ORIGINAL ARTICLE

Combination Therapy for Renal Cell Carcinoma using IFN- γ and OK-432: *in vitro* Study

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(Received for publication on November 15, 1989)

Abstract. Synergistic effect of recombinant IFN-gamma (IFN- γ) and OK-432, a streptococcal preparation, using chromium release assay was studied *in vitro* on killer cell induction. The target cells utilized for assay were a human leukemia cell line K562, a human renal carcinoma cell line KU-2, autologous normal kidney tissues and autologous renal cell carcinomas. Culture supernatant of peripheral blood lymphocytes (PBL) and OK-432 (designated as OK conditioned medium or OK-CM) demonstrated enhanced cytotoxicity of fresh PBL against these target cells. Killer cell activity against autologous cancer cells could be also induced from PBL of renal cell carcinoma patients. Pretreatment of PBL with IFN- γ revealed synergistic effect of OK-CM on killer cell induction. OK-CM derived from patients was shown to contain IL-2 activity as well as high titer of interferon. Neutralizing monoclonal antibody against IFN- γ and IL-2 receptor demonstrated reduction of cytotoxicity. These results suggested potential benefit of sequential use of IFN- γ and OK-432 for the treatment of metastatic renal cell carcinoma. (Keio J Med 39 (1) : 21–25, March 1990)

Key words: IFN- γ , streptococcal preparation, cytotoxicity

Introduction

Renal cell carcinoma (RCC) is known to be resistant to chemotherapy, radiation therapy or hormonal therapy and regarded as a cancer with poor prognosis.¹ However, immune mechanism of the host is suspected to relate to the progression of this tumor judging from the facts such as spontaneous regression or late recurrence of the tumor.^{2,3} A number of immunotherapies for metastatic RCC are clinically tried all over the world using several types of agents which include interferon and interleukin 2 (IL-2).^{4,5} Meanwhile we reported a good result of sequential immunotherapy using interferon (IFN) and OK-432 (Picibanil) or a streptococcal preparation against metastatic renal cell carcinoma.⁶ In order to decide the administration schedule of these biological response modifiers (BRMs) to obtain an optimal result, an *in vitro* study is required to investigate the synergistic effect of IFN and cytokines induced by OK-432. In this report, a mechanism of enhanced cytotoxicity of lymphocytes stimulated by culture supernatant of OK-432 and PBL (OK conditioned medium or OK-CM) and IFN- γ is

described and the killer cell activity induced by OK-CM was compared with lymphokine activated killer cell (LAK) activity against natural killer (NK) sensitive K562 cells and NK resistant KU-2 cells

Materials and methods

Target cells

A human renal cell carcinoma cell line KU-2,⁷ a human leukemia cell line K562,⁸ primary culture of fresh normal kidney tissue and fresh renal cell carcinoma, were prepared in a cell suspension, approximately 10^5 cells/ml in density, and utilized as target cells in cytotoxicity assay. These cells were cultured in RPMI 1640 culture medium with 10% fetal calf serum (FCS)(Gibco, USA). Primary culture of fresh normal kidney tissue or fresh renal cell carcinoma was minced with scissors and plastic adherent cells were cultured in FCS coated culture flasks. These cells were harvested after 4–7 days of culture using Trypsin-EDTA solution (Sigma, USA) and single cell suspension in culture medium was prepared

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for cytotoxicity assay.

Effector cells

Approximately 40ml of heparinized peripheral blood were obtained from normal healthy donors or patients with renal cell carcinoma. Mononuclear cells were separated by Ficoll-Isopaque gradient centrifugation method⁹ and washed in phosphate buffer solution (PBS) twice and used as peripheral blood lymphocytes (PBL). One million cells/ml of PBL were incubated for 48 hours under 5% CO₂ at 37°C either with 100 Japan Reference Units [JRU]/ml of a human recombinant IFN- γ (specific activity, 2.5×10^6 JRU/mg protein, Shionogi Pharmaceutical Co. Ltd., Osaka, Japan), 70 JRU/ml of a human recombinant IL-2 (specific activity, 7.0×10^6 JRU/mg protein, Shionogi Pharmaceutical Co. Ltd., Osaka, Japan), or a culture supernatant of PBL and OK-432 (OK conditioned medium or OK-CM). These cells were washed twice in plain RPMI 1640 medium and used as effector cells in cytotoxicity assay.

Cytotoxicity assay

Each target cell was labelled with 100 μ Ci of ⁵¹Cr Na₂O₄ (1Ci = 37 GBq, NEN, U.S.A.) for 1 hour and 10⁴ cells were added to 96 U bottom microtiter plate (Sterilin, England). One hundred μ l. of effector cell suspension was added to each well in order to adjust Effector/Target cell ratio as 50:1. The culture plate was incubated for 4 hours at 37°C under 5% CO₂. After the plate was centrifuged at 400 $\times$ g for 10 minutes, 100 μ l of supernatant was taken from each well and the radioactivity in the supernatant which was released from radioisotope labelled target cells was counted by gamma counter. Cytotoxicity was calculated by the formula as follows¹⁰: %Cytotoxicity = (experimental CPM - spontaneous CPM)/(maximal CPM - spontaneous CPM) $\times$ 100

Spontaneous count per minutes (CPM) was the CPM from wells incubated with 100 μ l of RPMI 1640 culture medium instead of effector cells and maximum CPM was obtained from 10% Triton-X treated wells instead of effector cells. Spontaneous CPM was always 5–10% of maximum CPM in all the experiments.

OK conditioned medium (OK-CM)

Five million cells/ml of PBL and 0.1 K.E./ml of OK-432 (1KE = 0.1g of Streptococcal preparation) were cultured for 2 days in 10% FCS containing RPMI 1640 culture medium. Culture supernatant was collected after centrifugation at 400 $\times$ g for 10 minutes and kept in a deep freezer at -80°C. One fifth dilution of the defrosted conditioned medium was utilized in the cytotoxicity assay.

Monoclonal antibody

Anti-IL-2 receptor monoclonal antibody was purchased from Becton and Dickinson Co. Ltd. (USA). Inhibition capability of DNA synthesis for one million lymphoblasts was 5 μ l. Anti-IFN- γ monoclonal antibody was a mouse IgG₁ and kindly provided by Dr. Ide of Shionogi research laboratory. Neutralizing titer was 5,000 JR Units of human IFN- γ per ml.

IL-2 and IFN titer

Five million cells/ml of PBL from 2 patients with RCC were cultured with 0.1 KE/ml of OK-432 and the culture supernatant was collected between 8–72 hours of incubation. IL-2 titer in the supernatant was measured by counting the radioactivity of incorporated [³H]-TdR in IL-2 dependent murine T cell line CTLL-2 by the method described by Gillis et al.¹¹ IFN titer was measured by a plaque reduction method using a murine fibroblast cell line L929.¹² Standard samples utilized were the human recombinant IFN- γ and IL-2 described as above and the value was expressed in JR Unit. Difference was determined by Student's t-test and p<0.05 was regarded as statistically significant.

Results

Cytotoxicity of PBL from healthy normal donors was enhanced by recombinant IFN- γ , recombinant IL-2 or OK-CM treatment when compared with PBL without stimulation against K562 cells (p<0.005), and the enhancement of cytotoxicity against KU-2 cells was the following order: IL-2 > OK-CM > IFN- γ (Fig. 1A and 1B). Strong cytotoxicity such as those of IL-2 activated killer cell (LAK cell) activity could not be induced by the addition of various concentrations of OK-CM. Cytotoxic effect of sample medium itself was not detected by 4 hour chromium release assay (data not shown).

Further experiment was performed whether killer cell activity can be induced from PBL of patients with RCC. Cytotoxicity was measured against autologous primary cultured kidney tissue, renal cell carcinoma or KU-2 cells. Cytotoxicity was not observed against the normal kidney tissue, however, enhanced cytotoxicity against autologous renal cell carcinoma was noted by stimulation with IL-2 or OK-CM. Strong cytotoxicity of PBL was induced from patients against KU-2 cells at a similar level from PBL of normal donors (Fig. 2).

In order to determine the mechanism of PBL activation by OK-CM, PBL was pretreated with anti-IFN- γ monoclonal antibody (neutralizing titer of 5,000 JRU/ml) and the OK-CM was added to the cell culture. Cytotoxicity was shown to be reduced both against K562 cells and KU-2 cells (Fig. 3).

Fig. 1A Cytotoxicity against K562 cells. PBL from 4 healthy donors was cultured either with culture medium (CONTROL), 100 JRU/ml of rIFN- γ , 70 JRU/ml of rIL-2, or 1/5 diluted culture supernatant of PBL and OK-432 (OK-CM) for 24 hours. Cytotoxicity against K562 was examined by ^{51}Cr release assay. PBL stimulated with IFN- γ , IL-2 or OK-CM was shown to have enhanced cytotoxicity when compared with non-stimulated PBL ($p < 0.005$). E/T ratio was 50:1 and the values and the bars indicate mean $\pm$ SD of the experiments.

Fig. 1B Cytotoxicity against KU-2 cells. PBL from 4 healthy donors was cultured either with culture medium (CONTROL), 100 JRU/ml of rIFN- γ , 70 J.R.U./ml of rIL-2, or 1/5 diluted OK-CM for 24 hours. Cytotoxicity against KU-2 were examined by ^{51}Cr release assay. PBL stimulated with IFN- γ , IL-2 or OK-CM was shown to have enhanced cytotoxicity when compared with non-stimulated PBL ($p < 0.01$). E/T ratio was 50:1 and the values and the bars indicate mean $\pm$ SD of the experiments.

Fig. 2 Autologous cell killing by PBL. PBL from 4 patients with renal cell carcinoma was cultured with culture medium, 100 JRU./ml of rIFN- γ , 70 JRU/ml of rIL-2, or 1/5 diluted OK-CM for 24 hours. Cytotoxicity was examined by ^{51}Cr release assay against autologous kidney tissue (AUTO.KIDNEY), autologous renal cell carcinoma (AUTO.RCC) obtained at the time of operation or KU-2 cells. E/T ratio was 50:1.

Fig. 3 Effect of anti-IFN- γ antibody treatment on PBL activation. PBL was treated with anti-IFN- γ monoclonal antibody at 4C for 1 hour (100 JRU/ml of neutralizing titer). Then PBL was activated with 1/5 dilution of OK-CM for the next 24 hours. Cytotoxicity was studied against K562 and KU-2. "CONTROL" shows cytotoxicity of PBL cultured with medium alone, and "OK-CM" indicates cytotoxicity of OK-CM treated PBL without anti-IFN- γ antibody treatment. E/T ratio was 50:1.

Fig. 4 Effect of anti-IL-2 receptor monoclonal antibody treatment on PBL activation. PBL from 4 healthy donors was cultured in 5 different conditions as follows:

	first 24 hours	wash twice	next 24 hours
1: CONTROL	culture medium	+	culture medium
2: IFN/MEDIUM	IFN- γ (100JRU)	+	culture medium
3: MEDIUM/CM	culture medium	+	OK-CM
4: IFN/CM	IFN- γ (100JRU)	+	OK-CM
5: IFN/IL2Rab/CM	IFN- γ (100JRU)	+	IL-2RMoAb treatment for 1 hour, followed by OK-CM

Activated PBL from each group (No. 1–5) was utilized for ^{51}Cr release cytotoxicity assay against K562 and KU-2. E/T ratio was 50:1 and the values and the bars indicate mean $\pm$ SD of the experiments.

Table 1 A IFN Production by OK-432 Activated PBL

	8 hours	16 hours	24 hours	48 hours	72 hours
Case.1	56.5	277.5	270.0	316.9	3,000.0
Case.2	149.1	241.4	271.5	253.2	1,801.0

*IFN titer (JRU/ml)

Table 1 B IL-2 Production by OK-432 Activated PBL

	8 hours	16 hours	24 hours	48 hours	72 hours
Case 1	38.5	54.9	69.9	41.6	1.4
Case 2	17.8	24.5	22.6	3.7	0.1

*IL-2 titer (J.R.U./ml)

5×10^6 cells/ml of PBL from 2 patients with RCC (Case 1 and case 2) was cultured with 0.1 KE/ml of OK-432 and the culture supernatant was collected at the time points indicated in the table. IFN and IL-2 titer was measured by the bioassay and expressed in Japan Reference Units described in Materials and Methods Section.

Based upon a good result of sequential immunotherapy against RCC using IFN and OK-432 in patients, synergism of these BRMs on killer cell induction and its mechanism was investigated whether it is accompanied by IL-2 and IL-2 receptor binding on PBL. PBL was incubated with IFN- γ , followed by stimulation with OK-CM and the cytotoxicity was determined by chromium release assay. A synergism of IFN- γ and OK-CM was observed both against KU-2 and K562 cells ($p < 0.005$) and this effect was reversed by the anti-IL-2 receptor monoclonal antibody treatment of PBL (KU-2; $p = 0.005$, K562; $p = 0.001$) (Fig. 4).

Further IL-2 and IFN titers were measured using bioassay directly in OK-CM derived from PBL of 2 patients with RCC (Case 1 and 2 in Table. 1). IL-2 and IFN activities were detected in the OK-CM, but not in the supernatant of non-stimulated PBL culture medium. A peak level of IL-2 was observed in the culture supernatant of PBL and OK-432 between 16–24 hours after the initiation of culture, while IFN level showed a gradient increase according to the culture period (Table 1).

Discussion

Several cytokines have been reported to be secreted by OK-432 activated PBL. It includes direct effector molecules for cytolysis such as tumor necrosis factor (TNF)¹³ and lymphotoxin, and regulatory cytokines such as IL-2¹⁴ and IFNs.¹⁵ Also it is reported that killer cells could be induced by OK-432 stimulation of PBL, however, the actual mechanism of killer cell induction and the responsible cytokines have not yet been determined. In this report, a detection of cytokines released by OK-432 stimulated PBL, and its effect on killer cell activity was investigated by the neutralization with monoclonal antibodies against IFN- γ and IL-2 receptor. OK-CM itself was shown to have no effect on cytotoxicity as determined by 4 hour chromium release assay and the induction of killer cells by cytokines was thought to be the mechanism of cytolysis rather than humoral factors such as TNF or lymphotoxin.

Enhancement of cytotoxicity by OK-CM was noted mainly against NK sensitive K562 cells, and enhancement against NK resistant KU-2 cells was not as strong as cytotoxicity of LAK cells (Fig.1.A. and 1.B.). Several concentrations of OK-CM failed to induce strong killer cell activity (data not shown). It may be due to insufficient concentration of IL-2 in the OK-CM because of the unpurified material or the contamination of various cytokines which suppress lymphocyte activation.¹⁶

There are several reports as for immunosuppression related to RCC. Proliferation of patients' PBL was inhibited and activation of fresh PBL was inhibited by the addition of patients' sera.¹⁷ A ratio of inducer, helper T cells/suppressor, killer T cells in PBL was reported to

decrease in patients with RCC.¹⁸ Also a direct evidence of immunosuppressive factor derived from RCC cells was reported.¹⁹ In this report, induction of killer cell activity from such immunosuppressed patients was studied. Cytotoxicity against autologous kidney tissue was minimal, but cytotoxicity could be induced against autologous renal cell carcinoma. The cytotoxicity was still weaker compared with the cytotoxicity against KU-2 cells that is allogeneic renal cell carcinoma. The mechanism of this phenomenon needs to be studied further for induction of autologous tumor killing.

Lymphocyte activation by IFN- γ was suggested to be necessary for killer cell induction judging from a high titer of IFN in the OK-CM (Table 1) and reduction of cytotoxicity by anti-IFN- γ , monoclonal antibody treatment (Fig. 3). After PBL was stimulated with IFN- γ , anti-IL-2 receptor monoclonal antibody was challenged to block the binding of IL-2 molecules to IL-2 receptors on the PBL surface. This treatment reversed the enhancement of cytotoxicity of PBL by OK-CM (Fig. 4). IL-2 receptor number on monocytes was reported to increase by IFN treatment in vitro,²⁰ but in vivo administration of IFN- γ failed to alter the expression of IL-2 receptors on PBL.²¹ It is partly because the ratio of monocytes in PBL is rather low and IFN itself does not have enough effect on PBL activation. Also, immunosuppressive factor secreted from RCC cells may block lymphocyte activation in vivo.¹⁹

Sequential cascade of PBL activation through (1) IL-2 receptor expression on monocytes by IFN, (2) IL-1 production by monocytes, and (3) T cell activation by IL-1, followed by secretion of IL-2 by T cells and activation of T cells by IL-2, was suggested to be a possible mechanism of killer cell induction by OK-432. Although OK-432 itself has the ability to induce IFN from PBL (Table 1), pretreatment of PBL with exogenous IFN- γ induced stronger killer cell activity (Fig. 4). This is probably because exogenous IFN- γ optimizes T cell activation cascade mainly through monocyte stimulation. This sequential immunotherapy may be beneficial for treatment of metastatic RCC cases which did not respond to ordinary immunotherapy.

Acknowledgments: The author is deeply indebted to Professor Hiroshi Tazaki, Chairman of the Department of Urology, School of Medicine, Keio University, for his great advice and encouragement throughout this study. Author's deep appreciation is also to Dr. Ken Marumo, Dr. Shiro Baba, Dr. Nobuhiro Deguchi, Dr. Masaaki Tachibana, Dr. Makoto Hata, Dr. Seido Jitsukawa, Miss Mari Nakai, and colleagues of the Department of Urology for their helpful suggestions and assistances.

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