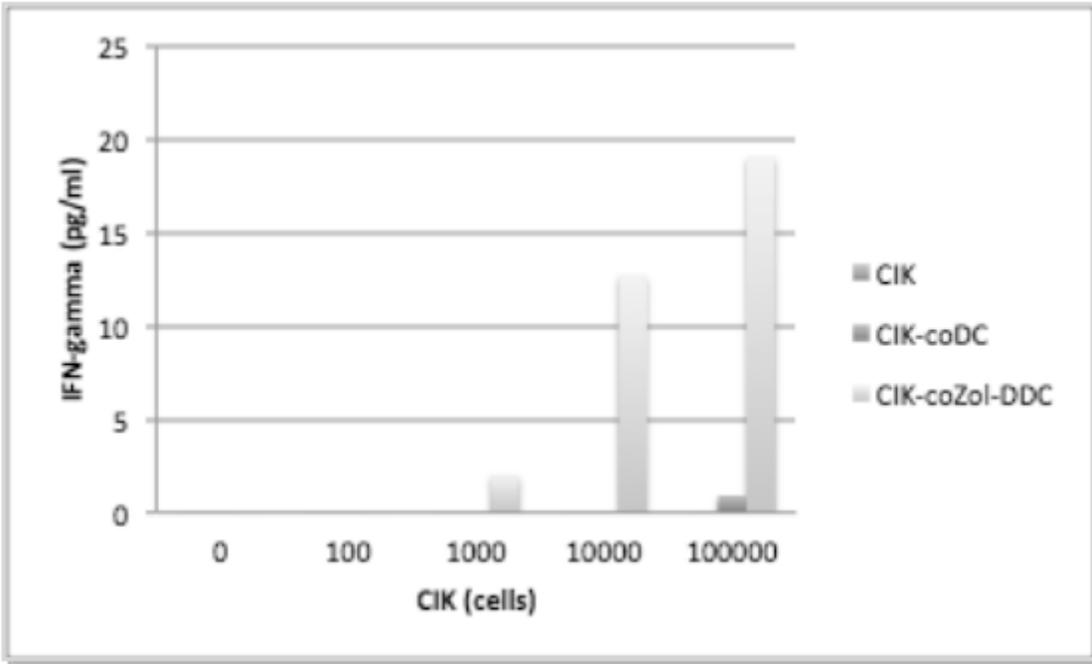


Diagram 3C: IFN-gamma levels measured in the culture media used in the co-culture of RMCCA1 cell lines and CIK cells under the normal CIK culture condition, the CIK coDC condition, and the CIK coZol-DC condition.



Clinical study results

After ethical approvals were granted from Ethical Review Committee for Research in Human Subjects of Ministry of Public Health and from Ethics Committee of Rajavithi Hospital, cholangiocarcinoma patients were screened for their eligibility to participate in the clinical trial over a one-year period from January to December 2011. Five patients aged 55-60 years (four males and one female), who had undergone bile duct cancer surgery, were recruited. In three out of the five patients, the cancer spread to lymph nodes while another patient had central nervous system metastasis. In the other patient, post-operative analysis showed that cancer had spread to peripheral areas of the liver.

The five patients were treated with CIK cell therapy after surgery. Three of them received all the four infusions of CIK cells at one-to-two-month intervals, while one patient received only one cycle of treatment. The details of enrolled patients are as follows.

Patient one

Patient one was a Thai man, aged 59, and was diagnosed with intrahepatic cholangiocarcinoma. The patient underwent a resection in February 2011 and post-operative pathological analysis revealed metastasis to central nervous system. During the study, he received 4 cycles of CIK cell treatment.

First infusion

Blood sample of 50ml was collected on March 18, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 28.5×10^6 cells to 1368×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 54.8% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on April 12, 2011 for the first intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C. The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Second infusion

Blood sample of 50ml was collected on April 26, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 29.4×10^6 cells to 7552×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 81.9% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on May 26, 2011 for the second intravenous infusion of CIK cells, after which no severe complications were observed.

Third infusion

Blood sample of 50ml was collected on May 26, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 30.0×10^6 cells to 1334.4×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 54.8% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on July 21, 2011 for the third intravenous infusion of CIK cells, after which the patient

developed a low fever of 37.5%. The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Fourth infusion

Blood sample of 50ml was collected on August 24, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 50.0×10^6 cells to 2822.4×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 74.2% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on September 12, 2011 for the fourth intravenous infusion of CIK cells, after which no severe complications were observed.

Post-therapy follow-up

The patient was examined with CT scan on the upper abdomen and checked for blood CEA and CA19-9 levels. The CT scan result showed no recurrence of the cancer and the blood CEA and CA19-9 levels were normal. Physical status score (PST) remained zero throughout the therapy. The last follow-up examination in March 2012 indicated that the patient was healthy enough to live a normal life.

Patient two

Patient two was a Thai man, aged 59, and was diagnosed with intrahepatic cholangiocarcinoma. The patient underwent a resection in March 2011 and post-operative pathological analysis revealed that cancer had spread to lymph nodes. During the study, he received 4 cycles of CIK cell treatment.

First infusion

Blood sample of 50ml was collected on March 15, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 20.0×10^6 cells to 1939.2×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 88.1% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on April 5, 2011 for the first intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C. The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Second infusion

Blood sample of 50ml was collected on April 26, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 22.0×10^6 cells to 8818.0×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 75.3% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on May 19, 2011 for the second intravenous infusion of CIK cells, after which no severe complications were observed.

Third infusion

Blood sample of 50ml was collected on July 12, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 40.0×10^6 cells to 2937.6×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 68.2% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on August 3, 2011 for the third intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C . The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Fourth infusion

Blood sample of 50ml was collected on August 9, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 33.4×10^6 cells to 1670.4×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 50.3% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on September 12, 2011 for the fourth intravenous infusion of CIK cells, after which no severe complications were observed.

Post-therapy follow-up

The patient was examined with CT scan on the upper abdomen and checked for blood CEA and CA19-9 levels. The CT scan result showed no recurrence of the cancer and the blood CEA and CA19-9 levels were normal. Physical status score (PST) remained zero throughout the therapy. The last follow-up examination in March 2012 indicated that the patient was healthy enough to live a normal life.

Patient three

Patient three was a Thai woman, aged 56, and was diagnosed with hilar cholangiocarcinoma. The patient underwent a resection in March 2011 and post-operative pathological analysis revealed that cancer had spread to lymph nodes. During the study, she received 4 cycles of CIK cell treatment.

First infusion

Blood sample of 50ml was collected on March 29, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 27.2×10^6 cells to 2918.4×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 59.5% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on April 26, 2011 for the first intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C . The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Second infusion

Blood sample of 50ml was collected on April 26, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 28.5×10^6 cells to 3283.0×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 54.5% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on June 6, 2011 for the second intravenous infusion of CIK cells, after which no severe complications were observed.

Third infusion

Blood sample of 50ml was collected on August 2, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 28.0×10^6 cells to 2035.6×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 57.1% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on August 31, 2011 for the third intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C . The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Fourth infusion

Blood sample of 50ml was collected on August 31, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 28.0×10^6 cells to 2059.8×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 72.8% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on September 29, 2011 for the fourth intravenous infusion of CIK cells, after which no severe complications were observed.

Post-therapy follow-up

The patient was examined with CT scan on the upper abdomen and checked for blood CEA and CA19-9 levels. The CT scan result showed no recurrence of the cancer and the blood CEA and CA19-9 levels were normal. Physical status score (PST) remained 0-1 throughout the therapy. The last follow-up revealed that the patient had died from pericarditis after being hospitalized for high fever, weakness and fatigue, and echocardiographic findings of pericardial effusion.

Patient four

Patient four was a Thai man, aged 60, and was diagnosed with hilar cholangiocarcinoma. The patient underwent a resection in March 2011 and post-operative pathological analysis revealed that cancer had spread to the remaining liver tissues. During the study, he received 1 cycle of CIK cell infusion.

First infusion

Blood sample of 50ml was collected on March 10, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 24.2×10^6 cells to 2352.0×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 71.8% of the total PBMC

cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on April 1, 2011 for the first intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C. The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Post-therapy follow-up

The patient was examined with CT scan on the upper abdomen and checked for blood CEA and CA19-9 levels. The CT scan result in May 2011 showed no recurrence of the cancer and the blood CEA and CA19-9 levels were normal. Physical status score (PST) was zero. However, further assessments were not feasible as the patient failed to come for the other three cycles of treatment due to his back pain and the need to stay at a local hospital.

Patient five

Patient five was a Thai man, aged 55, and was diagnosed with peripheral cholangiocarcinoma. The patient underwent a resection in July 2011 and post-operative pathological analysis revealed that cancer had spread to lymph nodes. During the study, he received 2 cycles of CIK cell treatment.

First infusion

Blood sample of 50ml was collected on August 26, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 40.0×10^6 cells to 4883.2×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 42.1% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on September 15, 2011 for the first intravenous infusion of CIK cells, after which the patient developed a low fever of 37.5°C. The fever dropped after treatment with paracetamol and the patient was discharged from the hospital the following day without any severe complication.

Second infusion

Blood sample of 50ml was collected on September 15, 2011. Peripheral blood mononuclear cells were then isolated and stimulated with cytokine. After the stimulation, the PBMC cells multiplied from 20.5×10^6 cells to 3763.0×10^6 cells and CD56+/3+ (CIK) cells were found to constitute 61.9% of the total PBMC cells. Testing of CIK solution sample showed no bacterial or fungal contaminants. The patient was hospitalized on October 5, 2011 for the second intravenous infusion of CIK cells, after which no severe complications were observed.

Post-therapy follow-up

The patient was examined with CT scan on the upper abdomen and checked for blood CEA and CA19-9 levels. The CT scan result in October 2011 showed no recurrence of the cancer and the blood CEA and CA19-9 levels were normal. Physical status score (PST) was zero. However, further assessments were not feasible as the patient failed to come for the remaining cycles of treatment due to the great floods in late 2011.

Study result analysis

Cholangiocarcinoma starts in epithelial cells of the bile duct lining and represents 10 to 25% of liver cancer. In western countries, the incidence rate of cholangiocarcinoma is 0.3 to 1.5 cases per 100,000 population, as opposed to far higher rates of 33.4:100000 for males and 12.3:100000 for females in Thailand. Cholangiocarcinoma is most prevalent in the Northeast Thailand where the infection of *O. viverrini* liver fluke is most commonly found. It is estimated that 100,000 persons out of 20 million population may be suffering from cholangiocarcinoma without any symptom. Generally, the prognosis for cholangiocarcinoma patients is very poor, with the 5-year survival rate of less than 5%. The median life expectancies for intrahepatic and perihilar cholangiocarcinoma patients are 18-30 months and 12-24 months respectively. Whereas surgery is the only cure for early-stage cholangiocarcinoma, recurrences are found within 5 years after surgery in 60-90% of the patients. Furthermore, a large number of the cholangiocarcinoma patients are first diagnosed at inoperable advanced or metastatic stages, and 75% of such cases die within 1 year of diagnosis due to the lack of chemotherapeutic drugs specific to the cancer. The study, therefore, was conducted to evaluate the effects of CIK cell therapy for cholangiocarcinoma.

CIK cells kill tumor cells without being restricted by major histocompatibility complex. These cytolytic cells are those that express CD3+ CD56+ molecular markers on cell surface. The CD3+ CD56+ molecules bind with ligand (MHC-unrestricted) on tumor cell surface, activating the release of perforin and granzyme from CIK cells and causing the target cell death. In vitro studies show that CIK cells can kill many types of tumor cells including those of lung cancer, gastrointestinal tract cancer, and liver cancer. Hence, the current study investigated CIK cells' efficacy and safety in treating cholangiocarcinoma patients after the in vitro cytolytic effect of CIK cells on cholangiocarcinoma cells had been established.

The current study planned to recruit 10 subjects at first, but due to the limited number of cholangiocarcinoma patients under treatment and the interruption of the great floods in late 2011, only 5 patients were able to participate in the project. It was found that all the 5 participants receiving CIK cell therapy had no severe adverse reactions, a result consistent with what had been reported in previous studies regarding immunotherapy safety. Previous research demonstrated that CIK cell therapy caused minimal side effects in patients with lymphatic, renal, and liver cancers, and even reported a complete response in some cases. Studies conducted in Germany also showed better disease-free-survival rate in CIK-treated patients than in control groups.

Nevertheless, one participant in the study died of pericarditis. Although the death occurred 2 months after the therapy completion, the relationship between pericarditis and CIK-therapy still needs to be scrutinized/explored/established. The popularity of cancer therapy with CIK cells is currently limited due to its high cost attributed to expensive chemicals and materials for CIK cell preparation and the strict requirement for expert scientists who can cultivate contamination-free CIK cells.

Summary

CIK cells (CD56+/3+) could be prepared from the blood of cholangiocarcinoma patients. After stimulated with cytokines, lymphocytes and CIK cells proliferated. CIK cells injected back to cholangiocarcinoma patients caused no severe adverse reactions and the infusions were well-tolerated with low fever. Three out of five patients participating in the study are still alive with no sign of recurrence. However, the relationship of CIK-cell therapy and the disease-free survival in cholangiocarcinoma patients are to be further assessed with a greater number of subjects.
