EXPERIMENTAL / LABORATORY STUDIES

Cytotoxicity of Low Dose Epirubicin-HCl Combined with Lymphokine Activated Killer Cells Against Hepatocellular Carcinoma Cell Line Hepatoma G2

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Abstract: Recent evidance supports the concept that Epirubicin-HCl and Lymphokine-activated killer cells (LAK) cytotoxicity may be mediated by free radical and oxyradical generation. We tested this hypothesis further by exposing Hep G2 cells to Epirubicin-HCl and LAK cells and subsequently monitored cell viability as a measure of cytotoxicity. The cytotoxicity of LAK alone and LAK together with low dose epirubicin-HCl (IC50 1/10) which is measured using the MTT cytotoxicity test on viability of Hep G2 cells and sensitization of target cells to effector cells was investigated. The cytotoxicity of LAK (IC50 value of 5LAK/Hep G2 in 24 hours) and LAK+epirubicin-HCl (IC50 value of 2.5LAK+epirubicin-HCl/Hep G2 cell in 24 hours) appeared to involve a free radical species production type of mechanism since free radicals scavenger enzymes activity, Mn-SOD, Cu,Zn-SOD, Se-dependent GPx and catalase, were increased (p<0.01). Preincubation of Hep G2 cell with SOD before adding LAK and LAK+epirubicin-HCl prevented increasing enzymes activity and cytotoxicity. Also, after LAK and LAK+epirubicin-HCl treatment, increasing expression of NADPH-dependent Cytochrome P450 reductase supported cytotoxicity results depending on free radical production. Increasing activity of Mn-SOD, Cu,Zn-SOD, Se-dependent GPx and catalase are higher in Hep G2 cell treated with combining epirubicin-HCl with LAK than LAK alone (p<0.01). The combining treatment made the Hep G2 cell more sensitive to free radical production and cytotoxicity than LAK treatment alone. SOD, catalase, glutathione peroxidase and NADPH-dependent Cytochrome P450 reductase must be considered as part of the intracellular antioxidant defense mechanism of Hep G2 cells against to single electron reducing quinone-containing anticancer antibiotics and free radical production as result of excess amount of NO synthesis.

Key Words: Hepatoma, epirubicin-HCl, cytotoxicity, enzymes

Introduction

Lymphokine-activated killer cells exhibit cytotoxic activity against virus-infected cells and tumor cells (1,2). LAK cells secrete inflammatory cytokines such as interferon-gamma (IFN-gamma) and tumor necrosis factor alpha (TNFalpha) that can induce nitric oxide (NO) synthesis. LAK cells induced NO synthesis in DLD-1 colon cancer cells in a dose dependent manner (3). NO has free-radical structure: it possesses an extra electron, making it highly chemically reactive. NO destroys tumor cells by inhibiting the energy-producing Krebs cycle and electron transport activities as well as DNA synthesis (4).

The antracycline analogue epirubicin-HCl is an intensely potent cytotoxic compound. It has less cardiac injury than doxorubicin derivatives at doses which produced equal antitumor activity (5). Antracyclines were considered to be cytotoxic by intercalating with DNA and inhibit DNA topoisomerase II activity (6). The proposed mechanism for their cytotoxicity involves formation of intracellular free radicals caused by quinone group of antracycline (7).

The activities of the enzymes glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase protect the cells from the effects of reactive oxygen species generated during the one-electron reduction of quinones (8,9). NADPH-Cytochrome P-450 reductase is an important enzyme in the dexofication mechanism of drugs. The reductase functions by catalysing electron transfer from NADPH to the hemeprotein during the catalysis (10). This enzyme is

found in both hepatic and extrahepatic tissues and is localized in the endoplasmic reticulum and nuclear membrane of the cell. Hepatoma G2 cells are valuable model to study hepatocellular carcinoma and liver metabolism where drugs are metabolised. The human Hep G2 cells (hepatocellular carcinoma cells) have adherent growth property and epithelial morphology. Hep G2 cells expres P-gp (P-glycoprotein). It is a 170 kDa membrane associated molecule that functions as an ATP-dependent drug efflux pump avoiding intracellular accumulation of the drug (11,12).

The liver is the major organ involved in drug biotransformation and the target of the toxic and carcinogenic effects of many compounds. We decided to evaluate cytotoxicity and metabolic pathways of LAK and LAK+epirubicin-HCl (1/10 IC50 concentration) in Hep G2 (Hepatoma G2 cell) cells. We also investigated an alternative mechanism of increasing sensitization of target cells to effector cells.

Materials and Methods

Hep G2 Cell Line: The Hep G2 cell line was purchased from American Type Culture Collection (Rockville, MD USA). Cells were routinely cultured in DMEM supplemented with 10% fetal calf serum, 1% antibiotic-antimycotic solution in a humidified atmosphere in 5% CO_2 at 37 °C. Hep G2 cells, treated with LAK, LAK+superoxide dismutase LAK+epirubicin-HCl and LAK+epirubicin-HCl+superoxide dismutase, were used as experimental groups. Untreated-Hep G2 cells were used as control group. All experiments were repeated eight times. Doubling time of Hep G2 cells was 26 and 28 hours in our experimental conditions.

LAK Cells: Venous blood was collected in heparinized vacutainer tubes from healthy adult donors. We prepared the mononuclear cells by centrifugation over Ficoll-Isopaque. They were depleted by incubation on plastic for 1 h at 37 °C. The nonadherent cell population was peripheral blood lymphocytes (PBL). PBL were activated by in vitro incubation for 4 days at 37 °C in RPMI 1640, 10 mM L-glutamine, 10% autologous plasma, and 1000 U/ml recombinant interleukin-2 (IL-2), at a concentration of $1-2x10^6$/ml in 25 cm^2 tissue culture flasks in a 5% CO_2 environment. rIL2 at 1000 U/ml (Cetus) had been predetermined to yield maximum LAK cell generation (13,14).

Epirubicin-HCl: It was a gift from Farmitalia Carlo Erba, (Milan/ Italy). IC50 (the concentration of a drug that inhibits cell growth by %50) was calculated for Hep G2 cells in 24 hour incubation.

MTT Assay: Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cytotoxicity. The MTT assay has been shown to be useful for measuring cytotoxic activities of human NK/LAK cells (15,16). This method was originally developed by Mossman (17). The cytotoxicity of LAK and LAK+epirubicin-HCl on Hep G2 cells was determined by the MTT assay. To determine cytotoxicity of, LAK and LAK+epirubicin-HCl, Hep G2 cells were plated onto a 96-well, treated with different numbers of LAK cells (effector cells) and LAK+epirubicin-HCl(1/10 IC50) and incubated in 5% CO_2 at 37 °C for 24, 48 and 72 hours. Hep G2 cells were treated with different concentration of epirubicin-HCl (0.8mg/ml, 1.2 mg/ml, 1.6 mg/ml, 2.4 mg/ml, 3.0 mg/ml) and incubated in 5% CO_2 at 37 °C for 24 hours. Hep G2 cells in number of wells was preincubated with SOD for 30 minute before LAK and LAK+epirubicin-HCl treatment for 24 hours incubation in order to show if epirubicin-HCl and LAK cause cytotoxicity generating reactive oxygen species. Untreated Hep G2 cells were used as control group. Test reagents were added to the culture medium. Briefly, 15% volume of dye solution was added to each well after the appropriate incubation time. In one hour of incubation at 37 °C, an equal volume of soluble/stop solution was added to each well for an additional in one hour of incubation. The absorbance of the reaction solution at 570 nm was recorded. The absorbance at 630 nm was used as a reference. The net $A_{570 nm}$-$A_{630 nm}$ was taken as the index of cell viability. The reading taken from the wells with cells cultured with control medium was used as 100% viability value. The percent viability was calculated by the formula $(A_{570 nm}-A_{630 nm})_{sample} / (A_{570 nm}-A_{630 nm})_{control}$ x 100.

Enzymatic Assays

For enzymatic assay, Hep G2 cells were plated at a density 3-5 x 10^3 cell/100 mm petri dish. The next day, cells were treated with LAK, LAK+epirubicin-HCl, LAK +100mg/ml SOD (cells were preincubated with SOD for 30 minute before adding the LAK) and LAK+epirubicin-HCl(1/10 IC50)+100 mg/ml SOD (Cells were preincubated with SOD for 30 minute before adding the

LAK+epirubicin-HCl) for 24 hours. Untreated Hep G2 cells used as control. After 24 hours, cells were scraped off culture plates with culture medium and were centrifuged with 400xg for 10 min. The cell pellets were washed with PBS and then sonicated (3x15 sec) in 50 mM potassium phosphate pH 7.2 containing 1 mM PMSF (Sigma) and 1 mg/ml leupeptin /Sigma) and centrifuged at 150,000 g for 1 hour. The supernatant (cytosolic fractions) was used for the enzymatic assays.

Glutathione reductase (GRX) was assayed spectrophotometrically by following NADPH oxidation at 340 nm (18). The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA. One unit of enzyme activity results in the reduction of one mmole GSSG/min. Glutathione transferase was determined according to Habig et al. (19) using 1-chloro-2,4-dinitrophenol as a substrate. One unit of enzyme activity results in the binding of one mmole GSH/min. Total SOD activity was measured spectrophotometrically using the method of McCord and Fridovich (20). Mn-SOD activity was distinguished from Cu,Zn-SOD activity by its resistance to 1 mM potassium cyanide.

GSH-Px activity was measured according to Paglia and Valentine (21). Selenium-dependent GSH-Px activity was assayed with 0.25 mM H_2O_2 as substrate whereas total GSH-Px was measured with 1.2 mM cumene hydroperoxide (CHP) (22). Catalase activity was measured spectrophotometrically using the method of Luck (23). Protein was determined by the Bradford method (24) with bovine serum albumin as a standard.

Western Blotting

Treated and untreated Hep G2 cells were collected with trypsinisation, washed with PBS, lysed with lysis buffer (RIPA + protease inhibitor), centrifuged (4000xg and 10 min) and the amount of protein was determined according to Bradford's method [24]. Suspended cells in the sample buffer (Reducing Sample Buffer) were boiled for 5 min at 95 °C, and 30 mg of protein per well was applied. The gel was electrophoresed for 1.5 h (90 V), and the protein was transferred onto Hybond-C nitrocellulose membrane. After blocking, nitrocellulose membrane was incubated with primer antibody (goat polyclonal antibodies, sc-8091 and sc-1517 Santa Cruz Biotechnology) followed by treatment with second antibody (donkey antigoat IgG) conjugated to horseradish peroxidase. Membrane were detected using the ECL system (ECL™ 1059243 batch 25 detection reagent I and 1059250 batch 25 detection reagent II, Amersham, USA) (25).

Densitometry was performed by scanning blots into a Power Macintosh 8100/100 computer with a Microtek ScanMaker IIsp scanner at a resolution of 150 lines per inch, with Adobe Photoshop 5.0 and NIH image 1.6.1 software. Western blotting result was discussed depend on densitometric analysis.

Statistical Analysis

Data concerning differences between the control group and the results of cytotoxicity, activity of enzymes and protein expressions experiments were evaluated using the variance analysis method (26). The multiple range test (27) was applied to determine the significance levels of these differences (P<0.05, P<0.01).

Results

LAK and LAK+Epirubicin-HCl Combination Cytotoxicity on Hep G2 Cells

The IC50 value of 24 h incubation were calculated 1.6 mg/ml epirubicin-HCl in Hep G2 cells. Hep G2 cells were treated with LAK and LAK+epirubicin-HCl (1/10 IC50) combinations at different E:T ratios (effector cell (LAK) / target cell (Hep G2 cell); 1/1, 1.25/1, 2.5/1, 5/1, 7.5/1, 10/1, 20/1, 40/1, 80/1) and incubation times (24, 48, 72 hours). The IC_{50} value of 24, 48, and 72 hours incubation was 5/1, 2.5/1 and 1.25/1 for LAK treatment and 2.5/1, 1.25/1, and 1/1 for LAK+epirubicin-HCl (1/10 IC50) combined treatment respectively (Table 1 and 2). The cytotoxicity of LAK and LAK+epirubicin-HCl (1/10 IC50) on Hep G2 cells changed when the cells were exposed to different E:T ratios, and time-intervals. The viability of Hep G2 cells decreased when the cells were treated with LAK until the ratios of 10/1, 10/1 and 20/1 for 24, 48 and 72 hours incubations respectively and did not change at the higher of these ratios (p< 0.05). The viability of Hep G2 cells decreased when the cells were treated with LAK+epirubicin-HCl(1/10 IC50) combination until the ratios of 10/1, 5/1 and 5/1 for 24, 48 and 72 hours incubations respectively and did not change at the these ratios or higher (p< 0.05). The treatment of Hep G2 cells with LAK+epirubicin-HCl (1/10 IC50) combination caused higher cytotoxicity than treatment of Hep G2 cells with LAK alone. The treatment of Hep G2

Table 1. Cytotoxicity of LAK cells on Hep G2 cells.

LAK / Hep G2 cells	Mean (X)[1] + SE Cytotoxicity (%) 24 hours incubation	Mean (X)[1] + SE Cytotoxicity (%) 48 hours incubation	Mean (X)[1] + SE Cytotoxicity (%) 72 hours incubation
1 / 1	8.15 + 2.1 ax	16.12 + 2.1 ay	23.55 + 2.1 aby
1.25 / 1	16.20 + 2.3 ay	37.40 + 1.7 by	49.67 + 2.7 cy
2.5 / 1	38.39 + 1.9 by	51.60 + 1.9 cy	66.50 + 1.9 dy
5 / 1	49.55 + 2.2 cy	60.55 + 3.2 cdy	78.99 + 2.2 ey
7.5 / 1	58.11 + 3.2 cdy	75.68 + 2.1 dey	83.56 + 2.5 ez
10 / 1	65.27 + 2.7 dy	79.15 + 2.7 ey	89.77 + 1.7 efz
20 / 1	71.12 + 2.3 dy	83.25 + 2.1 ez	93.44 + 2.2 fz
40 / 1	69.00 + 2.4 dy	82.55 + 1.5 ez	92.66 + 1.6 fz
80 / 1	70.10 + 2.6 dy	81.40 + 1.6 ez	90.50 + 1.6 fz

Data marked with the same letters in the same row or column are not different from each other at P < 0.05 probability level.
[1]X stands for the average value of eight repetitions cytotoxicity (%) at 24, 48 and 72 h.
SE, Standard Error

Table 2. Cytotoxicity of LAK+epirubicin-HCI (1/10 IC50) on Hep G2.

LAK+epi (1/10 LD50) / Hep G2 cell	Mean (X)[1] + SE Cytotoxicity (%) 24 hours incubation	Mean (X)[1] + SE Cytotoxicity (%) 48 hours incubation	Mean (X)[1] + SE Cytotoxicity (%) 72 hours incubation
1 / 1	15.54 + 1.7 ax	23.95 + 2.1 aby	50.55 + 3.1 cy
1.25 / 1	38.71 + 2.9 by	49.88 + 2.9 cy	67.52 + 2.9 dy
2.5 / 1	51.12 + 3.9 cy	61.45 + 3.9 cdy	76.55 + 1.7 edy
5 / 1	58.19 + 2.2 cdy	79.90 + 2.2 ey	89.54 + 2.3 efz
7.5 / 1	73.77 + 2.5 dy	88.55 + 2.7 efz	91.55 + 2.1 fz
10 / 1	82.88 + 2.7 ez	89.99 + 1.9 efz	96.55 + 1.7 fz
20 / 1	82.66 + 2.2 ez	88.77 + 2.5 efz	95.99 + 2.3 fz
40 / 1	83.77 + 3.6 ez	90.01 + 2.6 fz	97.98 + 1.5 fz
80 / 1	82.11 + 1.6 ez	89.98 + 1.7 efz	97.08 + 1.8 fz

Data marked with the same letters in the same row or column are not different from each other at P < 0.05 probability level.
[1]X stands for the average value of eight repetitions cytotoxicity (%) at 24, 48 and 72 h.
SE, Standard Error

cells with LAK+epirubicin-HCI (1/10 IC50) combination is more effective than treatment with LAK alone on cytotoxicity.

Preincubation of Hep G2 cells with SOD (100 mg/ml) for 30 minutes before LAK and LAK+epirubicin-HCI treatment result in 97% Hep G2 cell viability at the end of the 24 hours. Therefore, free radicals generated by LAK and LAK+epirubicin-HCI were considered to be the primary event in the cytotoxicity.

The result of our research indicate that Hep G2 cells are sensitive to LAK cytotoxicity, also, addition of low concentration of epirubicin-HCI caused the increasing sensitisation of Hep G2 cells to LAK cells.

Table 3. Activity of enzymes in Hep G2 cells.

	CAT U / mg + SE	GSSG-Rx mU / mg + SE	Se-GSH-Px mU / mg + SE	Non-Se. GSH-Px mU / mg+ SH	Mn-SOD mU / mg+ SE	Cu-Zn-SOD mU / mg+ SE	GST mU / mg+SE
Control	$40.41 + 13^{x}$	$117 + 14^{x}$	$477 + 7^{x}$	$642 + 17^{x}$	$830 + 8^{x}$	$630 + 5^{x}$	$31.16 + 5^{x}$
LAK	$63.61 + 8^{xy}$	$153 + 12^{x}$	$1450 + 13^{y}$	$860 + 19^{x}$	$2850 + 11^{y}$	$1300 + 22^{y}$	$52.00 + 10^{xy}$
LAK+SOD	$47.99 + 12^{x}$	$110 + 11^{x}$	$450 + 11^{x}$	$590 + 10^{x}$	$915 + 7^{x}$	$593 + 11^{x}$	$37.11 + 9^{x}$
LAK+Epi	$131 + 3^{z}$	$175 + 14^{x}$	$2932 + 19^{z}$	$966 + 18^{x}$	$5700 + 17^{z}$	$2950 + 13^{z}$	$57.05 + 9^{xy}$
LAK+Epi+SOD	$50.22 + 9^{x}$	$95.11 + 6^{x}$	$431 + 10^{x}$	$641 + 11^{x}$	$811 + 10^{x}$	$569 + 11^{x}$	$30.99 + 4^{x}$

Activities of enzymes in Hep G2 cells treated with 5LAK/Hep G2 cell, 2.5LAK+epirubicin-HCl (1/10IC50)/Hep G2 cell, 5LAK/Hep G2 preincubated with SOD (100 mg/ml for 30 minute before adding LAK cells) , 2.5LAK+epirubicin-HCl (1/10)/Hep G2 cells preincubated with SOD (100 mg/ml for 30 minute before adding LAK+ cells) and untreated cell (as a control) at 24 hours incubation.
CAT; Catalase, GSSG-Rx; glutathione reductase, Se-GSH-Px; selenium-dependent glutathione peroxidase, Non-Se-GSH-Px; selenium-independent glutathione peroxidase, Mn-SOD; mangan superoxide dismutase, Cu,Zn-SOD; Copper-Zinc superoxide dismutase, GST; glutathione S-transferase SE; standard Error,
x, y, z ; Data indicated by same letters in same vertical column are not different from each other on P<0.01 probability level.

Biochemical Parameters of Hep G2 Tumor Cells

Effects of LAK and LAK+epirubicin-HCl(1/10IC50) with IC50 value of 5LAK/Hep G2 and 2.5LAK/Hep G2 respectively at 24 hours on changes of catalase, Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, Se-dependent glutathione peroxidase, Se-independent glutathione peroxidase, glutathione reductase and glutathione S-transferase activities in Hep G2 cells were investigated. Table 3 gives means and their standard error for these enzymes.

Enzyme activities were measured in LAK and LAK+epirubicin-HCl(1/10IC50) treated Hep G2 cells and untreated Hep G2 cells as control for comparison. LAK treated Hep G2 cells have higher activity of Se-dependent glutathione peroxidase (3 times), Cu,Zn-superoxide dismutase (2 times), and Mn-superoxide dismutase (3.4 times) enzymes than control cells (p<0.01). LAK+epirubicin-HCl (1/10IC50) treated Hep G2 cells have increasing activity of Se-dependent glutathione peroxidase (6.2 times), Cu,Zn-superoxide dismutase (4.7 times) catalase (3.2 times) and Mn-superoxide dismutase (6.9 times) enzymes compared to control cells (p<0.01). The combined treatment makes Hep G2 cells more sensitive to LAK cells. The activity of catalase (2 times), Se-dependent glutathione peroxidase (2 times), Cu,Zn-SOD (2.3 times) and Mn-SOD (2 times) enzymes in Hep G2 cells treated with LAK+epirubicin-HCl(1/10IC50) combination are higher than the Hep G2 cells treated with LAK cell alone (p<0.01).

There are no significant differences of enzyme activities between LAK+SOD, LAK+epirubicin-HCl+SOD treated Hep G2 cells and control cells. But the activity of Se-dependent glutathione peroxidase, Cu,Zn-superoxide dismutase and Mn-superoxide dismutase in LAK treated Hep G2 cells are higher than that LAK+SOD treated cell. Also the activity of Se-dependent glutathione peroxidase, Cu,Zn-superoxide dismutase, catalase and Mn-superoxide dismutase in LAK+epirubicin-HCl treated Hep G2 cells are higher than LAK+epirubicin-HCl+SOD treated Hep G2 cells (p<0.01).

The Expression of NADPH-CYP 450 Reductase P-glycoprotein

Western blot analyses were carried out to study the expression of NADPH-CYP 450 reductase and P-gp. Immunoblotting of proteins extracted from LAK (5LAK/Hep G2) cell and LAK+epirubicin-HCl (2.5LAK+epirubicin-HCl(1/10IC50)/Hep G2 cell) treated Hep G2 cells and untreated Hep G2 cells as control with appropriate antibodies revealed bands of the expected molecular weights in each case (Figure 3). Expression of NADPH-CYP450 reductase increased in Hep G2 cells treated with LAK and LAK+epirubicin-HCl in 24 hours incubation period in respect to untreated Hep G2 cells (p<0.01). It can come from free radical production of LAK and LAK+epirubicin-HCl in Hep G2 cell. We found no the statistically significant change of P-gp expression between the treated and untreated Hep G2 cells (P < 0.01).
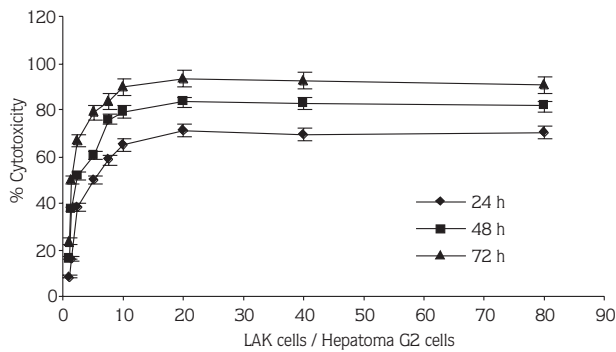
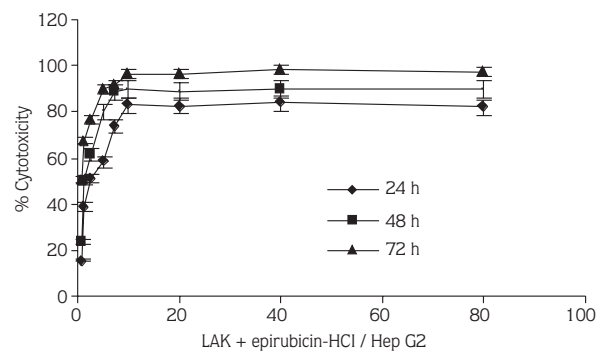Figure 1: Cytotoxicity of LAK cells on Hep G2 cells.

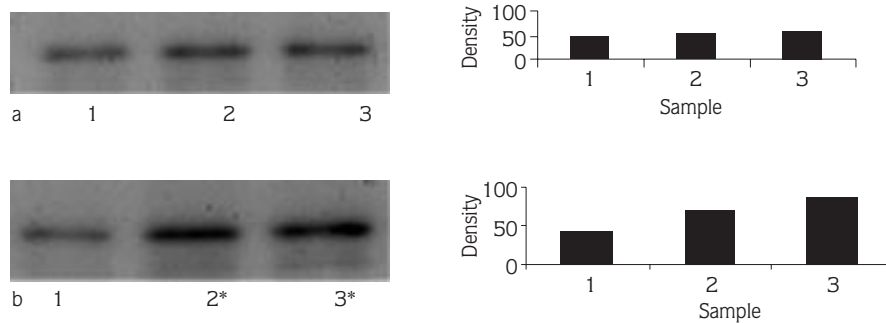Figure 2. Cytotoxicity of LAK+epirubicin-HCl (1/10 IC50) on Hep G2.

Figure 3. Effects of LAK and LAK + Epirubicin-HCl on P-gp (a) and NADPH-CYP 450 reductase (b)
expression in Hep G2 cells.
1; Control, 2; 5LAK/Hep G2 cell 3; 2.5LAK+epirubicin-HCl (1/10 IC50)/Hep G2 cell
*, values are statistically different from control (p<0.01) according to densitometric analysis

Discussion

In a recent paper, adoptive immunotherapy using LAK cells was proved to be benefical to patients survival (28). LAK cells induced NO synthesis in DLD-1 colon cancer cells in a dose dependent manner (3). On the other hand, NO destroys tumor cells by inhibiting the energy-producing Krebs cycle and electron transport activities as well as DNA synthesis(4). Epirubicin-HCl is an antracycline analogue which may go under one electron reduction through a metabolic activation due to NADPH-cytochrome P-450 reductase or other flavin containing enzyme and produce reactive reduced oxygen species such as superoxide anion, hydroxyl radical, and hydrogen peroxide (29).

LAK has cytotoxicity on Hep G2 cells with different E:T ratios and time-intervals (Table 1). The treatment of Hep G2 cells with LAK+epirubicin-HCl (1/10 IC50) combination is more effective than Hep G2 cells treated with LAK alone on cytotoxicity. The cytotoxicity of LAK and LAK+epirubicin-HCl (1/10 IC50) on Hep G2 cells was changed when the cells were exposed to different E:T ratios- and time-intervals (p<0.05). Increasing E:T ratios and incubation time caused increasing cytotoxicity to a certain extent (Table 1, 2). In Kamatsu and Ishigura' study, LAK cells can kill target cells not only early killing but also by late killing, and the late killing level against autotumour cell line (30). The considerably increased NK and LAK activity was seen in a human patient treated for generalized breast carcinoma with doxorubicin-PHPMA-IgG.(31). In one study, docetaxel treatment of HT-29 colon carcinoma cells reinforced the adhesion and immunocytotoxicity of peripheral blood lymphocytes in vitro (32). In another study, cisplatin induced Fas expression in oesophageal cancer cell lines and enhanced cytotoxicity in combination with LAK cells (33). Also, the studies on low dose anticancer agents taxol, cis-diamminedichloroplatin and 5-fluorouracil treated ovarian carcinoma cell line results indicated that some low dose anticancer agents can increase the sensitivity of

cancer cells to LAK cells and it would be useful in clinical practice (34). Cis-diamminedichloroplatin and 5-fluorouracil were indicated as potent inducers of cytokines and natural killer cell activity in vivo and in vitro (35).

Hep G2 cells treated with LAK with IC50 value of 5LAK/Hep G2 at 24 hours and enzyme activities were measured in the LAK treated Hep G2 cells and untreated Hep G2 cells as control for comparison (Table 3). Statistical analysis using student's t-test revealed significantly higher activity in LAK treated hep G2 cells than in untreated cells for Se-dependent glutathione peroxidase, Cu,Zn-superoxide dismutase and Mn-superoxide dismutase enzymes tested ($P < 0.01$). On the other hand, LAK+epirubicin-HCl (1/10IC50) treated Hep G2 cells have increasing activity of catalase, Se-dependent glutathione peroxidase, Cu,Zn-superoxide dismutase and Mn-superoxide dismutase enzymes compared to untreated Hep G2 cells ($p<0.01$). The treatment of Hep G2 cells with LAK(2.5 LAK/Hep G2)+epirubicin-HCl (1/10 IC50) in combination is more effective than Hep G2 cells treated with LAK(5/1) alone on generating free radicals. The combined treatment makes Hep G2 more sensitive to LAK cells. A nonpeptidyl mimic of superoxide dismutase, M40403, increased LAK cell cytotoxicity in vitro and in vivo (36). M38K cells treated with epirubicin had elevated glutathione peroxidase activity (37). Apoptosis of squamous cell carcinoma cells was enhanced by interleukin-2-activated cytotoxic lymphocytes combined with anticancer drugs and also LAK cells increased intracellular reactive oxygen intermediates (ROI) level and induced a decrease of mitochondrial membrane potential (38).

Doxorubicin, epirubicin-HCl and, idarubicin are substrates of P-gp. P-gp mediated drug-resistant cells exhibit elevated levels of drug resistance to vinca alkaloids and anthracyclines, colchicine and actinomycin D (39). The treatment with cytotoxic drugs appears to induce minimal expression of Pg-p in ovarian cancer (40). In our study, we couldn't find any significant differences for P-gp expression in LAK and LAK+epirubicin-HCl treated and untreated Hep G2 cells (Figure 3a). NADPH-CYP450 reductase band density increased in Hep G2 cells treated with LAK and LAK+epirubicin-HCl in 24 hours incubation in comparison to untreated Hep G2 cells (Figure 3b) ($p<0.01$).

In human hepatocytes detoxifying pathways remain to be characterised. Nevertheless, cultures of human hepatocytes are already used as a tool to evaluate the potential of novel drugs to induce cytochrome P450 and glutathione transferase activities (41). A comparable cytotoxicity was observed for the three investigated chloroacetanilide herbicides in both the rat and the human cell lines. Cytochrome P450-dependent enzyme activities increased to different degrees, and phase II GST activity increased in Fa32 (rat hepatoma) but not in hep G2 (human hepatoma) cells (42).

We can conclude that LAK generate reactive oxygen species as the epirubicin-HCl. Because, preincubation of Hep G2 cell with SOD prevented increasing free radical generation and cytotoxicity in Hep G2 cell. Increasing activities of Se-GPx, Cu,Zn-SOD and Mn-SOD and the expression of NADPH-CYP 450 reductase in Hep G2 cells treated with LAK and LAK+epirubicin-HCl(1/50 IC50) seem to be major mediators of epirubicin-HCl and LAK. LAK and Epirubicin-HCl combining treatment made Hep G2 cells more sensitive to LAK cells than LAK alone. Presence of P-gp in Hep G2 cells at the start of therapy may contribute to the inadequate response to anticancer drugs. Combining therapy (chemotherapy + immunotherapy) may become more effective than chemotherapy alone for treatment of drug resistance cancers. These results may lead to new insight on combining immunotherapy with chemotherapy.

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