Cell density-dependent alterations in tumorigenic potential of a murine T-cell lymphoma: implication in the evolution of multidrug resistance in tumor cells

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We have previously demonstrated that cells of murine T-cell lymphoma, when grown *in vivo* or *in vitro* in an environment of high cell density, undergo phenotypic alterations, providing them with survival benefits. However, it is unclear whether the acquisition of such growth-related phenotypic alterations is inheritable in successive cell generations and if these alterations are associated with an irreversible alteration in their tumorigenic ability and evolution of multidrug resistance. To investigate this, tumor cells of a murine model of a T-cell lymphoma, designated as Dalton's lymphoma, and obtained from high and low cell density environment *in vitro* and *in vivo*, were transplanted in mice with or without the administration of anticancer drugs followed by analysis of their phenotypic properties and tumorigenic potential as measured by kinetics of tumor growth and survival of the tumor-bearing host. Kinetics of tumor progression was comparatively rapid in tumor-bearing mice transplanted with tumor cells from a high cell density environment, causing an early death of the host. Moreover, under these conditions the antitumor response of anticancer drugs, cisplatin, doxorubicin, and methotrexate, was found to be less effective compared with mice transplanted with tumor cells from a low cell density environment. The tumor cells from a high cell density source showed a long-term alteration in their survival properties both *in vitro* and *in vivo*, indicating that such alterations were sustainable over successive cell cycles. The study also discusses the possible mechanisms indicating the role of MDR1, Hsp70 and 90, Bcl-2, IL-1, IL-6, IL-10, IFNγ, and TGFβ in the evolution of multidrug resistance in tumor cells obtained from a high cell density environment. *Anti-Cancer Drugs* 19:793–804 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Progressive tumor growth with respect to some types of cancers has been observed to be invariably associated with an evolution of subclones of tumor cells, possessing improved survival ability. Under such conditions tumor cells acquire the capacity to resist induction of cell death [1–5]. Evolution of such altered survival properties is reported to be dependent on stressful environmental conditions pertaining to the late tumor-bearing stage such as hypoxia, depletion of nutrition, and the availability of space for proper growth [6,7]. It is predicted that these altered survival properties of tumor cells may have an impact on the kinetics of tumor progression and the antitumor response of chemotherapeutic drugs, an effect that, remains to be investigated. Moreover, to the best of our knowledge, the existence of such survival-related alterations in hematological tumors such as leukemia and lymphoma, leading to their altered tumorogenicity ability, remains unexplored. It is essential to investigate this phenomenon in these tumor cells as 6% of total malignancies are of hematological origin [8] and these tumors are considered as one of the 'difficult malignancies' for clinical management [9].

As a consequence, for several years our laboratory has been investigating dimensions and mechanisms of host–tumor relationship with respect to the progressive growth of a T-cell lymphoma in a murine model designated as Dalton's lymphoma (DL) [5,10,11]. The DL can grow both in the form of an ascitic or a solid tumor and has been reported to possess chromosomal aberrations [12]. Like some other lymphoma and leukemia cells of human origin, DL cells do not metastasize to other lymphoid organs [13]. During the course of our previous investigations we have observed that the progression of the ascitic DL growth is rapid in syngeneic BALB/c ($H2^d$) mice, causing death of the host in a relatively short time [11]. Further, DL growth has been shown to be associated with the onset of thymic atrophy, modulation of macrophage antitumor activity, and inhibition of other cellular - and humoral immune responses associated with an alteration of Th1/Th2 cytokine balance [14–16]. Recently, we also demonstrated that interaction of the immune, endocrine, and nervous systems plays an important role in determining the host–tumor relationship with respect to tumor progression in mice bearing DL [5,10,11,15,16].

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In view of the aforesaid observations, we were also interested in investigating alterations in the survival ability of DL cells under high cell density environment *in vivo* and *in vitro*. In one of our earlier studies, we demonstrated that the progressive growth of DL was associated with improved DL cell survival along with acquired resistance against the induction of apoptosis because of the cytotoxic action of cisplatin and doxorubicin [5]. However, it remained unclear if such altered behavior of lymphoma cells depending on the high cell density-related environmental conditions had any impact on their tumorigenic potential. In addition, it also remained unclear whether such a phenomenon related to the altered survival of the tumor cells is also accompanied by the evolution of multidrug resistance in the lymphoma cells.

In view of these observations this study was undertaken to investigate whether progression of a T-cell lymphoma through a high cell density environment, *in vitro* or *in vivo*, is associated with the emergence of irreversible alterations in the survival properties and tumorigenic potential, leading to the evolution of a multi-drug-resistant phenotype. The study also focuses on some of the possible mechanisms.

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. 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 is maintained in an ascitic form by serial transplantation 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, where the yield of DL cells is higher, were used. Mice were transplanted intraperitoneally with 1×10^5 cells/mouse, in 0.5 ml phosphate-buffered saline (PBS), which normally survive for 30 ± 2 days [11].

Reagents

Tissue culture medium RPMI 1640 was purchased from Hyclone (Logan, Utah, USA). All culture media were supplemented with 20 μg ml^{-1} gentamycin, 100 μg ml^{-1} streptomycin, 100 IU penicillin purchased from Himedia (Mumbai, India), and 10% fetal calf serum from Hyclone (Logan, Utah, USA). Antibodies against IL-1, IL-2, IL-2R, IFNγ, TGFβ, Hsp70, p53, and caspase-activated DNase were purchased from Imgenex (San Diego, California, USA) and Chemicon (Hampshire, UK). Hsp90 (SPA835) obtained from Stressgen (Ann Arbor, Michigan, USA) was kindly gifted by Professor S.C. Lakhotia, Department of Zoology, Banaras Hindu University. Secondary antibodies conjugated to alkaline phosphatase were obtained from Bangalore Genie (Bangalore, Karnataka, India). BCIP/NBT was purchased from Amresco (Solon, Ohio, USA). The MDR1 primer was purchased from Metabion International AG (Martnsried, Germany).

Protocol for in-vivo treatment

DL cells, obtained from serially passaged stock, were incubated *in vitro* at cell densities of 10^5 and 10^9 cells per ml, henceforth referred to as low and high cell densities, respectively, for 48 h. This stock was maintained under the indicated cell density for consecutive in-vitro serial passage until further use. Mice in groups of six each were transplanted intraperitoneally with DL (1×10^5 cells/mouse in 0.5 ml of PBS). DL cells were harvested from mice 5 and 17 days after the transplantation; henceforth referred to as early and late tumor-bearing stages, respectively [5]. The basic protocol for investigating altered survival and tumorigenic properties of tumor cells is depicted in Fig. 1.

Study of tumor progression and survival of tumor-bearing mice

Tumor growth was monitored by measuring the change in body weight and monitoring the survival of tumor-bearing mice of control and experimental groups of DL-bearing mice, up to day 50 following a method described earlier [11]. The percentage increase in body weight was calculated as follows:

$$\text{Increase in body weight}(\%) = \frac{W_f - W_i}{W_i} \times 100$$

where W_f = weight of mice on day 21 of tumor transplantation; W_i = weight of mice on day 1 of tumor transplantation. A survival curve was plotted to record the span of survival of the tumor-bearing mice.

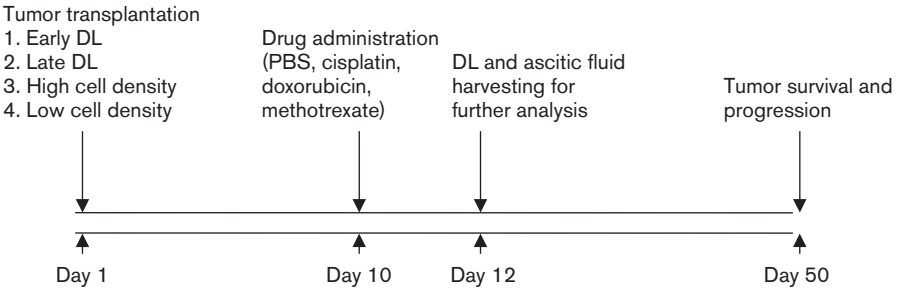
Preparation of Dalton's lymphoma cell-free ascitic fluid

DL cell-free ascitic fluid was prepared as described earlier [17]. DL was aspirated by peritoneal lavage on the indicated date as described in results following DL transplantation. The peritoneal exudates cells thus obtained were centrifuged at $200 \times g$ 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.

Morphological evaluation of apoptotic Dalton's lymphoma cells

Apoptotic cell population was enumerated by a method described earlier [5]. Cell suspension was smeared on a slide and air-dried, fixed in methanol, stained with Wright staining solution, and analyzed under a light microscope

Fig. 1



Experimental protocol for investigating alteration to tumorigenic potential of tumor cells and evolution of multidrug resistance. To investigate if tumor cells grown *in vitro* under high or low cell density conditions or *in vivo* at early or late tumor-bearing stage have altered tumorigenic ability, tumor cells taken from these sources were transplanted to mice followed by administration of PBS alone or cisplatin, doxorubicin, and methotrexate at a dose of 5 mg/kg body weight. DL cells were harvested on two days after drug administration and checked for altered phenotype. Other tumor-bearing mice were allowed to leave and tumor progression and survival was monitored until day 50. DL, Dalton's lymphoma; PBS, phosphate-buffered saline.

(Carl Zeiss, Gottingen, Germany) at 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.

Percentage of DNA fragmentation

Quantitative determination of DNA fragmentation was carried out following a method given by Sellins and Cohen [18] with slight modifications [5]. DL cells (1×10^6 cells/ml) from tumor-bearing mice 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 $13\,000 \times g$ at 4°C for 10 min. A 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 $13\,000 \times g$ at 4°C for 10 min. The supernatant was discarded and 80 µl of 5% TCA was added to each pellet. Heating at 90°C for 15 min hydrolyzed DNA. At this stage a blank was included containing 80 µl of 5% TCA. Then 160 µl of freshly prepared diphenylamine reagent (150 mg diphenylamine in 10 ml glacial acetic acid, 150 µl 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. This colored solution (100 µl) was transferred to the wells of a 96-well flat-bottomed ELISA plate and absorbance was measured at 600 nm in a microtiter ELISA plate reader (Labsystems, Helsinki, Finland). The percentage of DNA fragmentation was calculated as DNA fragmentation (%) = $[T/(T + B)] \times 100$.

ELISA for detection of cytokines in Dalton's lymphoma cell-free ascitic fluid

A standard ELISA was performed to detect the presence of IL-1, IL-6, IL-10, and TGF-β in the ascitic fluid of tumor-bearing mice following a method described earlier [5]. 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 wells of ELISA plates and the plate was processed for subsequent steps in the same way 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 standard preparation of the respective cytokines obtained from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK).

Assay for tumor cell survival *in vitro*

Survival of DL cells was assayed according to a method described earlier [5] with slight modification. The DL cells obtained were seeded (1.5×10^5 viable cells $200\,\mu l^{-1}\,ml^{-1}$), in a 96-well tissue culture plate and incubated at 37°C in a CO_2 incubator for 48 h. Cell survival was measured by standard MTT assay, described below.

MTT assay

The MTT assay was carried out to estimate DL cell proliferation, following a method described by Mosmann [19]. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl

tetrazolium bromide] was dissolved in PBS at a concentration of 5.0 mg/ml. MTT solution (50 µl) was added to each well of the culture plate containing 200 µl medium and incubated at 37°C for 4 h. The medium was then removed carefully, without disturbing the dark blue formazan crystals. Dimethylsulfoxide (50 µl) 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. The readings were presented as an optical density at 570 nm.

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 NaCl, 10% v/v glycerol, 1% v/v Triton X-100, 2 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride, 20 µmol/l leupeptin containing aprotinin at 0.15 U/ml) for 20 min at 4°C. The protein content in each sample was determined by using standard Bradford method [20]. Triton X-100-solubilized proteins (30 µg) were separated on 10% SDS-polyacrylamide gel at 20 mA as described by Davis [21]. The gel was either stained with Comassie brilliant blue (R250) for the analysis of proteins bands or was 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: anti-rabbit IgG conjugated to alkaline phosphatase and detected by a BCIP/NBT solution (Amresco, Solon, Ohio, USA). 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.

RT-PCR for expression of mRNA for MDR1 gene

RT-PCR analysis for the expression of mRNA for the MDR1 genes was carried out according to a method described earlier [11], using a one-step RT-PCR cell to cDNA kit (Ambion, Texas, USA). The primer sequence for MDR1-5′-GCCTGGCAGCTGGAAGACAAATACACA AAATT-3′ (forward); MDR1-1R-5′CAGACAGCAGCTGA CAGTCCAAGAACAGGACT-3′ (reverse). PCR was performed for 15 min to make cDNA at 50°C. The amplification was carried out for 35 cycles with the initial denaturation at 94°C for 2 min followed by annealing at 61°C for 30 s and elongation at 72°C for 30 s. The samples were separated on an agarose gel (1%) containing ethidium bromide (0.3 mg/ml). The band were visualized and analyzed on a UV transilluminator (Biorad, Milan, Italy).

Statistical analysis

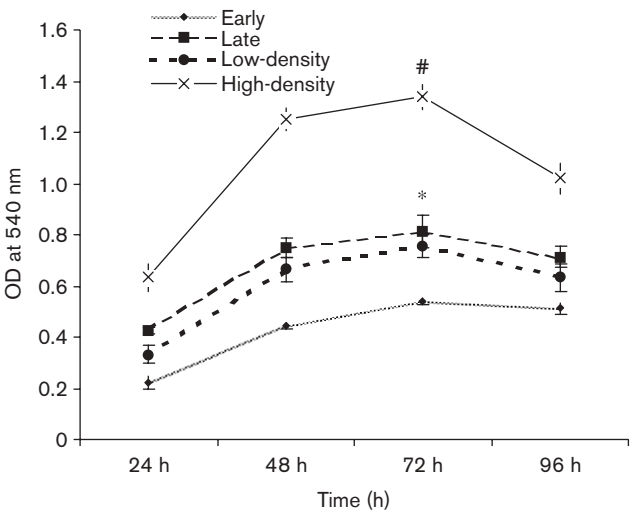
All experiments were conducted in triplicate at least three times. The statistical significance of differences between test groups was analyzed by one-way analysis of variance using all pair-wise multiple comparison procedures (Duncan's method). The difference was considered significant when P was less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

Results

Effect of the high cell density environment on long-term growth properties of tumor cells

Tumor cells (1×10^6) harvested from in-vitro cultures maintained at low (10^5) or high (10^9) cell densities for 48 h were equalized in number (1×10^6) and plated in 96-well culture plates for various time durations up to 96 h before the estimation of cell survival by MTT assay. Similarly, tumor cells obtained from tumor-bearing mice at early and late tumor-bearing stage were also harvested followed by equalization of cell number (1×10^6) and incubated *in vitro* under conditions as described above up to 96 h to estimate cell survival. Results are shown in Fig. 2. Tumor cells obtained from in-vitro stock of higher cell density or from late tumor-bearing stage showed a consistent and significantly higher survival compared with their respective controls of tumor cells obtained from low-density in-vitro cultures or early tumor-bearing stage.

Fig. 2



Long-term alteration in the survival properties of tumor cells obtained from high cell density environment *in vivo* and *in vitro*. Tumor cells initially obtained from early or late tumor-bearing stage and from in-vitro stock of DL cells cultured for 48 h under high-density (10^9) and low-density (10^5) culture were equalized in cell number followed by incubation *in vitro* (1×10^5 cells/well) in 96-well tissue culture plates for the indicated time duration followed by estimation of cell survival by MTT assay as described in the Materials and methods section. Values shown are mean ± SD of three independent experiments done in triplicate. $^*P < 0.05$ versus values for tumor cells obtained from early tumor-bearing stage. $^{\#}P < 0.05$ versus values for tumor cells obtained from low-density grown culture. DL, Dalton's lymphoma; OD, optical density.

Besides estimation of long-term survival alteration of tumor cells *in vitro*, we also investigated the permanency of such a phenomenon *in vivo* by transplanting DL cells (1×10^5) obtained from early or late tumor-bearing stage and after 12 days of transplantation followed by the quantification of apoptotic cells and estimation of survival by MTT assay. Results are shown in Table 1. Tumor cells of the late tumor-bearing stage consistently remained higher in their survival capacity compared with those from the early tumor-bearing stage concomitant with their lower level of apoptosis. These results show that over several cell generations the phenotype of tumor cells with better survival ability is a sustainable, permanent and an irreversible phenomenon both *in vitro* and *in vivo*.

Differential tumorigenic potential of Dalton's lymphoma cells depending on cell density-related environmental conditions

Considering the in-vitro and in-vivo observation shown in Fig. 2 and Table 2 regarding the development of a irreversible survival ability of tumor cells in the next experiment we investigated whether this was also associated with actual alteration in the tumorigenic potential of these cells. Tumor cells (1×10^5) obtained from in-vitro cultures maintained at low and high cell densities or those obtained from early and late tumor-bearing stages were transplanted to mice as described in the Materials and methods section followed by monitoring of tumor progression by estimating change of body weight [11] and survival time of the tumor-bearing host. Results are shown in Fig. 3a and b. Tumor progression was of a significantly less robust nature when mice were transplanted with tumor cells obtained from an early tumor-bearing stage or low cell density in-vitro culture stock compared with late tumor-bearing stage or high cell density culture, respectively (Fig. 3a). The survival period of mice transplanted with tumor cells from early tumor-bearing stage and low cell density culture was longer compared with mice transplanted with tumor cell from late tumor-bearing stage or high cell density culture.

Table 1 Survival of DL-bearing mice transplanted with DL cells

	% of surviving mice							
	DL from early tumor-bearing stage				DL from late tumor-bearing stage			
Days following DL transplantation	Cnt	CP	DR	MT	Cnt	CP	DR	MT
(a) DL cells obtained from early and late tumor-bearing mice following in-vivo administration of anticancer drugs								
15	100	100	100	100	100	100	100	100
25	50	100	100	75	100	100	100	100
30	50	75	75	75	100	75	80	50
35	0	75	50	50	0	25	80	50
40	0	75	50	50	0	25	40	40
45	0	50	40	30	0	10	30	40
50	0	20	10	0	0	0	0	0
	DL from low-density culture stock				DL from high-density culture stock			
	Cnt	CP	DR	MT	Cnt	CP	DR	MT
(b) DL cells obtained from high-density and low-density grown culture following in-vivo administration of anticancer drugs								
15	100	100	100	100	100	100	100	100
25	100	75	50	30	80	80	100	60
30	100	75	50	30	60	80	50	60
35	0	75	50	30	0	80	50	30
40	0	75	50	30	0	40	50	0
45	0	50	30	30	0	0	0	0
50	0	25	10	0	0	0	0	0

Cnt, control; CP, cisplatin; DL, Dalton's lymphoma; DR, doxorubicin; HD, high density; LD, low density; MT, methotrexate.

Table 2 Long-term alteration of tumor growth environment on the survival and induction of apoptosis in tumor cells

	Before transplantation in mice		12 days after transplantation in mice	
	Early TB DL	Late TB DL	Early TB DL	Late TB DL
(a) MTT (OD)	1.15 ± 0.16	1.61 ± 0.13*	0.838 ± 0.061**	1.278 ± 0.152*,**
(b) % apoptosis	27.9 ± 1.92	33.31 ± 2.42*	41.26 ± 3.92**	30.09 ± 2.81*,**
(c) % DNA fragmentation	35.8 ± 2.82	40.95 ± 3.31*	33.63 ± 2.44**	24.56 ± 1.94*,**

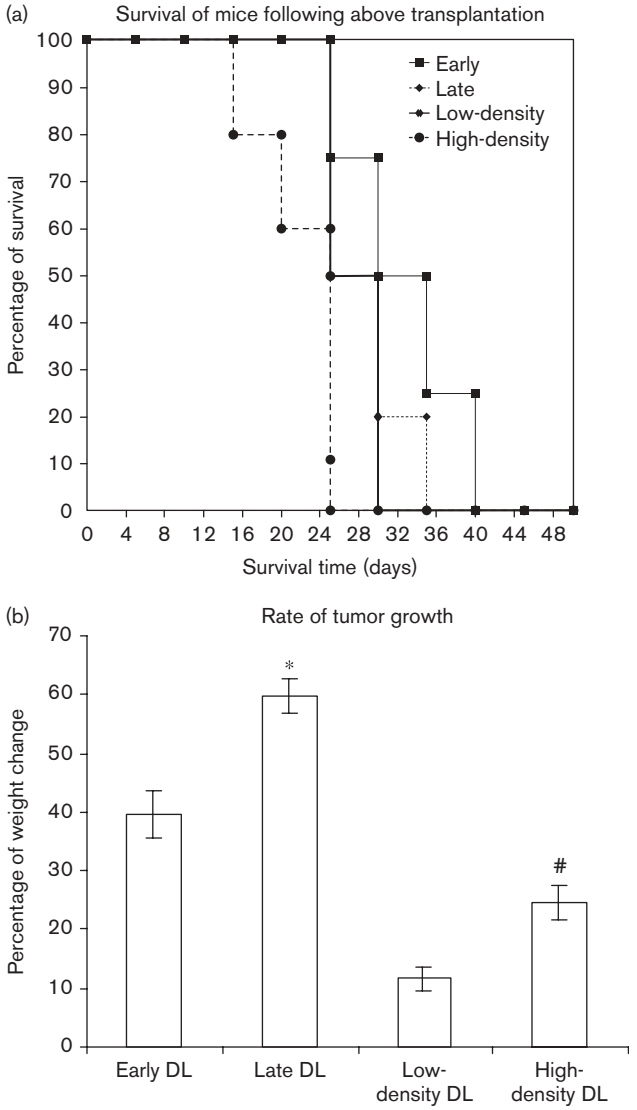
Tumor cells (1×10^6 cells/ml) obtained from early or late tumor-bearing stage were transplanted in mice after equalizing their cell number (1×10^5 cells/ml) as described in the Materials and methods section (Fig. 1). The tumor cells before transplantation and those obtained after 12 days of transplantation were processed for estimation of specific cell survival (a), enumeration of % apoptotic of tumor cells (b), and DNA fragmentation (c). Values shown are mean ± SD of three independent experiments done in triplicate.

OD, optical density; TB DL, tumor-bearing Dalton's lymphoma.

*$P < 0.05$ versus values for tumor cells obtained from early tumor-bearing stage.

**$P < 0.05$ versus values for tumor cells obtained from early and late tumor-bearing stage before transplantation.

Fig. 3

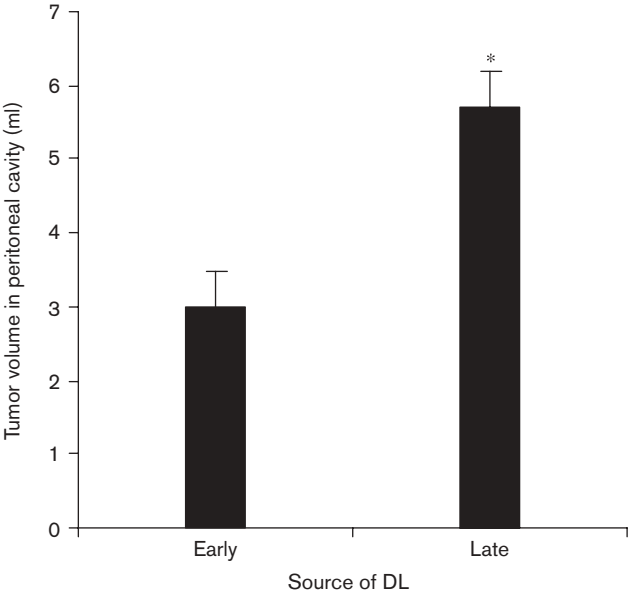


Differential ability of tumor cell from early and late tumor-bearing stage and from low-density and high-density grown culture to induce tumor growth. Tumor cells (1×10^6 cells/ml) obtained from early or late tumor-bearing stage and from in-vitro stock of DL cells cultured for 48 h under high-density (10^9) and low-density (10^5) culture for the indicated time duration *in vitro* were transplanted in mice after equalizing their cell number (1×10^6 cells/ml) to study tumor progression. (a) Kaplan–Meir plot for the survival of these mice was made. (b) Tumor-bearing mice were weighed regularly to monitor tumor progression, as described in the Materials and methods section. Values shown are mean ± SD of three independent experiments done in triplicate. *$P < 0.05$ versus values for tumor cells obtained from early tumor-bearing stage. #$P < 0.05$ versus values for tumor cells obtained from low-density grown culture. DL, Dalton's lymphoma.

Differential tumorigenic potential of tumor cell depending on cell density

Tumor cells (1×10^6 cell/ml) obtained from early or late tumor-bearing stage were transplanted in mice after equalizing their cell number (1×10^5 cells/ml) as described in the Materials and methods section (Fig. 1) and tumor volume was estimated on day 12 of tumor growth. Results are shown in Fig. 4. The volume of tumor cells was significantly higher in the tumor-bearing mice transplanted with tumor cells from late tumor-bearing stage compared with early tumor-bearing stage.

Fig. 4

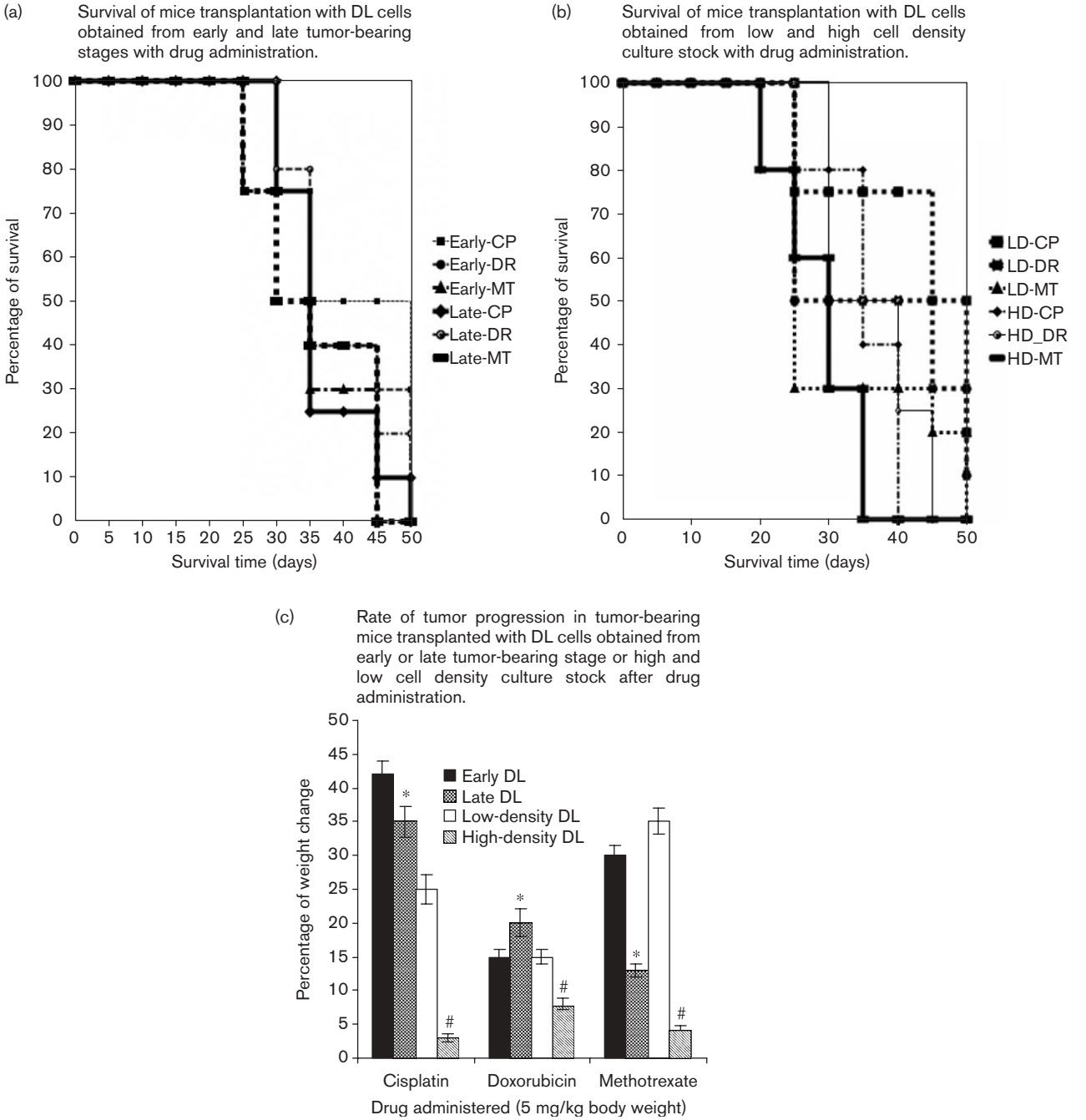


Differential tumorigenic potential of tumor cell depending on the cell density. Tumor cells (1×10^6 cells/ml) obtained from early or late tumor-bearing stage were transplanted in mice after equalizing their cell number (1×10^5 cells/ml) as described in Material and methods section (Fig. 1) and tumor volume was estimated on day 12 of tumor growth. Values shown are mean ± SD of three independent experiments done in triplicate *$P < 0.05$ versus values for tumor cells obtained from early tumor-bearing stage. DL, Dalton's lymphoma.

Evolution of resistance to anticancer drugs in tumor cells adapted to high cell density in-vivo and in-vitro environment

Mice were transplanted with tumor cells (1×10^5 cells/ml) followed by the administration of cisplatin, doxorubicin, or methotrexate (5 mg/kg body weight) on day 10 after tumor transplantation as shown in Fig. 1, followed by monitoring of tumor progression and survival of the tumor-bearing mice. The results are shown in Fig. 5a and b and Table 1. The administration of cisplatin, doxorubicin, or methotrexate was found to cause a better retardation of tumor growth in the tumor-bearing mice transplanted with tumor cells of low cell density in-vitro culture or early tumor-bearing stage compared with high cell density in-vitro culture or late tumor-bearing stage, respectively (Fig. 5a). Similarly, the efficacy of these anticancer drugs for the prolonged survival of tumor-bearing mice was found to be dependent on the type of tumor cells transplanted into the mice. Mice transplanted with tumor cells from high cell density in-vitro culture or late tumor-bearing stage showed a lesser response to the

Fig. 5



Differential effect of anticancer drugs on the tumorogenicity of DL cells obtained from low and high cell density environment. Tumor cells (1×10^6 cells/ml) obtained from early or late tumor-bearing stage and from in-vitro stock of DL cells cultured for 48 h under high-density (10^9) and low-density (10^5) culture for the indicated time duration *in vitro* were transplanted in mice after equalizing their cell number (1×10^5 cells/ml) and drug was administered (5 mg/kg body weight). (a, b) Kaplan–Meir plot and a tabular presentation (Table 1) for the survival of these mice were made. (c) Tumor-bearing mice were weighed regularly to monitor tumor progression, as described in the Materials and methods section. Values shown are mean ± SD of three independent experiments done in triplicate. $^*P<0.05$ versus values for tumor cells obtained from early tumor-bearing stage. $^{\#}P<0.05$ versus values for tumor cells obtained from low-density grown culture. CP, cisplatin; DL, Dalton's lymphoma; DR, doxorubicin; MT, methotrexate.

administration of anticancer drugs for the prolongation of survival compared with mice transplanted with tumor cells from low cell density in-vitro culture or early tumor-bearing stage.

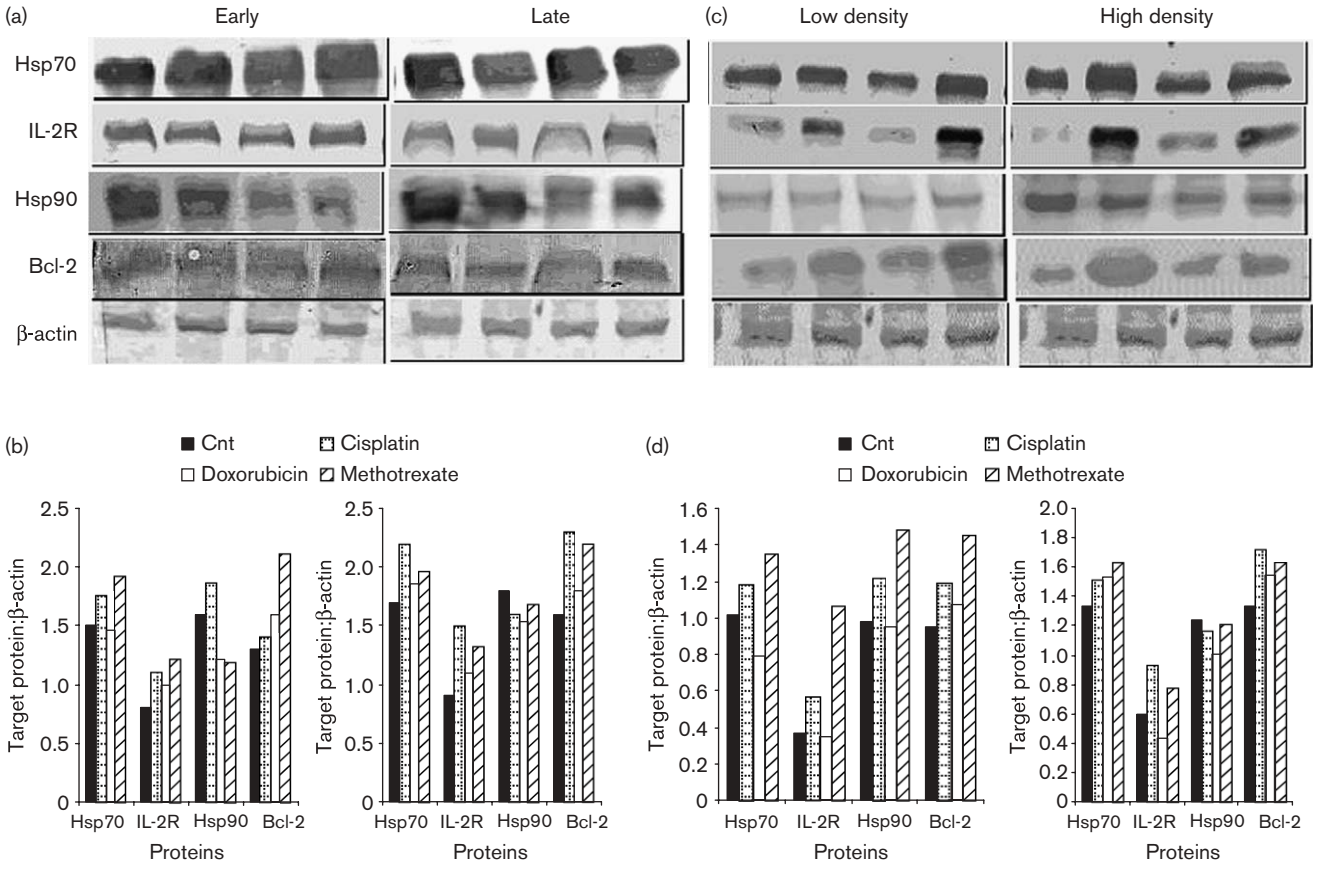
Differential expression of tumor growth-regulating proteins by DL cells depending on the cell density environment of the source cell: DL cells (1×10^6) obtained on day 12 after tumor transplantation from

tumor-bearing mice having been administered with DL cells obtained from early or late tumor-bearing stages or low or high cell density in-vitro culture stock followed by administration of PBS or cisplatin or doxorubicin or methotrexate (Fig. 1) were lysed and used for western blotting of Hsp70 and 90, IL-2R, Bcl-2, and β-actin. Results are shown in Fig. 6. The expression of Hsp70, Hsp90, IL-2R, and Bcl-2 was found to be upregulated in the DL cells obtained from the late tumor-bearing stage compared with those from an early tumor-bearing stage. Administration of anticancer drugs did not further decrease the expression of most of these proteins in DL cells of mice transplanted with DL cells from the late tumor-bearing stage compared with the early tumor-bearing stage. A similar pattern of expression of these proteins was observed in DL cells obtained from mice transplanted with DL cells from a high cell density culture (Fig. 6c).

Immunodetection of Il-1, IL-6, IL-10, IFNγ, and TGFβ in the ascitic fluid of Dalton's lymphoma-bearing mice

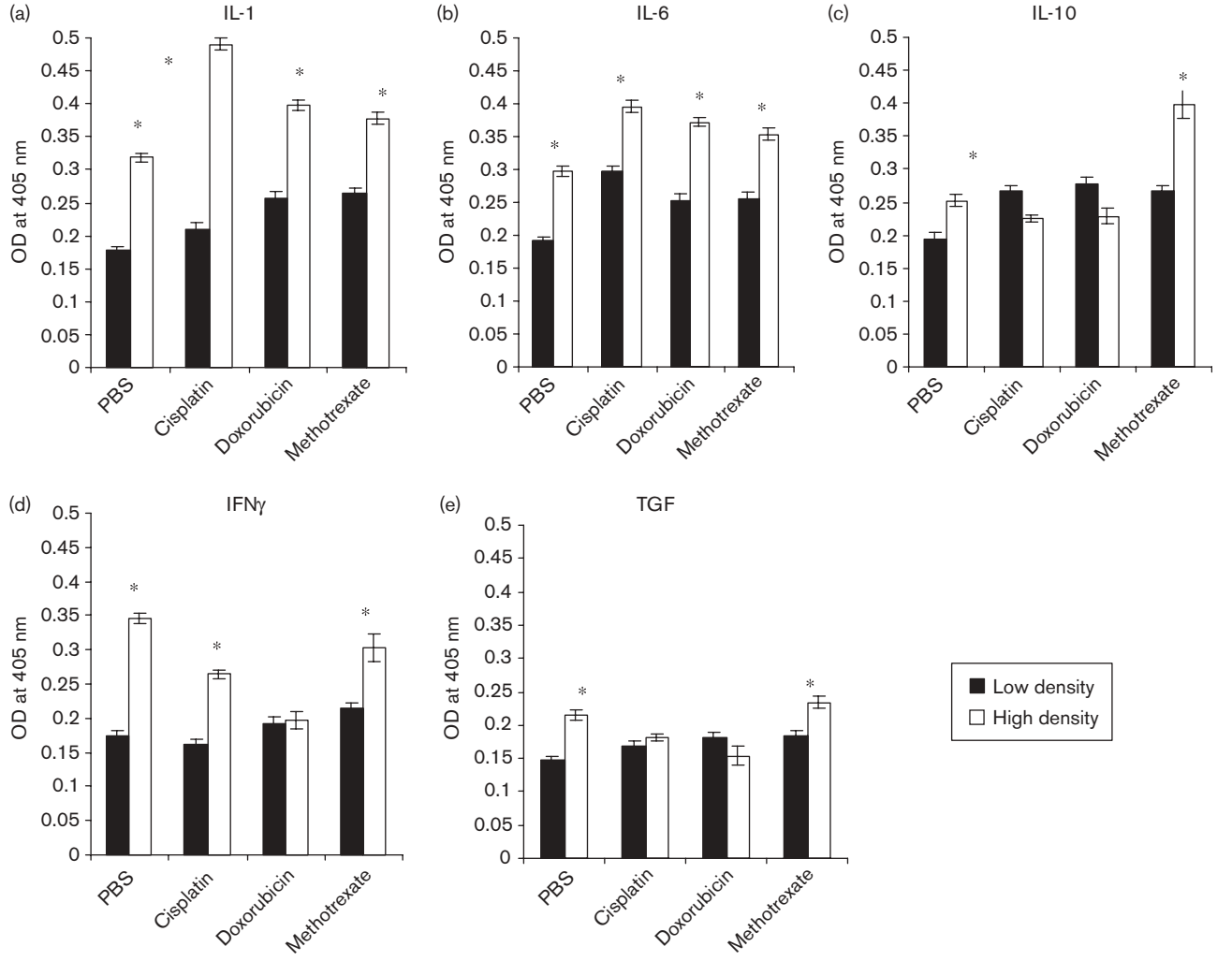
Mice were transplanted with DL cells grown at low or high cell density cultures following the administration of cisplatin, doxorubicin, or methotrexate (5 mg/kg body weight) according to the protocol shown in Fig. 1. The DL cell-free ascitic fluid was harvested on day 12, following tumor transplantation, and immunodetected by ELISA for the presence of IL-1, IL-6, IL-10, IFNγ, and TGFβ Results are shown in Fig. 7a-e. Transplantation of tumor cells to mice from a high cell density culture stock resulted in a significant augmentation in the level of IL-1, IL-6, IL-10, IFNγ, and TGFβ compared with the level of these cytokines in the ascitic fluid of mice transplanted with DL cells from low cell density culture. The administration of cisplatin and doxorubicin resulted in a significant augmentation of IL-1 and IL-10 levels in the ascitic fluid of mice transplanted with DL cells from a

Fig. 6



Differential expression of tumor growth-regulating proteins by DL cells depending on their source environment. Tumor cells obtained from early or late tumor-bearing stage (a) and in-vitro high-density and low-density grown culture (c) were transplanted in mice after equalizing their cell number (1×10^5 cells/ml) and then DL was harvested from these mice after 10 days of tumor growth with or without administration of drugs (5 mg/kg body weight), followed by equalizing the cell number and preparation of lysate 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 detection of the expression of β-actin. (b, d) Densiometric scans of the immunoblots shown in Fig. 6a and c showing relative intensity of different bands (target protein: β-actin). Cnt, control; DL, Dalton's lymphoma; IL-2R, interleukin 2 receptor.

Fig. 7



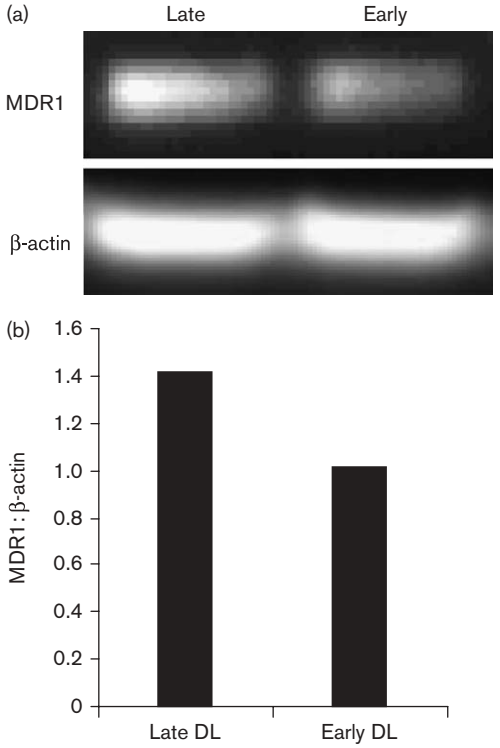
Cytokine profile in ascitic fluid of tumor mice transplanted with DL from various sources after drug administration. Tumor cells obtained from early or late tumor-bearing stage and in-vitro high-density and low-density grown culture were transplanted in mice after equalizing their cell number (1×10^5 cells/ml). Cytokine profile was checked in the ascitic fluid after 10 days with or without drug administration (5 mg/ kg body weight) in these mice as described in the Materials and methods section. Values shown are mean ± SD of three independent experiments done in triplicate. *$P < 0.05$ versus values for tumor cells obtained from low-density grown culture. DL, Dalton's lymphoma; IFNγ, interferon gamma; IL, interleukin; OD, optical density; PBS, phosphate-buffered saline; TGF, tumor growth factor.

high cell density culture stock compared with DL cells of low cell density culture (Fig. 6a and b). Similarly, a significant surge in the level of IFNγ was detected in the ascitic fluid of mice transplanted with tumor cells from high cell density culture compared with low cell density culture administered with cisplatin or methotrexate (Fig. 7d). However, following the administration of methotrexate, cisplatin, or doxorubicin to DL by mice transplanted with DL cells from high cell density, a significant inhibition in the level of IL-10 was detected compared with mice transplanted with DL cells from low cell density culture (Fig. 7c). The administration of any of these anticancer drugs did not alter the level of TGFβ in the ascitic fluid in either condition except methotrexate, where a significant rise in the level of TGFβ was observed in the case of the ascitic fluid of mice transplanted with DL from high cell density culture compared with low cell density culture (Fig. 7e).

Effect of tumor progression on the expression of MDR1 gene in tumor cells

DL cells obtained from early and late tumor-bearing stages were analyzed for the expression of MDR1 mRNA by RT-PCR as described in the Materials and methods section. Results are shown in Fig. 8a and b. The expression of MDR1 mRNA was found to be higher in the tumor cell obtained from the late tumor-bearing stage compared with the early tumor-bearing stage.

Fig. 8



RT-PCR to check differential expression of MDR1 gene by DL cells depending on the cell density of tumor microenvironment. (a) DL cells obtained from early or late tumor-bearing stages were checked for the expression of MDR1 mRNA by RT-PCR as described in the Materials and methods section. Results shown are from representative experiments out of three independent experiments done in triplicate with similar results. (b) Densiometric scans of the figure shown in (a), showing relative intensity of different bands (target gene: β-actin). DL, Dalton's lymphoma.

Discussion

Tumor growth during the late tumor-bearing stages is invariably difficult to contain therapeutically, making it essential to investigate whether tumor progression is associated with the evolution of tumor cells that are better adapted for survival under harsher environmental conditions. Moreover, the molecular mechanisms and clinical implications of such survival and cell death-related adaptation of tumor cells during the later course of tumor progression are poorly understood and are essential for investigation on an individual tumor basis to optimize the therapeutic efficacy of anticancer strategies. Some sporadic studies have indicated that during the progression of tumor growth, conditions related to hypoxia and depleted nutrition lead to the evolution of apoptosis-resistant tumor cells [6,7,22]. On the same line, we also demonstrated in a previous study that the progressive growth of DL was associated with such adaptive alteration in their tumor phenotype that rendered the tumor cells better adapted for survival *in vitro* and resistant to the cytotoxic actions of anticancer drugs [5]. However, it has remained unclear whether such phenotypic alterations in the survival ability of tumor cells were sustainable and irreversible, and if the same had any implication in the tumorogenicity of the lymphoma cells. To investigate this problem, tumor cells were grown in low or high cell density cultures or obtained from early and late tumor-bearing stages followed by estimating their long-term survival and tumorigenicity. The results of the present investigation indicate that transplantation of tumor cells adapted to a low or high cell density environment had a cell density-dependent differential potential for survival *in vitro*, which was sustainable over a longer duration of in-vitro incubation. We have previously reported that average specific growth rate of DL cells *in vitro* is 0.68/h [11]. Tumor cells obtained from a high cell density environment were consistently superior in their long-term survival in culture, indicating that the behavioral alteration in the growth properties of these cells was of irreversible nature, even if the cell density is reverted to low. This was further corroborated by the experimental evidence showing a differential survival ability of tumor cells, following in-vivo transplantation of an equal number of tumor cells from a high cell density environment that was unaltered when they were harvested after 12 days of transplantation (Table 2). Nevertheless, transplantation of tumor cells, obtained from higher cell density environmental conditions *in vivo* or *in vitro* to mice also reflected their differential ability for inducing tumor growth and thus the survival of the tumor-bearing mice (Fig. 3). Thus these results again support the conclusion that tumor cells grown under a high cell density environment become better adapted to survival, which is an irreversible phenomenon with implications for their tumorogenicity.

The next related aspect of this study was to investigate whether such sustainable alteration in the tumorigenicity of the tumor cells was dependent on their adaptations for survival under high cell density environmental conditions. Moreover, we also investigated whether the same had any bearing on the antitumor response of multiple anticancer drugs, having distinct mechanisms of tumor cytotoxicity. The results of this study indicate that tumor cells grown in a high cell density environment are not only superior in their tumorigenic potential, but are also resistant to the antitumor action of multiple anticancer drugs (Fig. 5). Such acquired resistance of tumor cells could be because of several mechanisms inducing (i) 'switching off' of apoptotic pathways in tumor cells and (ii) augmented expression of cytokines and other prosurvival proteins. Indeed, the evidence from this study indicates that an increased expression of IL-1, IL-6, IL-10, and IFNγ was observed in the ascitic fluid of the DL-bearing mice transplanted with tumor cells from a high cell density environment, showing the likelihood of a role of these

cytokines in regulating the improved survival of these tumor cells within the tumor microenvironment. Moreover, cells harvested from tumor-bearing mice, transplanted with tumor cells from a high cell density source showed a higher level in the expression of Hsp70 and Hsp90, which have been shown to be key regulators of stress adaptations of tumor cells [23,24]. Hsp70 is also implicated in the upregulation of Bcl-2 and IL-2R, which have a T-cell growth-inducing ability [25,26]. Hsp90 has also been shown to be expressed in a variety of tumor cells and has been associated with the evolution of drug resistance [27,28]. IFNγ could act through its ability to modulate the production of IL-1 and IL-6 from macrophages in the tumor environment [29]. We have shown earlier that IL-1 derived from macrophages acts as a tumor growth factor [12,17]. Thus, the expression of heat shock proteins seems to be a key player in the 'switching on' or 'off' of other cell growth-regulating proteins [30]. In addition, studies in a wide range of human cancers such as breast, endometrial, uterine cervical, and bladder carcinomas have revealed that the expression of heat shock proteins under stressful environmental conditions is an irreversible phenomenon [31]. Nevertheless, reports have also indicated that IL-6, IL-10, IL-1, and IFNγ are prime tumor cells for an increased or decreased susceptibility against the cytotoxic action of chemotherapeutic drugs [32–34]. IL-6, IL-10, and heat shock proteins have been implicated in the regulation of the expression of MDR genes in tumor cells [35–37]. Interestingly our observations indicated that a high cell density environment augments expression of MDR1 genes in DL cells (Fig. 8). However, further studies will be necessary to understand the sequence of molecular events including that of tumor and host-derived factors leading to the evolution of the MDR phenotype of tumor cells under a stressful high cell density environment.

Taken together, the results of this study indicate that an interplay of cytokines and cell cycle-regulating proteins at high cell density environment cause the tumor cells to undergo irreversible alterations in their tumorigenicity, rendering them resistant to the cytotoxicity of chemotherapeutic drugs. Thus, these results need to be considered in the design of specific antitumor therapy for tumor cells of late tumor-bearing stage.

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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. 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 Singh V, Singh SM. Effect of high cell density on the growth properties of tumor cells: a role in tumor cytotoxicity of chemotherapeutic drugs. *Anticancer Drugs* 2007; **18**:1123–1132.
6 Sprague LD, Mengele K, Schilling D, Geurts-Moespot A, Sweep FC, Stadler P. Effect of reoxygenation on the hypoxia-induced up-regulation of serine protease inhibitor PAI-1 in head and neck cancer cells. *Oncology* 2006; **71**:282–291.
7 Pfäfflin A, Brodbeck K, Heilig C, Häring H, Schleicher E, Weigert C. Increased glucose uptake and metabolism in mesangial cells overexpressing glucose transporter 1 increases interleukin-6 and vascular endothelial growth factor production: role of AP-1 and HIF-1α. *Cell Physiol Biochem* 2006; **18**:199–210.
8 Armitage JO, Vose JM, Weisenburger DD. Towards understanding the peripheral T-cell lymphomas. *Ann Oncol* 2004; **15**:1447–1449.
9 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.
10 Gupta V, Singh SM. Gender dimorphism in the myeloid differentiation of bone marrow precursor cells in a murine host bearing a T cell lymphoma. *J Reprod Immunol* 2007; **74**:90–102.
11 Gupta V, Singh SM. Gender dimorphism of tumor growth: role of gonadal hormones in differential regulation of apoptosis of a murine T cell lymphoma. *J Biomed Sci* 2007;**15**:147–168.
12 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.
13 Kumar A, Singh SM. Effect of tumor growth on the blastogenic response of splenocytes: a role of macrophage derived nitric oxide. *Immunol Invest* 1996; **25**:413–423.
14 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.
15 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.
16 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.
17 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.
18 Sellins KS, Cohen JJ. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol* 1987; **139**:199–206.
19 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Methods* 1983; **65**:55–63.
20 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.
21 Davis BJ. Disc electrophoresis-II. Method and application to human serum proteins. *Ann N Y Acad Sci* 1964; **121**:404–414.
22 Simons AL, Ahmad IM, Mattson DM, Dornfeld KJ, Spitz DR. 2-deoxy-D-glucose combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cells. *Cancer Res* 2007; **67**:3364–3370.
23 Tanimura S, Hirano AI, Hashizume J, Yasunaga M, Kawabata T, Ozaki K, Kohno M. Anticancer drugs up-regulate HspBP1 and thereby antagonize the prosurvival function of Hsp70 in tumor cells. *J Biol Chem* 2007; **282**:35430–35439.
24 Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *J Leukocyte Biol* 2007; **81**:15–27.
25 Charo J, Finkelstein SE, Grewal N, Restifo NP, Robbins PF, Rosenberg SA. Bcl-2 overexpression enhances tumor-specific T-cell survival. *Cancer Res* 2005; **65**:2001–2008.
26 Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, *et al*. The chaperone function of hsp70 is required for protection against stress induced apoptosis. *Mol Cell Biol* 2000; **20**:7146–7159.

27 Goetz MP, Toft DO, Ames MM, Erlichman C. The Hsp90 chaperone complex as a novel target for cancer therapy. *Ann Oncol* 2003; **14**:1169–1176.
28 Sreedhar AS, Kalmár E, Csermely P, Shen YF. Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett* 2004; **562**:11–15.
29 Mantovani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. *Eur J Immunol* 2007; **37**:14–16.
30 Davidoff AM, Iglehart JD, Marks JR. Immune response to p53 is dependent upon p53/HSP70 complexes in breast cancers. *Proc Natl Acad Sci* 1992; **89**:3439–3442.
31 Ciocca DR, Calderwood SK. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* 2005; **10**:263–274.
32 Borsellino N, Belldegrun A, Bonavida B. Endogenous interleukin 6 is a resistance factor for cis-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res* 1995; **55**:4633–4639.
33 Yusuf RZ, Duan Z, Lamendola DE, Penson RT, Seiden MV. Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. *Curr Cancer Drug Targets* 2003; **3**:1–19.
34 Steiner T, Junker U, Henzgen B, Nuske K, Durum SK, Schubert J. Interferon-alpha suppresses the antiapoptotic effect of NF-kB and sensitizes renal cell carcinoma cells in vitro to chemotherapeutic drugs. *Eur Urol* 2001; **39**: 478–483.
35 Kim SH, Hur WY, Kang CD, Lim YS, Kim DW, Chung BS. Involvement of heat shock factor in regulating transcriptional activation of MDR1 gene in multidrug-resistant cells. *Cancer Lett* 1997; **115**:9–14.
36 Shen F, Chu S, Bence AK, Bailey B, Xue X, Erickson PA, *et al*. Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells. *J Pharmacol Exp Ther* 2008; **324**:95–102.
37 Niethammer AG, Wodrich H, Loeffler M, Lode HN, Emmerich K, Abdollahi A, *et al*. Multidrug resistance-1 (MDR-1): a new target for T cell-based immunotherapy. *FASEB J* 2005; **19**:158–169.