Effect of high cell density on the growth properties of tumor cells: a role in tumor cytotoxicity of chemotherapeutic drugs

Vivek Singh and Sukh Mahendra Singh

The aim of this study was to understand the role of tumor progression in the growth properties of tumor cells and their susceptibility to the cytotoxicity of chemotherapeutic drugs. A murine transplantable T cell lymphoma of spontaneous origin, designated as Dalton's lymphoma, was used as a model tumor for this investigation. Tumor cells were harvested from the early (5 days after tumor transplantation) and late tumor-bearing stages (17 days after tumor transplantation), with or without in-vivo administration of the chemotherapeutic drugs, cisplatin or doxorubicin. Tumor cells harvested at the late tumor-bearing stages showed a higher proliferative ability *in vitro*. Tumor progression was found to be associated with a decline in the tumor cytotoxicity of the chemotherapeutic drugs. Similar results were also obtained when tumor cells were cultured at low (10^5 cells/ml) and high (10^9 cells/ml) cell densities *in vitro* in medium alone or in one containing the chemotherapeutic drugs. An increase in the expression of heat shock protein (Hsp70), vascular endothelial growth factor, interleukin-2 receptor and interleukin-2 proteins along with an inhibition in the expression of caspase-activated DNase and p53 proteins was observed during the late tumor-bearing stage and also in the Dalton's lymphoma cells when cultured *in vitro* at a higher cell density. The ascitic fluid obtained from the late tumor-bearing stage and the culture supernatant of tumor cells incubated *in vitro* at high cell density showed high levels of cell growth-regulating cytokines: interleukin-1, interleukin-2, interferon-γ, vascular endothelial growth factor, tumor growth factor-β and interleukin-10. In-vivo administration of cisplatin in tumor-bearing mice at the late tumor-bearing stage did not alter the level of these cytokines in the ascitic fluid. In view of the results of this investigation, it is suggested that under high cellular density-associated environmental conditions the tumor cells alter their growth properties depending on an alteration in the expression of cell growth and apoptosis-regulating proteins. Tumor cells, thus, switch to a high level of proliferation, which renders them resistant to the cytotoxicity of chemotherapeutic drugs. *Anti-Cancer Drugs* 18:1123–1132 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:1123–1132

Keywords: apoptosis, cisplatin, doxorubicin, proliferation, T cell lymphoma

School of Biotechnology, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Correspondence to Professor Sukh Mahendra Singh, PhD, School of Biotechnology, Banaras Hindu University, Varanasi 221 005, Uttar Pradesh, India
Tel: +91 542 2368331; fax: +91 542 2368693;
e-mail: sukhmahendrasingh@yahoo.com

Received 21 February 2007 **Revised form accepted** 25 June 2007

Introduction

Tumor progression, with respect to many types of tumors, has been demonstrated to be associated with a concomitant evolution of tumor subclones, which possess a better capability to tolerate stressful environmental conditions associated with the late tumor-bearing stage [1–4]. The molecular mechanism underlying the emergence of such 'better-adapted' tumor cells under stressful environmental conditions remains largely unclear and is likely to vary in a tumor-specific manner. Moreover, it also needs to be understood if such adaptive responses can aid in the survival of tumor cells by antagonizing the induction of apoptosis and by acquiring a higher proliferative ability, both of which contribute to a rapid tumor growth. It, nevertheless, also remains unclear whether such adaptive responses of cancer cells during the late tumor-bearing stages render protection to the tumor cells against the cytotoxicity of anticancer drugs and the host's antitumor immune responses. Therefore, to optimize the efficacy of antitumor therapies, it will be essential to understand the mechanisms of such stress responses of tumor cells in a tumor-specific manner.

Despite the fact that tumors of lymphocytic origin are some of the most complicated tumors for clinical management [5–7], little is known regarding their adaptability to high cell density-dependent environmental conditions. Studies using nonlymphoid tumor cells have, however, suggested that under stress conditions an altered expression of heat shock proteins (Hsps), and of those regulating apoptosis, angiogenesis and cell cycle is observed [8–12]. Not much is, however, understood even in the case of nonlymphoid tumor cells regarding high cell density-associated stress adaptations.

It has been observed that spontaneous animal tumors mimic human malignancy most closely; therefore, they serve as ideal tumor models for various investigative purposes to understand host–tumor relationships [13]. Therefore, our laboratory has been engaged in the understanding of host–tumor relationships with respect to the progressive growth of a transplantable T-cell lymphoma of spontaneous origin, designated as Dalton's lymphoma (DL). DL is a T cell tumor that originated in the thymus of the DBA strain (H-2^d) of mice [14,15]. DL

can grow both in the form of an ascite or a solid tumor and has been reported to possess chromosomal aberrations [16]. Similar to some lymphomas and leukemias of human origin, DL cells do not metastasize to other lymphoid organs [17]. During the course of our previous investigations, we observed that the progression of the ascitic growth of DL is rapid in syngenic BALB/c (H-2^d) mice, causing the death of the host in a relatively shorter time. Further, DL growth has been shown to cause thymus regression, and to modulate macrophage and T cell-mediated immune responses associated with an alteration of the T helper 1/2 cytokine balance [17,18]. Recently, we have demonstrated that the interactions of immune, endocrine and nervous systems play a role in determining the host–tumor relationship in mice bearing DL [16,19,20].

In view of the aforesaid observations on the host–tumor relationship in a lymphoma-bearing host, in this study we investigated the effect of high cell density on the growth properties of T cell lymphoma. Further, we also investigated whether such high cell density-dependent altered growth properties of tumor cells provide them survival benefits against the cytotoxic action of anticancer drugs. In our investigation, we used the cancer chemotherapeutic drugs cisplatin and doxorubicin as representative anticancer drugs for tumoricidal activity. Both these drugs have been shown to have a wide spectrum of anticancer activity [21,22].

To the best of our knowledge this is the first report of its kind showing that lymphoma cells when grown under higher cell density conditions evolve into a population with a higher proliferative ability and a decreased rate of cell death; this plays a role in making these tumor cells resistant to the cytotoxic action of the anticancer drugs, cisplatin and doxorubicin. The study also explores the possible mechanisms underlying this phenomenon.

Materials and methods

Mice and tumor system

Pathogen-free inbred adult mice of the BALB/c (H-2^d) strain were used at 8–12 weeks of age. The mice received food and water *ad libitum*, and were treated with utmost human care in an approved and certified animal room facility of the Banaras Hindu University at the Institute of Medical Sciences. DL was being maintained in the ascitic form by serial transplantations in BALB/c mice or in an in-vitro cell culture system by serial passage. Irrespective of whether the DL cells were obtained from the in-vitro culture system or from the ascitic fluid they exhibited similar phenotypic features. A stock of DL cells was also maintained in a cryopreserved state for reference. In all the experiments, the cells obtained from the ascitic fluid, in which the yield of DL cells was higher, were used. Mice were transplanted intraperitoneally with 1×10^5 DL cells/mouse in 0.5 ml of phosphate-buffered saline (PBS), following which the mice normally survive for 20 ± 2days (18).

Reagents

Tissue culture medium RPMI-1640 was purchased from Hyclone (Logan, Utah, USA). The culture medium was supplemented with 20 μg/ml gentamycin, 100 μg/ml streptomycin, 100 IU of penicillin purchased from Himedia (Mumbai, India) and 10% fetal calf serum from Hyclone (Logan, Utah, USA). Antibodies against interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-2 receptor (IL-2R), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β), Hsp70, vascular endothelial growth factor (VEGF), p53 and caspase-activated DNase (CAD) were purchased from Imgenex (San Diego, California, USA) and Chemicon (Chandbers Ford, UK). Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (Bangalore, Karnataka, India). BCIP/NBT was purchased from Amresco (Solon, Ohio, USA).

Preparation of Dalton's lymphoma cell-free ascitic fluid

DL cell-free ascitic fluid was prepared as described earlier [23]. The ascitic fluid of the DL was aspirated by peritoneal lavage after 5 or 17 days following the DL transplantation. The peritoneal exudate cells thus obtained were centrifuged at 200*g* for 10 min at 4°C. The cell-free supernatant was collected and passed through a 0.22-μm membrane filter and stored at −20°C until use.

Protocol for in-vivo treatment

Mice in groups of six each were transplanted intraperitoneally with DL (1×10^5 cells/mouse in 0.5 ml of PBS). The DL cells were harvested from mice 5 and 17 days after the transplantation, henceforth referred to as early and late tumor-bearing stages, respectively. The mice were injected intraperitoneally with 0.5 ml of PBS, alone or containing cisplatin or doxorubicin at a dose of 5 mg/kg body weight, 48 h before the harvesting of the DL cells [24]. A flow chart showing the in-vivo treatment protocol is shown in Fig. 1.

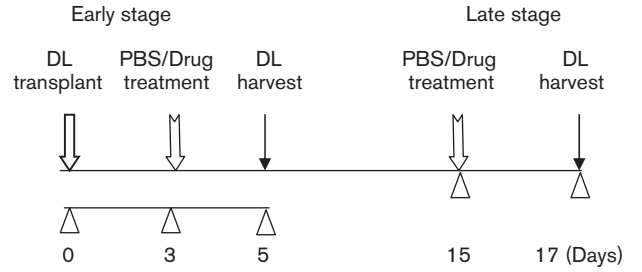
Protocol for in-vitro treatment

The DL cells, obtained from a serially passaged stock, were incubated for 48 h *in vitro* at cell densities of 10^5 and 10^9 cells/ml, henceforth referred to as low and high cell densities, respectively. Cisplatin (10 μg/ml) or doxorubicin (10 μg/ml) was added to the culture media followed by the washing of the cells twice with warm serum-free medium before their further use.

Morphological evaluation of apoptotic Dalton's lymphoma cells

The apoptotic cell population was enumerated following a method described earlier [25]. Cell suspension was

Fig. 1



Experimental protocol for tumor transplantation and in-vivo administration of chemotherapeutics drugs. Diagrammatic representation of the protocol for in-vivo drug administration to Dalton's lymphoma (DL)-bearing mice before the harvesting of DL cells. DL-bearing mice (six in each group) were administered intraperitoneally either 0.5 ml of phosphate-buffered saline (PBS) alone or PBS containing drugs at a dose of 5 mg/kg body weight on the indicated days after DL transplantation, followed by the harvesting of DL cells (on day 5 or 17) (designated as early and late tumor-bearing stages, respectively) for further experiments.

smeared on a glass slide, air-dried, fixed in methanol, stained with Wright staining solution and analyzed under light microscopy (Carl Zeiss, Gottingen, Germany) at $\times$ 400 magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed and densely stained chromatin, and membrane-bound apoptotic bodies containing one or more nuclear fragments. The percentage of apoptotic cells was determined by counting more than 300 cells in at least three separate microscopic fields.

Percent DNA fragmentation

Quantitative determination of DNA fragmentation was carried out following a method given by Sellins and Cohen [26] with slight modifications [24]. DL cells (1×10^6 cells/ml) from tumor-bearing mice or in-vitro culture were lysed in 0.5 ml of Tris–EDTA buffer (pH 7.4) containing 0.2% (v/v) Triton X-100 and the fragmented DNA was separated from intact chromatin in a microfuge tube (labeled as B) by centrifugation at 13 000*g* at 4°C for 10 min. Supernatant containing the fragmented DNA was transferred to another microfuge tube (labeled as T). A volume of 0.5 ml of 25% TCA was added to each T and B tube and vortexed vigorously. DNA was precipitated overnight at 4°C and collected at 13 000*g* at 4°C for 10 min. Supernatant was discarded and 80 µl of 5% TCA was added to each pellet. Heating at 90°C for 15 min hydrolyzed the DNA. At this stage a blank was included containing 80 µl of 5% TCA. Then 160 µl of freshly prepared diphenylamine reagent (150 mg of diphenylamine in 10 ml of glacial acetic acid, 150 µl of concentrated H_2SO_4 and 50 µl of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop color. Later 100 µl of this colored solution was transferred to the wells of a 96-well flat-bottomed enzyme-linked immunosorbent assay (ELISA) plate and absorbance was read at 600 nm in a microtiter ELISA plate reader (Labsystems, Helsinki, Finland). The percentage of DNA fragmentation was calculated as:

$$\text{DNA fragmentation}\,(\%) = [\mathrm{T}/(\mathrm{T}+\mathrm{B})] \times 100.$$

SDS–PAGE and Western immunoblot analysis

DL cells were washed with chilled PBS and then lysed in 50 µl of lysis buffer [20 mmol/l Tris–HCl (pH 8.0), 137 mmol/l of NaCl, 10% v/v glycerol, 1% (v)/v Triton X-100, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride and 20 µmol/l leupeptin containing aprotinin at 0.15 U/ml] for 20 min at 4°C. Protein content in each sample was determined by using the standard Bradford method [27]. Thirty micrograms of Triton X-100-solubilized proteins was separated on 10% SDS–polyacrylamide gel at 20 mA as described by Davis [28]. The gel was either stained with Comassie Brilliant Blue (R250) for analysis of proteins bands or processed further for Western immunoblotting. The separated proteins were transferred onto a nitrocellulose membrane (Sartorius, Gottingen, Germany) (1.5 h at 150 mA), immunoblotted with antibodies against indicated proteins and probed with a secondary antibody, antirabbit IgG, conjugated to alkaline phosphatase and detected by a BCIP/NBT solution (Amresco). The blotting membrane was striped and reprobed with anti-β-actin primary and secondary antibody, and detected with the BCIP/NBT solution for equal loading of protein.

Enzyme-linked immunosorbent assay for detection of proteins in Dalton's lymphoma cell-free ascitic fluid and Dalton's lymphoma cell culture supernatant

A standard ELISA was performed to detect the presence of IL-1, IL-2, interleukin-10 (IL-10), TGF-β and VEGF in ascitic fluid of tumor-bearing mice or supernatant of in-vitro culture following a method described earlier [29]. Briefly, polystyrene microwell plates (Tarsons, Kolkata, West Bengal, India) were coated with 10 µg of cell lysate protein and incubated overnight at 4°C. In the negative control test, samples were not added to the wells of ELISA plates and the plate was processed for subsequent steps in the same ways as described for experimental sets. The plates were then washed with 0.15 mol/l PBS containing 0.1% Tween-20 (PBS–Tween). Unbound sites were saturated with PBS containing 1% bovine serum albumin. The plates were again washed with PBS–Tween followed by the addition of antibodies against the indicated proteins at a dilution of 1 : 1000. The plates were incubated at 37°C for 60 min followed by the addition of 50 µl of *p*-nitrophenyl phosphate (1 mg/ml in enzyme substrate buffer). The absorbance was measured after 10 min at 405 nm in an ELISA plate reader (Labsystems). ELISA for cytokines was compared with

the standard preparation of the respective cytokines obtained from the National Institute for Biological Standards and Control (Potters Bar, UK).

Assay for tumor cell survival and proliferation

Cell proliferation of DL cells was estimated by an enumeration of viable cells using a standard Trypan blue dye exclusion test followed by calculation of specific growth rate following a method described by Kim *et al.* [30]. An estimation of cell survival and drug cytotoxicity was also carried out by a standard MTT assay [31] described below:

$$\mu = 1/x(\mathrm{d}x/\mathrm{d}t)h^{-1},$$

where μ = specific growth rate and x = initial cell number.

MTT assay

MTT assay was carried out to estimate DL cell survival following a method described by Mosmann [32]. MTT was dissolved in PBS at a concentration of 5.0 mg/ml. Fifty microliters of MTT solution was added to each well of the culture plate containing 200 µl of medium and incubated at 37°C for 4 h. The medium then was removed carefully without disturbing the dark-blue formazan crystals. Fifty microliters of dimethylsulfoxide was added to each well and mixed thoroughly to dissolve the formazan crystals. The plates were then read on a microplate reader (Labsystems) at a wavelength of 570 nm. Readings were presented as optical density at 570 nm.

Statistical analysis

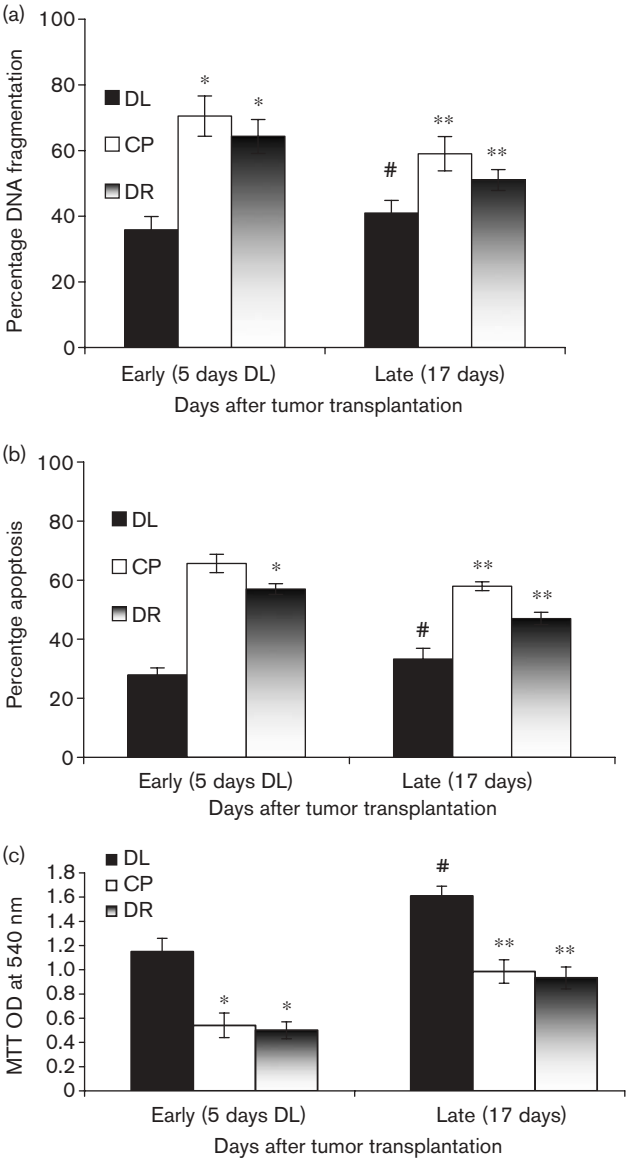
All experiments were conducted in triplicate at least three times. The statistical significance of differences between test groups was analyzed by one-way ANOVA using all pairwise multiple comparison procedures (Duncan's method). The difference was considered significant when P was less than 0.05. The level of variation in ELISA for cytokines was determined by calculating the coefficient of variation, which is defined as the relevant SD divided by the overall mean and expressed as a percentage.

Results

Effect of tumor growth on the tumoricidal activity of chemotherapeutic drugs, cisplatin and doxorubicin

DL cells (1×10^5) obtained from the DL-bearing mice with or without administration of cisplatin or doxorubicin as shown in Fig. 1 were processed for the estimation of the percentage of specific DNA fragmentation (Fig. 2a) and enumeration of the percentage of apoptotic cells (Fig. 2b). A significant increase in the population of apoptotic cells along with an increase in percentage of specific DNA fragmentation was observed in the DL cells obtained from the late tumor-bearing stage compared with that observed in the case of the DL cells of the early tumor-bearing stage (Fig. 2a and b). In-vivo administration of cisplatin (5 mg/kg body weight, a dose determined to be optimum in earlier experiments) or doxorubicin (5 mg/kg body weight) resulted in an augmentation in the percentages of apoptotic cells and of DNA fragmentation

Fig. 2



Effect of tumor growth and in-vivo administration of cisplatin (CP) and doxorubicin (DR) on the proliferative ability and induction of apoptosis in tumor cells. Tumor cells harvested from tumor-bearing mice on the indicated days after tumor transplantation and drug administrations, as described in Materials and methods (Fig. 1), were processed for the estimation of specific DNA fragmentation (a), enumeration of percentages of apoptotic tumor cells (b) and cell proliferation (c). Values shown are mean ± SD of three independent experiments done in triplicate. #*P<0.05 vs. values for tumor cells obtained from early tumor-bearing stage. **P<0.05 vs. values for tumor cells obtained from late tumor-bearing stage. DL, Dalton's lymphoma; OD, optical density.

in the DL cells obtained from both early and late tumor-bearing stages. This augmentation was, however, significantly lower in the DL cells obtained from the late tumor-bearing stage compared with that in the DL cells obtained from the early tumor-bearing stages. Similarly the proliferative ability of the DL cells obtained from the early and late tumor bearing stages, with or without in-vivo administration of cisplatin or doxorubicin, was also checked by the MTT assay (Fig. 2c), the counting of viable cells and the estimation of specific growth rate (Table 1). The proliferative ability of the DL cells obtained from the late tumor-bearing stages was significantly higher compared with that of cells from early tumor-bearing stages. In-vivo administration of cisplatin or doxorubicin causes up to a 2.1-fold decrease in the survival of DL cells obtained from early tumor-bearing stages compared with the approximately 1.7-fold inhibition in those from the late tumor-bearing stage (Fig. 2c and Table 1). Specific growth rate was also found to show a similar trend (Table 1).

Effect of in-vitro incubation of tumor cells at different cell densities and treatment with cisplatin and doxorubicin on tumor cell growth

To understand the mechanism of the differential rate of apoptosis and proliferation in DL cells at the early and late tumor-bearing stages, and their differential susceptibility to chemotherapeutic drugs, DL cells were incubated *in vitro* at the indicated cell densities (Fig. 3) in medium alone or in medium containing cisplatin (10 μg/ml) or doxorubicin (10 μg/ml) for 48 h, followed by the equalization of cell numbers and the estimation of percentages of DNA fragmentation (Fig. 3a) and cell proliferation (Fig. 3c) along with an enumeration of the percentage of apoptotic cells (Fig. 3b). Induction of apoptosis was found to be significantly higher in DL cells when incubated at low cell density *in vitro* compared with those incubated at a high cell density (Fig. 3a and b), whereas the proliferative ability was found to be lower (2.2-fold) when DL cells were incubated at low cell density compared with those at high cell density (Fig. 3c, Table 2). Addition of cisplatin or doxorubicin to the culture medium during the initial 48 h of in-vitro incubation resulted in an increase in apoptosis (1.6-fold at low density and 1.9-fold at higher density) and inhibition of proliferation (1.8-fold at low density and 1.3-fold at high density).

Effect of tumor growth on the expression of interleukin-2 receptor, heat shock protein 70, vascular endothelial growth factor, p53 and caspase-activated DNase proteins in tumor cells

Expression of IL-2R, Hsp70, VEGF, p53 and CAD proteins in DL cells obtained from early and late tumor-bearing stages was analyzed by immunoblotting (Fig. 4). Expression of IL-2R, Hsp70 and VEGF proteins was found to increase, whereas the expression of p53 and CAD proteins decreased in DL cells obtained from late tumor-bearing stage.

Effect of in-vitro incubation of tumor cells at different cell densities on the expression of caspase-activated DNase, p53, heat shock proteins 70, interleukin-2 receptor and vascular endothelial growth factor proteins

DL cells were incubated *in vitro* at low (10^5 cells/ml) or high (10^9 cells/ml) cell densities for 48 h followed by equalization of cell numbers and estimation of the expression of IL-2R, VEGF, Hsp70, p53 and CAD proteins by immunoblotting (Fig. 5a and b). Expression of p53 and CAD proteins was found to decrease, whereas that of VEGF, Hsp70 and IL-2R increased in DL cells incubated at high cell density compared with those at low cell density.

In-vitro cell density-dependent alteration in the secretion of interleukin-2, vascular endothelial growth factor and tumor growth factor-β by lymphoma cells

DL cells were incubated *in vitro* at low (10^5 cells/ml) or high (10^9 cells/ml) cell densities for 24 h followed by equalization of cell numbers and further incubation for 48 h. The culture supernatant was then harvested and assayed by ELISA to detect the presence of IL-2, VEGF and TGF-β (Fig. 6). The levels of all these proteins were

Table 1 Effect of tumor growth and in-vivo administration of cisplatin and doxorubicin on the proliferative ability and specific growth rate of tumor cells

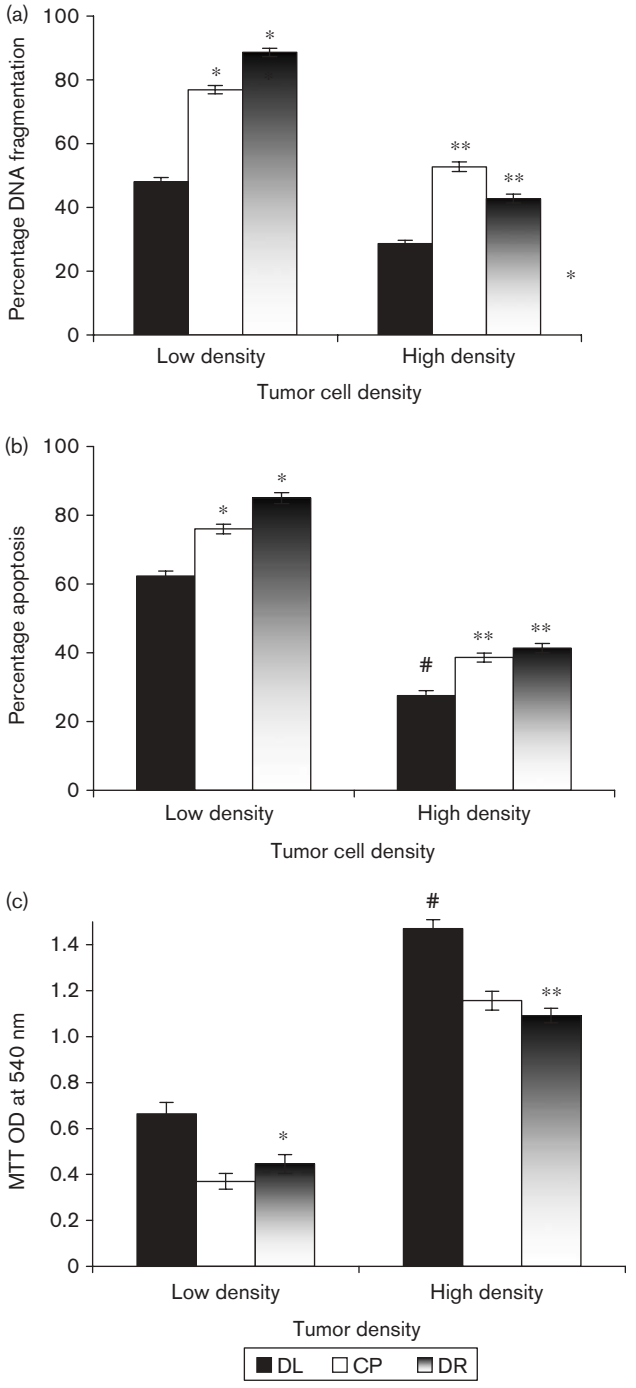
	In-vivo treatment of tumor-bearing mice					
	Early DL (5 days)			Late DL (17 days)		
	Without drugs	Cisplatin-treated	Doxorubicin-treated	Without drugs	Cisplatin-treated	Doxorubicin-treated
Cell count after 48 h of culture ($\times 10^5$ cells/ml)	102.4 ± 2.2	5.4 ± 1.4	72.8 ± 3.2	114.4 ± 2.6	55.2 ± 3.1	102.8 ± 2.4
Specific growth rate (h^{-1})	2.11 ± 0.02	0.092 ± 0.008*	1.50 ± 0.03*	2.36 ± 0.02	1.13 ± 0.03**	2.12 ± 0.03**

Tumor cells harvested from the tumor-bearing mice on the indicated days after tumor transplantation and drug administration were incubated for 48 h *in vitro* as described in Materials and methods followed by estimation of cell count and specific growth rate. Values shown are mean ± SD of three independent experiments done in triplicate. DL, Dalton's lymphoma.

*$P < 0.05$ vs. values for tumor cells obtained from early tumor-bearing stage without drug treatment.

**$P < 0.05$ vs. values for tumor cells obtained from late tumor-bearing stage without drug treatment.

Fig. 3



Effect of in-vitro incubation of tumor cells at different cell densities on the proliferation ability and induction of apoptosis. (a–c). In-vitro serially passaged tumor cells were incubated *in vitro* at low (10^5 cells/ml) and high (10^9 cells/ml) cell densities and treated with cisplatin (CP, 10 μg/ml) or doxorubicin (DR, 10 μg/ml) as described in Materials and methods, before the estimation of percentages of specific DNA fragmentation (a), apoptotic of tumor cells (b), and cell proliferation (c). Values shown are mean ± SD of three independent experiments done in triplicate. *#$P<0.05$ vs. values for tumor cells cultured at low density. **$P<0.05$ vs. values for tumor cells cultured at high density. DL, Dalton's lymphoma; OD, optical density.

found to increase approximately 2.9, 2.8 and 3.2 times, respectively, in the culture supernatant of DL cells incubated at high cell density compared with that in the DL cells incubated at lower cell density.

Effect of progressive tumor growth on the level of interleukin-1, interleukin-2, interferon-γ, vascular endothelial growth factor, tumor growth factor-β and interleukin-10 in the ascitic fluid of tumor after drug treatment

The level of cytokines, i.e. IL-1, IL-2, IFN-γ, VEGF, IL-10 and TGF-β, in the ascitic fluid of DL-bearing mice that were obtained from early and late tumor-bearing stages was estimated by ELISA. The levels of all of these cytokines were found to be significantly higher in the ascitic fluid of late tumor-bearing stage compared with that of the early stage; however, their levels declined significantly following in-vitro treatment with cisplatin (Fig. 7) and doxorubicin (results not shown).

Discussion

The results of this study suggest that tumor cells show a differential susceptibility to killing by antitumor drugs cisplatin and doxorubicin depending on the stage of tumor growth. Tumor cells obtained from the late tumor-bearing stages are more resistant to the cytotoxic action of antitumor drugs compared with those obtained from the early tumor-bearing stages. The precise mechanisms underlying the differential cytotoxic actions of cisplatin and doxorubicin depending on the stage of tumor growth remain unclear; however, some of the possibilities were considered. The differential cytotoxic responses of tumor cells to the anticancer drugs could be due to an altered growth potential of the tumor cells at the later stages of tumor progression. Indeed, our results demonstrate that tumor cells obtained from the late tumor-bearing stages show a higher proliferative ability compared with those from the early tumor-bearing stages. This could be owing to a major fraction of the tumor cell population at the late tumor-bearing stages switching to the proliferative pathway of the cell cycle and consequently showing an increased resistance to induction of apoptosis. We also observed, however, that although the tumor cells from late tumor-bearing stages showed a higher proliferative ability, the percentage of apoptotic cells increased concomitantly. This increase in the percentage of apoptotic tumor cells at the late stage could also be attributed to an increased crowding of tumor cells, leading to an accumulation of proapoptotic factors. Indeed, other workers have shown that during tumor progression, an increased cell death of tumor cells is observed owing to increased hypoxic conditions [33].

To understand the role of high cell density in altering the growth potential of tumor cells during the late

Table 2 Effect of in-vitro incubation of tumor cells at different cell densities on the proliferative ability and specific growth rate

	In-vitro treatment of tumor cells					
	Low density			High density		
	Medium	Cisplatin-treated	Doxorubicin-treated	Medium	Cisplatin-treated	Doxorubicin-treated
Cell count after 48 h of culture ($\times 10^5$ cells/ml)	11.5 ± 1.7	0.15 ± 0.02	4.5 ± 01.0	56 ± 3.2	4.2 ± 0.8	9.5 ± 1.1
Specific growth rate (h^{-1})	0.22 ± 0.02	(−) 0.018 ± 0.004*	0.073 ± 0.01*	1.15 ± 0.03	0.067 ± 0.007**	0.177 ± 0.01**

In-vitro serially passaged tumor cells were incubated *in vitro* at low (10^5/ml) and high (10^9/ml) cell densities and treated with cisplatin (10 μg/ml) or doxorubicin (10 μg/ml) followed by enumeration of the cell number and calculation of specific growth rate as described in Materials and methods. Values shown are mean ± SD of three independent experiments done in triplicate.
*$P<0.05$ vs. values for tumor cells cultured at low density in medium alone without drugs.
**$P<0.05$ vs. values for tumor cells cultured at high density in medium alone without drugs.

tumor-bearing stages, in-vitro simulating experiments were carried out. DL cell grown *in vitro* at a high cell density showed an increased proliferative ability; however, apoptosis was found to decrease. It is thus likely that when growing at a high cellular density, the lymphoma cells undergo a phenotypic change by the 'switching on' of the pathways leading to an upregulation of cell proliferation. Further, the discrepancy between in-vivo and in-vitro results could be attributed to the differences in the environmental conditions pertaining to the in-vitro and in-vivo growth of tumor cells. In the in-vivo conditions, a more heterogeneous internal environment exists in the vicinity of tumor compared with a more homogenous culture condition *in vitro*. Indeed, we have previously shown that during late tumor-bearing stages *in vivo*, an increased influx of tumor-associated macrophages (TAM) into the growing tumor mass helps in tumor progression [24]. In contrast, in-vitro culture of tumor cells did not result in an influx of TAM. Our previous observations have shown that DLs infiltrating the TAM produce enhanced amounts of IL-1, which plays an important role in augmentation of tumor growth [16,24]. The ascitic fluid of late tumor-bearing stage, nevertheless, showed an enhanced level of IL-1 and IFN-γ [34]. IFN-γ has been reported to activate macrophages to produce enhanced amounts of IL-1 [35].

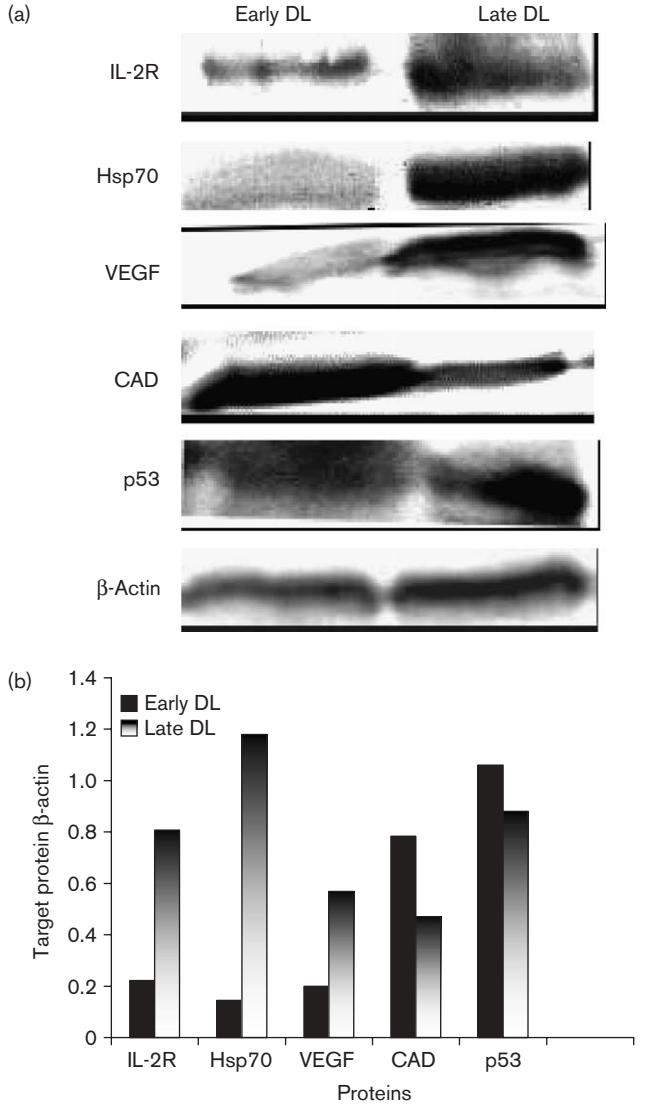
The role of cytokines and other cell growth-regulating proteins that contribute to the differential growth properties of tumor cells was also investigated. Indeed, we found an increase in the expression of Hsp70, VEGF and IL-2R in the tumor cells incubated under high density *in vitro* or late stage of tumor growth *in vivo*, along with a decrease in the expression of CAD and p53 proteins. Hsp70, VEGF and IL-2R have been reported to regulate cell division and apoptosis in a variety of cells [12,36]. IL-2 and IL-2R are, nevertheless, considered to be one of the key regulators of T lymphocyte proliferation [37]. VEGF has, nevertheless, been reported to augment the growth of several types of tumor cells and to antagonize the induction of apoptosis [38]. Moreover, Hsp70 expression is reported to increase in tumor cells under conditions like hypoxia, nutrition depletion and oxidative stress [39]. Studies have shown that Hsp70 plays an indispensable role in generating a protective environment for tumor cells under stressful conditions, thereby augmenting the survival of tumor cells [40]. It is thus possible that DL cells during the late tumor-bearing stages were triggered to express Hsp70, which could in turn be one of the key factors in generating tumor growth-promoting environment. Further studies will, however, be required to establish a link between Hsp70 expression and the modulation of other tumor growth-regulating mechanisms observed in our system.

The above suggestions are also corroborated by the results showing that the ascitic fluid as well as the culture supernatant of lymphoma-bearing mice showed elevated levels of IL-1, IL-2, IFN-γ, IL-10, VEGF and TGF-β, which have been demonstrated to not only augment the proliferative ability of lymphocytes, but also to play a protective role in antagonizing the induction of apoptosis [41–43]. These cytokines could be of both tumor and/or host origin [44].

Further, this study also indicates that tumor cells under a high cell density environment *in vitro* and *in vivo*, owing to their expression of a unique repertoire of cell growth-promoting cytokines, acquire an ability to resist the cytotoxicity of chemotherapeutic drugs. This might also suggest a novel mechanism about how tumor cells during tumor progression can acquire multidrug resistance, despite the fact that cisplatin and doxorubicin induce tumor cell death through independent pathways of cytotoxicity [45]. In-vitro administration of cisplatin could not lower the level of tumor growth-promoting cytokines to the control level, indicating that these cytokines can indeed play a role in the resistance of tumor cells against the cytotoxicity of antitumor drugs.

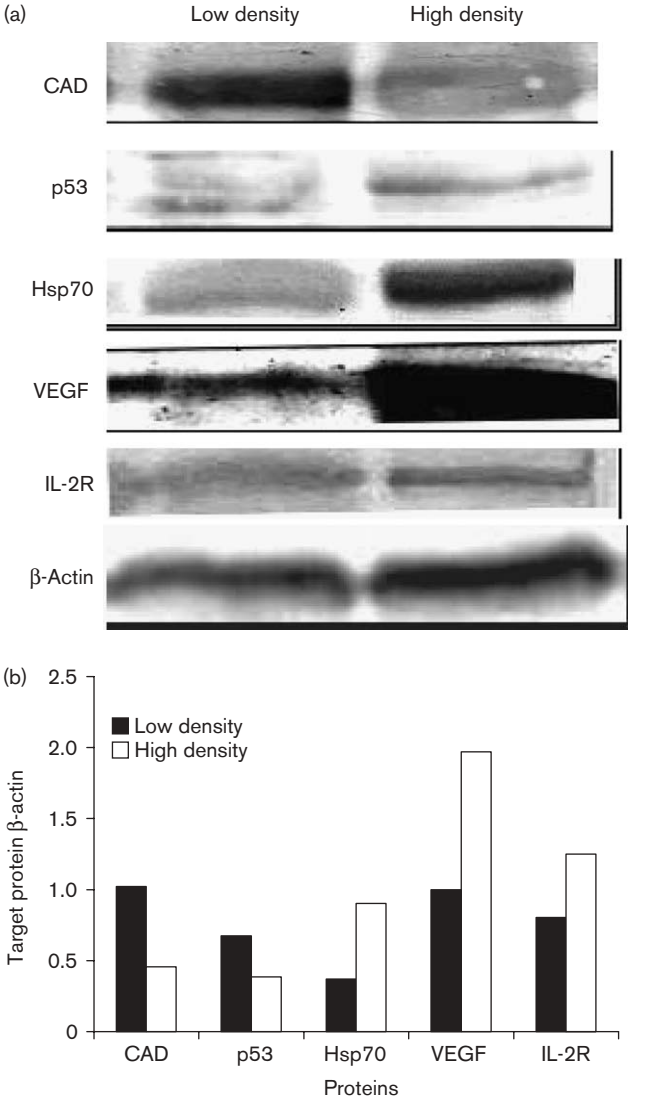
Taken together, the observations of this study indicate for the first time that lymphoma cells, during a progressive tumor growth, undergo genetic and phenotypic

Fig. 4



Progressive tumor growth alters the expression of cell cycle-regulating and apoptosis-regulating proteins. (a) Tumor cells harvested from tumor-bearing mice at early and late tumor-bearing stages were lysed and the lysates were immunoblotted for detection of interleukin-2 receptor (IL-2R), heat shock protein (Hsp70), vascular endothelial growth factor (VEGF), caspase-activated DNase (CAD) and p53 proteins as described in Materials and methods. Results shown are from representative experiments out of three independent experiments done in triplicate with similar results. Equal loading of proteins was checked by detection of the expression of β-actin. (b) Densiometric scan of the immunoblots shown in (a), showing relative intensity of different bands (target protein: β-actin). DL, Dalton's lymphoma.

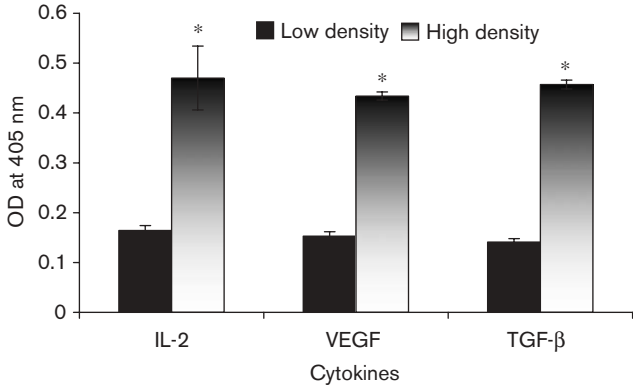
Fig. 5



Effect of in-vitro incubation of tumor cells at different cell densities on the expression of caspase-activated DNase (CAD), p53, heat shock proteins (Hsp70), vascular endothelial growth factor (VEGF) and interleukin-2 receptor (IL-2R) proteins. (a) Tumor cells obtained from serially passaged samples were incubated for 48 h *in vitro* at the low (10^5 cells/ml) and high (10^9 cells/ml) cell density followed by equalizing the cell numbers and preparation of lysates for immunodetection of the indicated proteins. Results shown are from representative experiments out of three independent experiments done in triplicate with similar results. Equal loading of proteins was checked by the detection of the expression of β-actin. (b) Densiometric scans of the immunoblots shown in (a), showing relative intensities of different bands (target protein: β-actin).

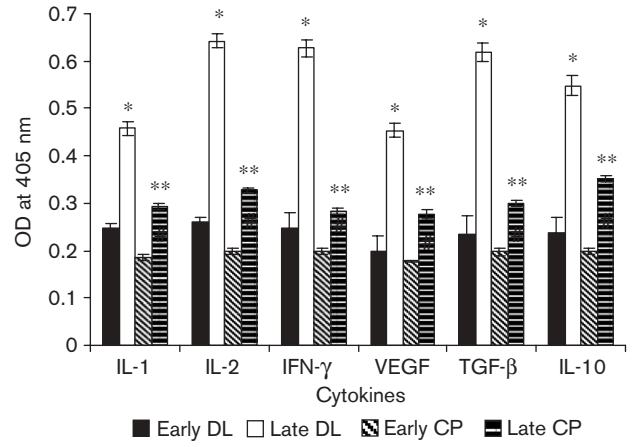
alterations that help them to acquire multiple direct or indirect mechanisms to ensure an unhindered and rapid tumor progression resulting from a 'switching off' of the apoptotis-inducing pathway and a 'switching on' of the pathway promoting cell growth. This might also enable the tumor cells to resist the cytotoxicity of anticancer drugs. These results will thus have long-lasting clinical implications in designing specific approaches to therapeutic strategies during the late tumor-bearing stage. This study will thus have a novel impact on the development of new cancer therapies, depending on the stage of tumor growth.

Fig. 6



In-vitro incubation of tumor cells at high density (10^9 cells/ml) increases the production of interleukin-2 (IL-2), vascular endothelial growth factor (VEGF) and tumor growth factor-β (TGF-β) in the culture supernatant as compared with low density-incubated (10^5 cells/ml) culture. In-vitro serially passaged tumor cells were incubated at the indicated cell densities in the tissue culture flask for 48 h and the culture supernatant (10 μg of protein/well) was plated in 96-well enzyme-linked immunosorbent assay (ELISA) plate for immunodetection of the indicated cytokines. Values shown are mean ± SD from three independent experiments done in triplicate. *$P<0.05$ vs values for tumor cells obtained at lower density. Range of interassay coefficient of variation was 4.09–6.12% and range of intra-assay coefficient of variation was 1.34–7.51%. OD, optical density.

Fig. 7



Effect of progressive tumor growth on the level of cytokines in the ascitic fluid in response to in-vivo administration of cisplatin (CP). Ascitic fluid aspirated from tumor-bearing mice on the indicated days after tumor transplantation with or without cisplatin treatment was plated at a protein concentration of 10 μg/well and processed for detection of the indicated cytokines as described in Materials and methods by enzyme-linked immunosorbent assay (ELISA). Values shown are mean ± SD from three independent experiments done in triplicate. *#$P<0.05$ vs. values for tumor cells obtained from early tumor-bearing stage. **$P<0.05$ vs. values for tumor cells obtained from late tumor-bearing stage. Range of interassay coefficient of variation was 4.09–6.12% and range of intra-assay coefficient of variation was 1.34–7.51%. IFN-γ, interferon-γ; IL-1, interleukin-1; IL-2, interleukin-2; IL-10, interleukin-10; OD, optical density; TGF-β, tumor growth factor-β; VEGF, vascular endothelial growth factor.

Acknowledgements

The authors are grateful to the Department of Biotechnology, Government of India for the research grant to S.M.S and the Junior Research Fellowship support to V.S.

References

1 Klaunig JE, Xu Y, Isenberg JS, Bachowski S, Kolaja KL, Jiang J, *et al*. The role of oxidative stress in chemical carcinogenesis. *Environ Health Perspect* 1998; **106**:289–295.
2 Bishopric NH, Discher DJ, Kaiser S, Hernandez O, Sato B, Zang J, *et al*. Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. *J Clin Invest* 1999; **104**:239–252.
3 Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep* 2002; **3**:420–425.
4 Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. *Blood* 2001; **98**:2603–2614.
5 Zhou J, Mauerer K, Farina L, Gribben JG. The role of the tumor microenvironment in hematological malignancies and implication for therapy. *Front Biosci* 2005; **10**:1581–1596.
6 Moll A, Niwald A, Gratek M, Stolarska M. Ocular complications in leukemias and malignant lymphomas in children. *Klin Oczna* 2004; **106**:783–787.
7 Selby P. Acquired resistance to cancer chemotherapy. *Br Med J (Clin Res Ed)* 1984; **288**:1252–1253.
8 Nicholson SE, Metcalf D, Sprigg NS, Columbus R, Walker F, Silva A, *et al*. Suppressor of cytokine signaling (SOCS)-5 is a potential negative regulator of epidermal growth factor signaling. *Proc Natl Acad Sci U S A* 2005; **102**:2328–2333.
9 Mercurio AM, Lipscomb EA, Bachelder RE. Non-angiogenic functions of VEGF in breast cancer. *J Mammary Gland Biol Neoplasia* 2005; **10**: 283–290.
10 French TO, Morelon E. Cancer immunosuppression: pro- and antitumoral effects of immunosuppressive drugs. *Nephrol Ther* 2005; **1**:23–30.
11 Atre N, Thomas L, Mistry R, Pathak K, Chiplunkar S. Role of nitric oxide in heat shock protein induced apoptosis of gammadeltaT cells. *Int J Cancer* 2006; **119**:1368–1376.
12 Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol* 2000; **20**:7146–7159.
13 Ben-Effrain S. One hundred years of cancer immunotherapy: a critical appraisal. *Tumor Biol* 1999; **20**:1–24.
14 Klein G. Comparative studies of mouse tumors with respect to their capacity for growth as 'ascitic tumors' and their average nucleic acid content. *Exp Cell Res* 1951; **2**:518–524.
15 Goldie H, Felix MD. Growth characteristics of free tumor cells transformed serially in the peritoneal fluid of mouse. *Cancer Res* 1951; **11**:73–80.
16 Singh MP, Rai AK, Singh SM. Gender dimorphism in the progressive in-vivo growth of a T cell lymphoma: involvement of cytokines and gonadal hormones. *J Reprod Immunol* 2005; **65**:17–32.
17 Kumar A, Singh SM. Effect of tumor growth on the blastogenic response of splenocytes: a role of macrophage derived nitric oxide. *Immunological Invest* 1996; **25**:413–423.
18 Bharti A, Singh SM. Inhibition of macrophage nitric oxide production by gangliosides derived from a spontaneous T-cell lymphoma: the involved mechanisms. *Nitric Oxide* 2003; **8**:75–82.
19 Singh MP, Sharma H, Singh SM. Prolactin promotes growth of a spontaneous T cell lymphoma: role of tumor and host derived cytokines. *Cancer Invest* 2006; **24**:601–610.
20 Singh MP, Singh G, Singh SM. Paraneoplastic neurodegeneration in a murine host following progressive growth of a spontaneous T-cell lymphoma: role of proinflammatory internal responses. *Neuroimmunomodulation* 2006; **13**:122–132.
21 Nishio S, Katsumata N, Tanabe H, Matsumoto K, Yonemori K, Kouno T, *et al*. A feasibility study of doxorubicin/cisplatin (AP) for postoperative chemotherapy in patients with advanced endometrial cancer. *Gan To Kagaku Ryoho* 2006; **33**:1589–1593.
22 Langer F, Wintzer HO, Werner M, Weber C, Brummendorf TH, Bokemeyer C. A case of pulmonary carcinosarcoma (squamous cell carcinoma and osteosarcoma) treated with cisplatin and doxorubicin. *Anticancer Res* 2006; **26**:3595–3600.
23 Parajuli P, Singh SM, Kumar A. Effect of cisplatin and FK565 on the activation of tumor-associated and bone marrow-derived macrophages by Dalton's lymphoma. *Int J Immunopharmacol* 1995; **17**:1–7.

24 Parajuli P, Singh SM. Alteration in IL-1 and arginase activity of tumor-associated macrophages: a role in the promotion of tumor growth. *Cancer Lett* 1996; **107**:249–256.
25 Bharti A, Singh SM. Induction of apoptosis in bone marrow cells by gangliosides produced by a T cell lymphoma. *Immunol Letters* 2000; **72**:39–48.
26 Sellins KS, Cohen JJ. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol* 1987; **139**:199–206.
27 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem* 1976; **72**:248–254.
28 Davis BJ. Disc electrophoresis – II. Method and application to human serum proteins. *Ann N Y Acad Sci* 1964; **121**:404–414.
29 Ogushi F, Sone S, Singh SM, Tani K, Ozaki T, Honda M. Elevated level of soluble interleukin-2 receptor in bronchoalveolar lavage fluid from asrcoidosis patients. *Jpn J Med* 1991; **30**:113–117.
30 Kim KM, Song JJ, An JY, Kwon YT, Lee YJ. Pretreatment of acetylsalicylic acid promotes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by down-regulating BCL-2 gene expression. *J Biol Chem* 2005; **280**:41047–41056.
31 Khwaja F, Allen J, Lynch J, Andrews P, Djakiew D. Ibuprofen inhibits survival of bladder p75NTR tumor suppressor protein. *Cancer Res* 2004; **64**: 6207–6213.
32 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Meth* 1983; **65**:55–63.
33 Kiang JG, Tsen KT. Biology of hypoxia. *Chin J Physiol* 2006; **49**:223–233.
34 Shanker A, Singh SM, Sodhi A. Impairment of T-cell functions with the progressive ascitic growth of a transplantable T-cell lymphoma of spontaneous origin. *FEMS Immunol Med Microbiol* 2000; **27**:247–255.
35 Mantovani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. *Eur J Immunol* 2007; **37**:14–16.
36 Cabillic F, Bouet-Toussaint F, Toutirais O, Rioux-Leclercq N, Fergelot P, de la Pintiere CT, *et al*. Interleukin-6 and vascular endothelial growth factor release by renal cell carcinoma cells impedes lymphocyte–dendritic cell cross-talk. *Clin Exp Immunol* 2006; **146**:518–523.
37 Yasumura S, Lin WC, Weidmann E, Hebda P, Whiteside TL. Expression of interleukin 2 receptors on human carcinoma cell lines and tumor growth inhibition by interleukin 2. *Int J Cancer* 1994; **59**:225–234.
38 Ye L, Haider HK, Guo C, Sim EK. Cell-based VEGF delivery prevents donor cell apoptosis after transplantation. *Ann Thorac Surg* 2007; **83**: 1233–1234.
39 Kim EK, Park JD, Shim SY, Kim HS, Kim BI, Choi JH, Kim JE. Effect of chronic hypoxia on proliferation, apoptosis, and HSP70 expression in mouse bronchiolar epithelial cells. *Physiol Res* 2006; **55**:405–411.
40 Nylandsted J, Gyrd-Hansen M, Danielewicz A, Fehrenbacher N, Lademann U, Høyer-Hansen M, *et al*. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J Exp Med* 2004; **200**:425–435.
41 Gold LI. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Crit Rev Oncog* 1999; **10**:303–360.
42 Masztalerz A, van Luyn M, Werner N, Molema G, Everse LA, Otter WD. Treatment with IL-2 and IL-12 inhibits tumour cell division in SL2 lymphoma. *Anticancer Res* 2004; **24**:2633–2642.
43 Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. *J Leukoc Biol* 2005; **78**: 1043–1051.
44 Pinkas J, Teicher BA. TGF-beta in cancer and as a therapeutic target. *Biochem Pharmacol* 2006; **72**:523–529.
45 Osbild S, Brault L, Battaglia E, Bagrel D. Resistance to cisplatin and adriamycin is associated with the inhibition of glutathione efflux in MCF-7-derived cells. *Anticancer Res* 2006; **26**:3595–3600.