Breast Cancer Research and Treatment **69:** 39–51, 2001.

Report

Apoptotic cell death in mammary adenocarcinoma cells is prevented by soluble factors present in the target organ of metastasis

Virginia Ladeda, Alejandro P. Adam, Lydia Puricelli, and Elisa Bal De Kier Joffé
Cell Biology Department, Research Area, Institute of Oncology 'Angel H. Roffo', University of Buenos Aires, Buenos Aires, Argentina

Key words: apoptosis, breast cancer, microenvironment, organ-specific metastasis, survival factors

Summary

Target organ of metastasis determines the fate of metastasis. The soluble factors released from one or more cell types in the new stroma may influence growth and survival of metastatic cells. In the present study, we used conditioned media from the kidney, liver and lung, the latter being the target organ of metastasis of murine mammary adenocarcinoma cell lines LM3, LMM3 and F3II, to assess whether the soluble factors released from these organs could modulate *in vitro* survival of these cell lines after apoptosis-inducing treatments and to investigate the mechanisms involved in this effect. We demonstrate that conditioned medium from lung, but not from liver or kidney, promotes survival of these cells after doxorubicin, cisplatin, agonistic anti-Fas antibody and serum withdrawal treatments. Furthermore, LMM3 cells treated with lung conditioned medium after doxorubicin exposure maintained their tumorigenic capacity and metastatic potential. Neither IGF nor EGF could promote survival but, surprisingly, TGF-β could reduce sensitivity of LMM3 cells to doxorubicin *in vitro*. Doxorubicin treatment induced Bax expression and down-regulated Bcl-2 expression. In contrast, lung conditioned medium increased Bcl-2 expression and inhibited doxorubicin-mediated Bcl-2 down-regulation. Neither of those treatments alone modified Bcl-x_L expression, although co-treatment induced a 3- to 5-fold increase of its expression. These results suggest that the lung microenvironment could promote metastasis of these adenocarcinoma cell lines by increasing survival of metastatic cells, possibly by modulation of Bcl-2 protein family expression.

Introduction

The main cause of cancer death is the natural or acquired resistance of metastasis to conventional therapies. The process of metastasis involves the release of cells from the primary tumor, their dissemination to distant sites, their arrest in the microcirculation in organs and, finally, their survival and growth into new tumor colonies [1]. It has been recognized that tumor progression is not only determined by events intrinsic to the tumor cells but also largely influenced by factors present in tumor microenvironment [2].

Tumor cell growth and apoptosis in the target organ may be key events that limit the ability to form metastasis [3]. Many compounds used for the treatment of malignant tumors are cytotoxic drugs that can induce tumor cell death by apoptosis [4] and the intracellular damage induced by these drugs is generally well characterized. Genetic modifications intrinsic of the tumor cell itself are key regulators of apoptosis and suggested as the main mechanism of tumor drug resistance. They include the mutation of the *p53* gene [5], overexpression of Bcl-2 or inactivation of Bax [6, 7] or lack of function of the Fas/FasL system [8]. Furthermore, the organ microenvironment may influence the biological phenotype of metastatic tumor cells modulating cell adhesion, invasiveness, migration and growth as well as response to different therapies [9–15]. Dong et al. [16] have shown that resistance to doxorubicin (Dox) in lung metastases from CT-26 murine colon carcinoma was due to the induction of a transient expression of *mdr* gene in lung microenvironment. It was proposed that metastatic cells, even damaged by cytotoxic treatments, might acquire survival advantages

in certain microenvironments that provide them with survival factors that regulate the rate of apoptosis [12, 17]. It has also been reported [18] that the successful metastatic growth of the malignant T lymphoma Cs-21 cell line in the lymph nodes is mediated by direct contact between Cs-21 cells and Ca-12 stromal cells, which prevented apoptotic death and enhanced cell proliferation.

Bcl-2 protein family is tightly involved in control of apoptosis. It consists in two sets of proteins, which inhibit (Bcl-2, Bcl-x_L) or promote (Bax, Bad, Bid) apoptosis (reviewed in [19]). It is believed that, rather than levels of individual proteins, the balance between apoptosis inducing and inhibiting proteins is what determines the cell fate [20]. In a recent report, Wong et al. [21] demonstrated that apoptosis is an early event involved in the selection of metastatic cells in the lungs, and that Bcl-2 overexpression can greatly reduce apoptosis of cancer cells and augment macroscopic lung nodules. In addition, Liu et al. [22] showed that Bcl-x_L overexpression promotes survival of mammary cancer cells after anticancer drugs treatment *in vivo*.

In a previous work we demonstrated that soluble factors from lung, the target organ for a murine mammary adenocarcinoma dissemination, were able to enhance local and metastatic growth when inoculated together with the tumor cells via iv, or separately via ip route [11]. We also showed that the conditioned media from lung (LCM) also stimulated *in vitro* growth and migration of the tumor cells and secreted uPA activity, as well as enhanced tumor angiogenesis *in vivo* [9, 11, 23].

The purpose of this study was to determine whether the target organ microenvironment influences the tumor cells response to apoptosis-inducing treatments *in vitro* and some of the mechanisms involved. We demonstrate that the treatment of murine mammary adenocarcinoma cell lines with soluble factors from normal lung, but not from other organs such as kidney or liver, enhanced the survival of cells previously exposed to various apoptosis-inducing treatments, and that this inhibition of apoptosis may be mediated by modulation of the expression of the Bcl-2 protein family.

Materials and methods

Mice and tumor cell lines

Ten week old male Balb/c mice were obtained from the Animal Care Facility of the Institute of Oncology 'A. H. Roffo'. Food and water were administered *ad libitum*. All animal studies were conducted in accordance with the NIH 'Guide for the Care and the Use of Laboratory Animals'.

LMM3 and LM3 cell lines were established in our laboratory [24] from primary cultures of MM3 and M3 related murine mammary adenocarcinoma, with different metastatic ability [25]. F3II cell line was obtained from the parental adenocarcinoma M3 by clonal dilution [26]. The three cell lines share a high capacity to metastasize in lungs. Cells are cultured at 37°C in humidified 5% CO_2/air atmosphere, in minimum essential medium (MEM) (41500, Gibco BRL) supplemented with 5% fetal calf serum (FCS) (Gen, Argentina) and 80 μg/ml gentamicin (complete medium). All experiments in LMM3 cells were carried out within 160–170 *in vitro* passages. Routine controls were periodically done to check the absence of *Mycoplasma* infection by Hoescht staining.

Organ conditioned media

Conditioned media (CM) were prepared from lung, or from kidney or liver as control organs. The organs were obtained from 10 week old Balb/c normal mice, sliced into 1 mm^3 fragments and carefully washed. Homologous fragments from at least 10 animals were pooled and weighted. They were aliquoted at 0.125 g tissue per ml medium MEM containing 2 mM L-glutamine and 80 μg/ml gentamicin. After 24 h of incubation at 37°C in 5% CO_2 in air, the CM were collected and spun at 720 g for 20 min. The supernatants were centrifuged again at 25,000 g for 30 min. CM were aliquoted and stored at −40°C. Non-conditioned medium alone was also processed in the same way to be used as control.

Cytotoxicity assays

Pure doxorubicin (Dox) was kindly provided by Gador Laboratories (Buenos Aires, Argentina). Cisplatin (Cis) and paclitaxel (Pac) (Sigma) were solubilized in DMSO to a 1000 × solution. In the corresponding experiments, controls were 0.1% DMSO in MEM.

Tumor cells were seeded at a density of 10^4 cells/200 μl of complete medium per well into 96-well plates in triplicate. 24 h later, subconfluent cell cultures were washed with PBS and treated for 2 h with Dox, Cis or Pac. After treatment, cells were thoroughly washed and further incubated with or without 10% v/v of the different organ CM in serum free MEM

or the purified growth factors TGF-β_1 and -β_2 (Calbiochem), EGF (Sigma) or IGF-I (kindly provided by Dr. H. Werner).

To study Fas-dependent apoptosis, LMM3 cells were treated for 48–72 h with an agonistic monoclonal anti-Fas antibody (clone Jo2, Pharmingen) in the presence or absence of LCM. An isotypic antibody (anti-trinitrophenol, Pharmingen) was employed as control.

To investigate serum withdrawal effect, 5–20 $\times 10^3$ cells were seeded in a 96-well plate in complete medium. After 24 h, cells were washed three times with PBS and cultured in serum free MEM with or without 10% LCM.

At different times (24–96 h), cytotoxicity was indirectly evaluated with the Celltiter 96^{TM} non radioactive proliferation assay (Promega). The extent of apoptosis was determined as the amount of cell death after that treatment versus incubation with MEM alone.

Long term viability

The effect of LCM on the ability of LMM3 cells to survive and maintain the proliferative capacity was assessed. Semiconfluent LMM3 monolayers treated with or without 1.2 μM Dox for 2 h at 37°C were washed and further incubated with MEM added with 10% LCM, or 5% FCS or serum-free MEM as controls. 72 h later cells were washed with PBS and fed with MEM added with 10% FCS. The number of surviving cells in each condition was evaluated after 10 days. Then, 10^3–10^4 cells/well were seeded in 96-well plates and the ability to regrow was determined after 48 h by densitometric analysis of toluidine blue stained cells as an indirect measure of cell content.

Assessment of DNA degradation

1×10^6 LMM3 cells were treated for 2 h with 2 μM Dox and further grown for 24–48 h in the presence or absence of LCM. Then attached and detached cells were collected, washed in ice-cold 0.01 M Tris–HCl pH 8.2 and spun at 3000 rpm for 5 min. Both pellets were resuspended in 20 μl of digestion buffer (0.5% w/v SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0), mixed and incubated at 50°C for 2 h with 40 μg proteinase K and then 1 h with 40 μg DNase-free RNase. After incubation, 20 μl of loading buffer were added and 30 μl were then analyzed by a 2% (w/v) agarose gel electrophoresis with ethidium bromide.

Identification of apoptotic cells by acridine orange (AO) staining

After removal of the medium, cells were washed with PBS and one drop of AO solution (10 μg/ml in PBS) was added to unfixed cell monolayers that were immediately examined with a Nikon fluorescence microscope. Rounded cells exhibiting bright green fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic and expressed as a percentage of the total cell number. More than 200 cells were scored for each plate in triplicate.

Flow cytometric analysis of apoptosis

After 24–72 h treatment with Dox and organ CM, adherent and non-adherent cells of each dish were combined, washed with PBS and fixed in 70% ethanol, stained with propidium iodide and analyzed with a flow cytometer (Becton Dickinson). For each treatment, 1200–2500 events were recorded.

Effect of LCM on the tumorigenic and metastatic capacity of Dox-treated LMM3 cells

Subconfluent LMM3 monolayers were treated for 2 h with 1.2 μM Dox, washed and further incubated overnight with MEM with 10% v/v LCM, or 5% FCS or serum-free MEM. 5×10^5 viable cells were injected sc into the left flank of syngeneic Balb/c female mice. Mice were followed along 4 months evaluating tumor take and growth rate.

Western blot assay

Cells were treated with or without Dox and/or LCM for 8 or 24 h and then lysed in 1% Triton-X100 in PBS plus protease inhibitors mix (Sigma). Protein content in cell extracts were determined by the Bradford assay (Bio-Rad), boiled with β-mercaptoethanol and 30 μg of total protein per lane were separated by 12–15% SDS-PAGE and transferred to PDVF membranes (Amersham), which were incubated with either anti-Bcl-2, anti-Bax, anti-Bcl-x or anti-Cyclin-D_1 antibodies (Santa Cruz Biotechnology, cat n°. sc-783, sc-6236, sc-634 and sc-6281, respectively) and detected with the enhanced chemiluminiscence kit (ECL, Amersham). Quantification was done with a GS-700 Densitometer (Bio-Rad). To determine the presence of TGF-β_2 in CM, the CM were concentrated 10 times with a centricon 10 membrane (Chemicon) and 20 μl of concentrated LCM were boiled and separated on a

15% SDS-PAGE. PDVF membranes were incubated with an anti-TGF-β_2 antibody (Santa Cruz, sc-146), and detected as described above. The observed band co-migrated with a purified TGF-β_2 control lane.

Results

Antineoplastic drug-induced apoptosis

A brief exposure (2–4 h) of exponentially growing LMM3 cells to 2 μM Dox, followed by a further incubation in serum-free medium, first showed a marked hypertrophic morphology and cytoplasmic granules surrounding the nuclear envelope. After 48 h, typical apoptosis features, namely, condensed chromatin, nuclear fragmentation, membrane budding and appearance of apoptotic bodies were evident (Figure 1A). Apoptotic death was further confirmed by DNA laddering assay, as shown in Figure 1B. Furthermore, Dox treatment induced cell death in a dose-dependent fashion. An IC_{50} dose of about 1.5 μM was determined after 48 h by the MTS assay (Figure 1C). The number of apoptotic cells was also dose-dependent (data not shown). Similar results were obtained in LMM3 cells treated with Cis (Figure 1D) and Pac (Figure 1E).

Effect of LCM on drug-induced cytotoxicity

Incubation of LMM3 cell line in the presence of LCM after Dox treatment significantly inhibited apoptosis (Figures 1A and 1B). This ability to suppress apoptosis was evident even at high Dox doses, up to 4 μM (Figure 1C), and persisted for at least 72 h after drug treatment (data not shown). The same results were obtained after Cis treatment (Figure 1D and data not shown). Interestingly, LCM was unable to modify Pac-induced cell death (Figure 1E).

As can be seen in Figure 2A, the related LM3 and F3II cell lines were protected from Dox induced apoptosis by LCM similarly to LMM3 cells. In the following figures, only results for LMM3 cells are shown.

Counting of apoptotic cells in monolayers stained with AO at 48 h after Dox exposure revealed a significant reduction in the number of apoptotic cells when cultures were treated with LCM (Figure 1A and 2B). Cells receiving both Dox and LCM treatments exhibited an important augment in size compared to controls or single treated cells, suggesting that a very large fraction of cells could be in the G_2 phase of cell cycle (Figure 1A and data not shown). LCM also diminished apoptotic cell number in control cultures, to a level similar to FCS treatment (Figure 2B). In contrast, pre-treatment of LMM3 cells with LCM 6–120 h before Dox exposure did not prevent Dox-induced apoptosis (Figure 2C).

As can be seen in Figure 2B, FCS is a potent inhibitor of apoptosis in these cells. Thus, induction of apoptosis and inhibition assays were done in the absence of serum to avoid interference.

Effect of conditioned media from non-target organs

To determine if the activity of LCM could be mimicked by other organ CM, similar experiments to those shown in Figure 2 were done, but cells were incubated in the presence or absence of 10% or 50% v/v kidney CM (KCM) or liver CM (HCM) after Dox treatment (Figure 3 and data not shown). Neither of both CM was able to inhibit or reduce Dox cytotoxic effect on LMM3 cells, as measured by MTS assay (Figure 3A) or AO staining (Figure 3B). Furthermore, these experiments showed that HCM has itself cytotoxic activity on LMM3 cells.

Effect of CM on cell cycle distribution of Dox-treated LMM3 cells

Cell cycle distribution and loss of DNA fragments were revealed by propidium-iodide staining followed by flow cytometer analysis. LMM3 cells treated with Dox and incubated for 72 h in serum-free medium exhibited a lowering of G_1 peak associated to an important subdiploid (sub-G_1) peak, typical of apoptotic cells, together with an increase in the percentage of cells in G_2/M when compared to control non treated cells (Table 1). LMM3 cells exposed to Dox and post-treated with KCM or HCM showed a marked enhancement of sub-G_1 peak, associated to a significant reduction of the other peaks (Table 1 and data not shown). In contrast, the combined treatment with Dox followed by LCM resulted in an important reduction in the apoptotic sub-G_1 peak together with a marked retardation of cells in the G_2/M stage, in agreement to the prolonged survival and resistance to apoptosis *in vitro* (Table 1). The same results were obtained after evaluation on a single-cell basis, using a computer-assisted evaluation cell analysis system (CAS) of cultures grown on coverslips (data not shown).

As no mitosis was observed at the phase-contrast or fluorescence microscopy up to 72 h after Dox ex-

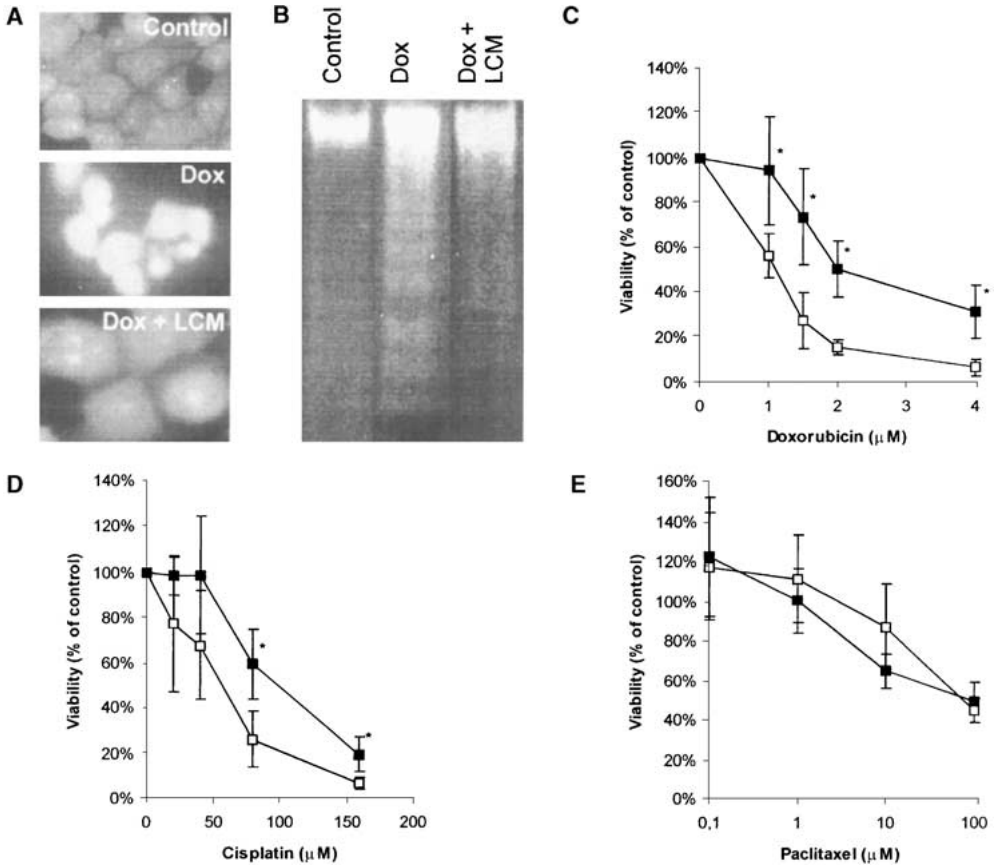

Figure 1. Induction of apoptosis by antineoplastic drug treatment and effect of LCM. (A) Representative fields from Acridine Orange-stained cultures treated with or without 2 μM Dox and 10% LCM (original magnification, 400 ×). (B) DNA laddering assay from cells treated as in (A). (C–E) Dose-dependent cytotoxicity of LMM3 cultures treated with Dox (C), Cis (D) or Pac (E) (□) or with the drug plus LCM (■), determined by MTS assay. *, $p < 0.05$ LCM versus control for each drug concentration (Tuckey test), $n = 3$. These results are representative of at least three independent experiments.

posure, the raise in G_2/M peak suggests a specific block of cells in G_2. Incubation with 10% LCM did not prevent this block. However, incubation with 50% LCM induced a small percentage of mitotic figures after 72 h (data not shown).

Inhibition of Fas-induced apoptosis by LCM

The Fas death receptor is a well-known inducer of apoptosis upon specific ligation and is one of the mediators of cytotoxic T lymphocytes cytotoxicity [27]. A monoclonal Fas antibody induced apoptosis in LMM3 cells in agreement with other reports [28, 29], although the response was delayed compared to other systems (Figure 4A). In a similar way to Dox-, Cis- and serum withdrawal-induced cell death, LCM can significantly prevent Fas-induced apoptosis, as shown by MTS assay (Figure 4B) and AO staining (Figure 2B). The level of protection was similar to that observed after FCS treatment (Figure 2B). No loss of viability was observed after treatment with 0.5 μg/ml isotypic control antibody (data not shown).

LCM-induced long term viability

Although the level of apoptosis induced by 2 or 3 days incubation without serum is low in LMM3 cells (Figure 2B), long-term serum withdrawal promotes high levels of cell death. Incubation of these cells for 10 days with LCM in the absence of serum completely inhibited the induced apoptosis. Furthermore, cells were able to grow and form monolayers in these conditions (data not shown).

To study the ability of LCM to protect cells from drug induced apoptosis on a long-term basis, exponentially growing LMM3 cells were treated with Dox

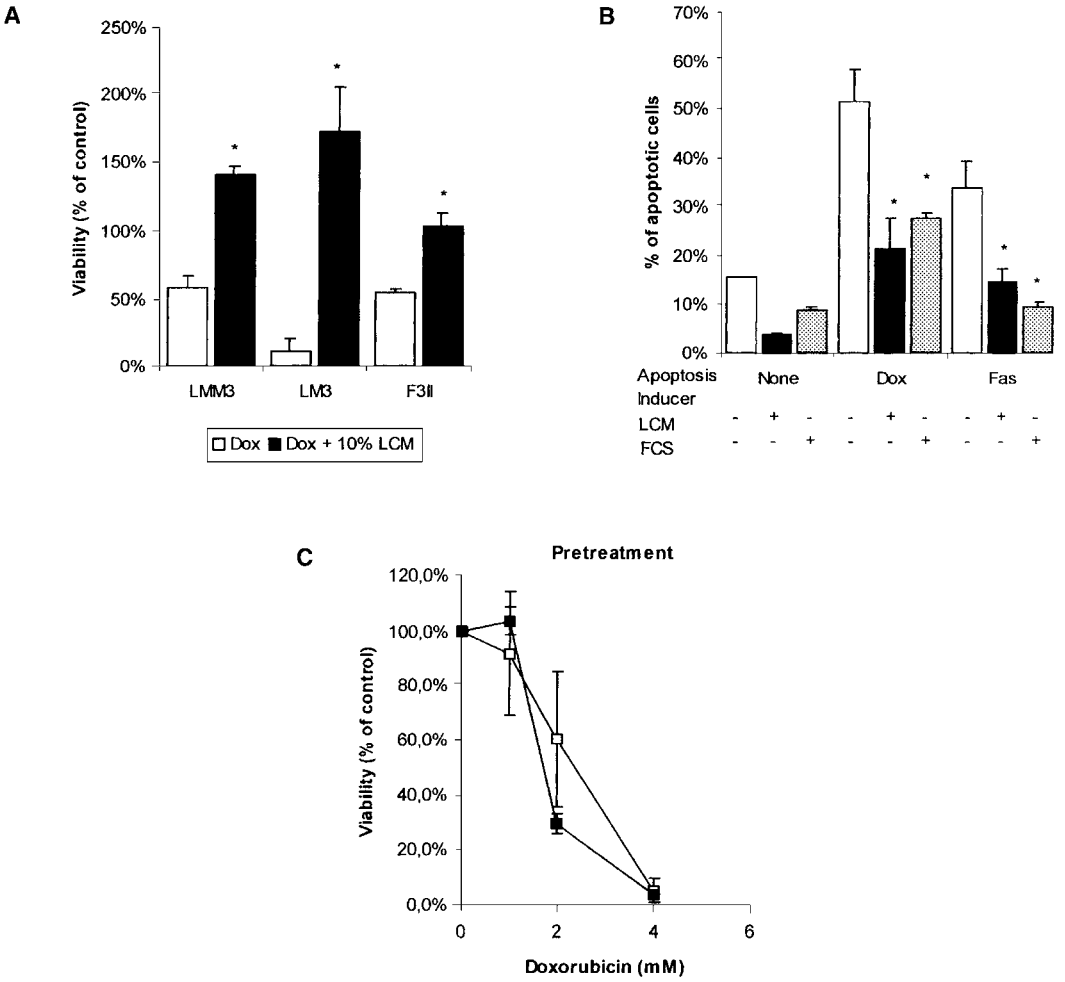

Figure 2. (A) Comparison of LCM effect on LMM3, LM3 and F3II cell lines survival after 2 μM Dox treatment, as measured by MTS assay. (B) Percentage of apoptotic cells measured by AO staining. Cells were left untreated or treated with 2 μM Dox or with 0.5 μg/ml of an agonistic anti-Fas antibody in the presence or absence of 10% LCM or 5% FCS. At least 250 cells in random fields were recorded in each culture. (C) Lack of effect of LCM pretreatment on Dox-induced apoptosis. LMM3 cells were pretreated with 10% LCM (■) or MEM alone (□). After 6 h, medium was replaced with MEM with or without Dox and cell viability was determined by MTS assay after 48 h of incubation. *, $p < 0.05$ versus MEM for each apoptosis-inducing treatment (Two Way ANOVA), (A) $n = 3$, (B) $n = 2$, (C) $n = 3$. These results are representative of three independent experiments.

for 2 h and then were incubated with 10% of LCM, or 5% FCS or serum-free MEM (control). After 72 h, only scarce cells were alive in the control group while about 50% did so in the other two groups. Cells remaining attached were washed and fed every two days with MEM plus 10% FCS. After 10 days, a high percentage of control and FCS treated cells had died, while LMM3 cells treated with LCM not only survived but also proliferated and gave rise to colonies with mitotic figures and no sign of apoptosis as observed in a phase-contrast microscope. Count of viable cells of these cultures confirmed this observation (data not shown). The cells were then trypsinized and seeded at 10^3–10^4 cells/well in 96 well plates in complete medium. As shown in Figure 5, cells treated with LCM or FCS showed a three-fold increase in their capacity to regrow after Dox treatment, compared to control cells treated with MEM alone.

Tumorigenic capacity

To examine whether the prevention of Dox-induced apoptosis exhibited *in vitro* by LCM had any *in vivo* correlate, we have tested the tumorigenic capacity of

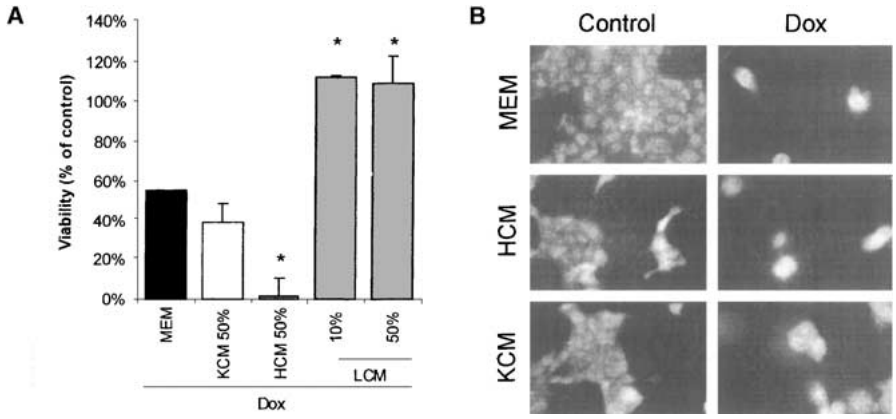

Figure 3. Lack of effect of HCM and KCM on Dox-induced apoptosis. LMM3 cells were treated or not with 2 μM Dox and then incubated in the presence or absence of KCM, HCM or LCM. (A) Viability as measured by MTS assay. (B) Representative fields of AO-stained cultures treated with or without 10% HCM or 10% KCM. *, $p < 0.05$ versus Control (Tuckey test), $n = 3$. These results are representative of three independent experiments.

Table 1. Flow cytometry analysis of LMM3 cells showing the effect of LCM and KCM on DNA content after Dox treatment

Dox	CM	Stage			
		Pre G_1	G_1	S	G_2/M
−	−	21.5	65.3	4.2	7.0
+	−	36.1	12.8	14.4	35.3
+	KCM	60.5	13.7	13.7	11.4
+	LCM	24.7	10.9	12.3	50.3

Cells were treated with or without 2 μM Dox and then incubated with MEM alone, LCM or KCM as indicated. The cell cycle distribution and the percentage of apoptotic cells (Pre-G_1) are shown for each group. These results are representative of at least three independent experiments.

LMM3 cells after Dox exposure. LMM3 cultures were incubated with Dox and then treated with LCM, FCS or MEM overnight. Cells were then trypsinized and injected subcutaneously into syngeneic (Balb/c) mice. After 4 months, growing tumors were observed in four of six (66%) mice injected with LCM treated cells, whereas FCS or MEM-treated cells were incapable (zero out of six mice each) to produce any tumors or metastasis. The histopathological analysis of tumors and lung metastasis showed the characteristic features of a poorly differentiated adenocarcinoma (Figure 6), suggesting that LMM3 cells treated with LCM after Dox exposure maintain a similar phenotype than that of the untreated LMM3 cell line [24]. All mice bearing tumors showed spontaneous lung metastasis, but no metastasis in other organs were observed.

Effect of purified growth factors on Dox-induced apoptosis

In an attempt to identify the molecule/s responsible for the LCM apoptosis-protecting effect, we next investigated whether purified growth factors with known apoptosis-modulating activity and known to be present in the lungs, were capable to protect these cells against Dox-induced apoptosis. Figure 7A–C shows experiments in which IGF-I, EGF or TGF-β activities were compared with LCM and FCS protective capacity. While neither IGF-I nor EGF protected LMM3 cells against apoptosis, surprisingly, TGF-β_1 and -β_2 significantly diminished apoptosis induced by Dox treatment (Figure 7C and data not shown). TGF-β_1 or -β_2 treatments, while promoting survival, did not promote growth in the absence of serum (not shown). TGF-β_2 presence in LCM was detected by western blot (Figure 7C, inset).

Modulation of Bcl-2 protein family and Cyclin D_1 by LCM

To get insight into the mechanisms of protection by LCM treatment, analysis of Bcl-2 protein family expression was done by western blot. As shown in Figure 8, while the treatment with Dox of LMM3 cells induced a 2-fold reduction Bcl-2 expression, post Dox incubation with LCM for 8 h inhibited Dox-induced reduction of Bcl-2 expression. Furthermore, 24 h incubation of LMM3 cells with LCM induced a marked enhancement of Bcl-2 expression by itself, to a level similar to that observed with FCS treat-

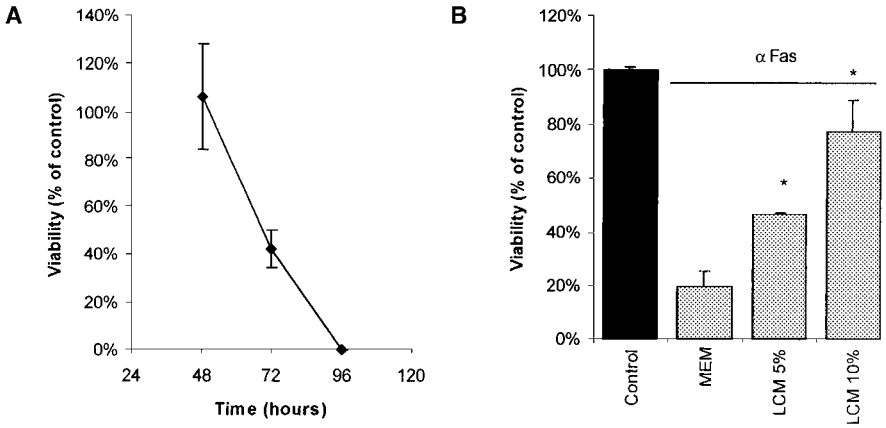

Figure 4. Effect of LCM on Fas-induced apoptosis. (A) Time course of apoptosis induced by an agonistic anti-Fas antibody (0.5 μg/ml). At 96 h there were no living cells in the wells treated with the antibody. (B) Effect of 5% or 10% LCM treatment in the presence or absence of the same antibody. *, $p < 0.05$ versus anti-Fas alone (MSD test), $n = 3$. This experiment was repeated with similar results.

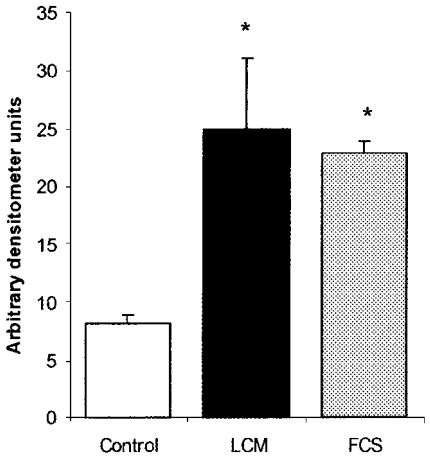

Figure 5. Effect of LCM on the regrowth capacity of LMM3 cells after Dox treatment. LMM3 cell cultures were treated with Dox 1.2 μM and then incubated 3 days with or without 10% LCM or 5% FCS and then with 5% FCS. After another 7 days, the cells were trypsinized, counted, replated and incubated to allow regrowing. Densitometric analysis of cells stained with toluidine blue is shown. *, $p < 0.05$ versus MEM (Student t Test), $n = 3$.

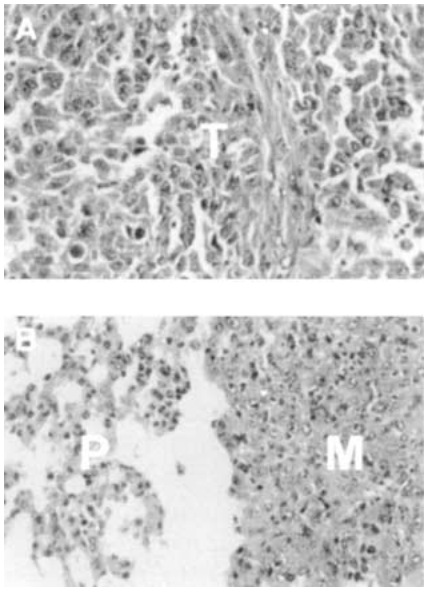

Figure 6. Tumorigenic capacity of LMM3 cells treated with 1.2 μM Dox and 10% LCM. When injected subcutaneously into the flank of syngeneic (Balb/c) mice, these cells formed poorly differentiated adenocarcinomas (A) and were able to spontaneously metastasize the lungs (B) as shown in sections stained with hematoxylin and eosin (400 X). T: tumor; P: lung parenchyma; M: metastasis. Results are representative of two independent experiments.

ment (Figure 8A). Dox also induced 2-fold increase on Bax expression, which could be inhibited partially by posterior treatment with LCM in some experiments (Figure 8B and data not shown). Neither Dox nor LCM could by itself modulate Bcl-X_L expression, but, surprisingly, co-treatment with Dox and LCM induced a three to five-fold increase Bcl-X_L expression (Figure 8C).

Dox induced a 2-fold decrease in Cyclin-D_1 expression in similar experiments (Figure 8D). In agreement to the absence of mitosis observed after 8 h Dox and LCM co-treatment, this reduction could not be inhibited by incubation of these cells with LCM.

Discussion

Several observations have suggested that the organ microenvironment can influence the response of tumors

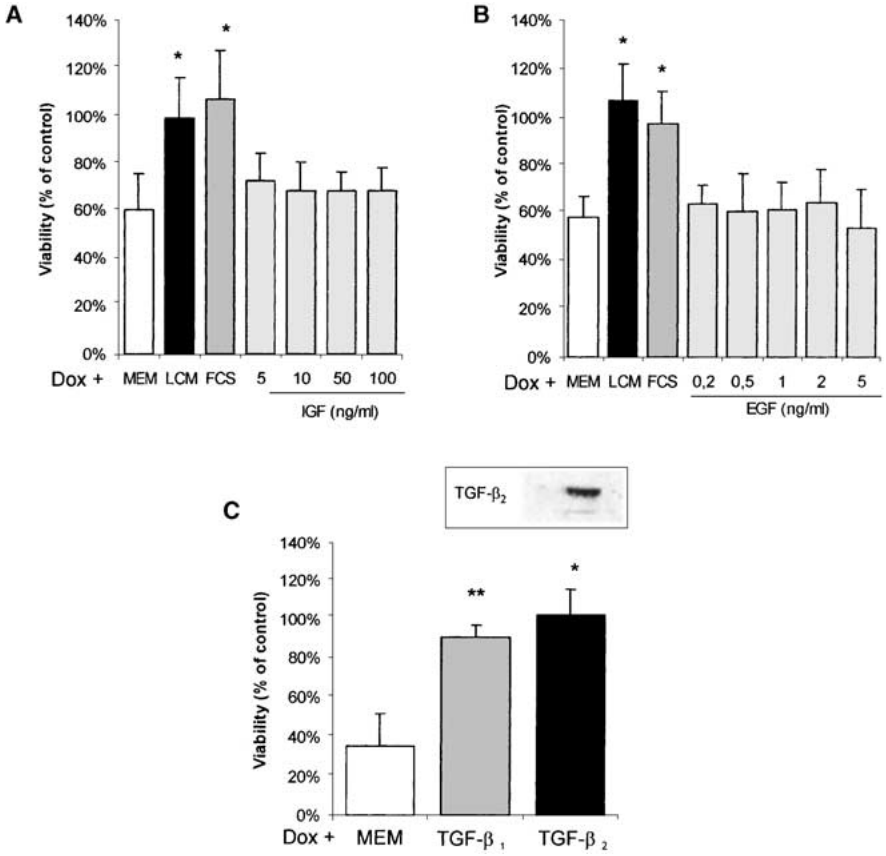

Figure 7. Effect of purified growth factors on LMM3 viability after Dox treatment. LMM3 cells were treated with 2 μM Dox and then incubated with different concentrations of IGF-I (A) or EGF (B). Positive and negative controls were incubated with 10% LCM, 5% FCS or MEM alone, as indicated. (C) Cells were treated with or without Dox 2 μM and then incubated in MEM alone or MEM with 4 ng/ml TGF-β_1 or TGF-β_2 *, $p < 0.05$ (Tuckey test), $n = 3$. Similar results were obtained in other two independent experiments. Inset in (C) shows the presence of TGF-β_2 in LCM as revealed by western blot assay.

to chemotherapy. Experimental studies on mice have demonstrated that direct interaction of tumor cells with target stromal cells [18], paracrine modulation from the latter [9, 13, 16, 17] or ECM components [30] can profoundly influence tumor cell behavior. Furthermore, direct *in vivo* studies demonstrate that metastatic cells can survive and grow in the target organ, while non-metastatic cells cannot [3]. Aoudjit et al. [31] have reported that two murine lymphoma cell lines with different metastatic capacity in kidney, liver and spleen have the same ability to colonize those organs after 48 h post-injection, suggesting that their different metastatic ability is a post-homing characteristic.

The present study was designed to determine the ability of soluble factors present in target organ of metastasis for regulating tumor cell response to various apoptosis-inducing treatments. LMM3 tumor cells are highly metastatic in lung [24]. We showed that Dox, Cis and Pac were capable to induce apoptosis in LMM3 cells in a dose dependent fashion. However, treatment of LMM3 cell cultures with LCM during 24–72 h significantly enhanced cell survival after Dox and Cis treatments with doses up to those corresponding to the IC_{90} for each drug. CM from liver and kidney, which are never colonized by these cells, were not able to rescue LMM3 cells from apoptosis.

Apoptosis is characterized by a decrease in the nuclear DNA content. The cytometric analysis of LMM3 cultures exposed to Dox showed a strong sub-G_1 peak that was markedly reduced when cells were incubated with LCM. Instead, cells treated with CM from kidney and liver showed an important sub-G_1 peak, confirming the inability of these CM to overcome Dox-induced apoptosis in LMM3 cells.

LCM can also inhibit Fas- and serum withdrawal-induced apoptosis. Fas has recently been suggested

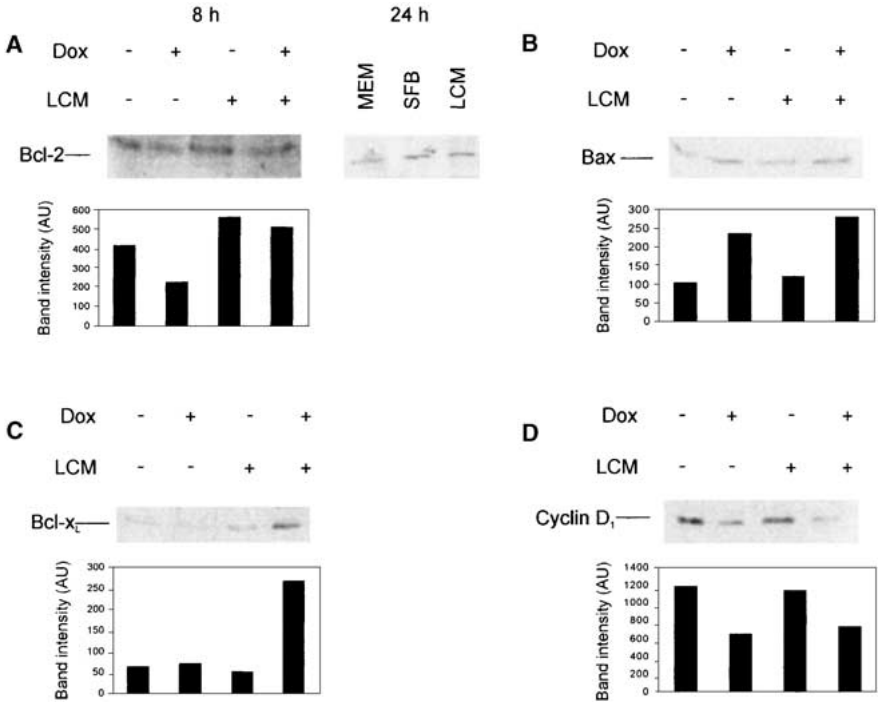

Figure 8. Effects of Dox and LCM on protein expression. Whole cell lysates from LMM3 cell cultures, treated with or without 2 μM Dox and/or 10% LCM as indicated, were assayed for Bcl-2 (A), Bax (B), Bcl-x_L (C) and Cyclin D_1 (D) expression. The graphs below the gels show the densitometric analysis of the corresponding band. (A) Bcl-2 expression after 8 h (left) or 24 h (right). (B–D) Expression after 8 h of incubation. This experiment was repeated with similar results.

to mediate drug, p53 and serum withdrawal-induced apoptosis as well [32–34], and studies are undergoing to know whether the Fas pathway is involved in LMM3 Dox- and serum withdrawal-induced apoptosis. If indirect Fas activation is involved, LCM could prevent apoptosis induced by several distinct inducers in a common step. Alternatively, the survival factors present in LCM could inhibit apoptosis by different, stimulus-dependent mechanisms.

IGF-I and EGF are well-known factors that can inhibit apoptosis in different systems [35–37], although EGF may increase antineoplastic drug sensitivity in some models [38]. We studied whether purified growth factors could mimic the effect of LCM on LMM3 growth and survival. We demonstrated that IGF-I and EGF lacked of any effect on these cells. We have previously established that the parental tumor MM3 expresses both IGFs and functional IGF-Rs [39], and recently we found that LMM3 cell line expresses at least IGF-R 1 (unpublished data), so we expected that IGF would be able to reduce Dox and Fas sensitivity. A possible explanation for the absence of protecting activity is that these cells may have a fully active IGF autocrine loop, so addition of more IGF would not increase survival. In fact, the sensitivity of these cells to Dox is very low, compared to other systems [30, 40, 41]. TGF-β, in contrast, is known to inhibit growth and promote apoptosis in a variety of normal and tumor cells [42–44]. Surprisingly, TGF-β could inhibit, at least in part, Dox induced apoptosis. We also showed that TGF-β_2 is present in LCM, although this molecule was found in KCM and HCM also (not shown). In addition, TGF-β did not allow growth in serum deprived media (unpublished data), suggesting that other(s) factor(s) with survival and/or proliferative activity may be present in LCM and that not only one molecule, but a sum of many, may be responsible for the observed effects.

Bcl-2 family proteins localize mainly in intracellular membranes, where they interact with each other [6, 19], and it is believed that the overall state of these proteins determines whether apoptosis will occur [20]. Exposure of LMM3 cultures to Dox reduces the expression of Bcl-2, while augments expression of Bax. This shift from an antiapoptotic state to a proapoptotic one could be necessary for the induction of apoptosis by the drug [20]. LCM only slightly modulated the increase in the expression of Bax, but it inhibited the

reduction of, and enhanced by itself, Bcl-2 expression. If apoptosis is induced by the shift in Bcl-2 protein family expression, this effect of LCM on Bcl-2 expression could be sufficient to inhibit Dox induced apoptosis. Furthermore, the 3- to 5-fold increase in Bcl-X_L expression in co-treated cells could be important in maintaining the antiapoptotic state. Little is known about the biochemical function of these proteins and to what extent they modulate apoptosis. In fact, Bcl-2 and Bcl-x_L can be cleaved and inactivated by caspases [45, 46] and overexpression of a caspase-resistant form of Bcl-x_L, but not wild type Bcl-x_L, promotes clonogenic survival after ionizing radiation [47]. Bcl-2 can also be inactivated by phosphorylation after Pac exposure [48]. Interestingly, LCM was unable to inhibit Pac-induced apoptosis, raising the possibility that LCM-induced survival is dependent on the non-phosphorylated form of Bcl-2. In some models, Bcl-2 is capable to delay, but not inhibit, apoptosis [49]. We have detected a ∼15 kDa band that may correspond to a proteolytic fragment of Bcl-2 (not shown), but we always detected full length Bcl-2 protein, so the significance of proteolysis in this model is not known.

Flow cytometry analysis showed that Dox treatment induces an augment in the percentage of cells in the G_2/M that was further increased by LCM co-treatment, suggesting that cells surviving the Dox treatment are arrested in the G_2 stage of the cell cycle, at least up to 72 h of post-treatment. We also found that LCM treatment prolonged survival in the long term and increased the regrowth ability and the tumorigenic and metastatic capacity of LMM3 cultures exposed to Dox. The significant increase on long-term survival, both *in vitro* and *in vivo*, demonstrates that LCM acts indeed by reducing apoptosis and not merely by delaying the effect of the apoptosis-inducing treatments. The cell cycle arrest after Dox and LCM co-treatment could allow these cells to trigger DNA damage responses and permit the correction of the damages before the next mitotic cycle, thus promoting long term survival. In agreement with this hypothesis, mitotic figures in Dox and LCM treated cells appear only after several days of culture. In addition, we showed that Dox treatment, either in the presence or absence of LCM, induced a two-fold decrease in Cyclin-D_1 expression. In agreement with this hypothesis, the latency period was strongly increased from ∼11 days in untreated cells [24] to 3–4 months after the combined treatment.

Nevertheless, LCM may promote survival by other mechanisms. This is supported by the fact that LCM inhibits not only Dox-induced cytotoxicity, but also Fas- and serum withdrawal-induced apoptosis, suggesting that LCM activity may be exerted at a common level of apoptosis regulation, such as the modulation of NF-κB, PKB, IAPs or Bcl-2 protein family [50–52], and we have shown that LCM influences Bcl-2 protein family expression in a complex manner.

We have previously shown that the factors released from the lung promote growth, migration, enhancement of secreted uPA activity and angiogenesis [9, 11, 23], and this work shows that it can also inhibit apoptosis, induced by diverse mechanisms, of tumor cell lines metastatic to lung. Our results contribute to the knowledge that survival and growth in the target organ of metastasis are key steps in metastasis formation and that the new microenvironment plays an important role modulating the fate of the metastatic cell and thus 'deciding' whether the metastatic cell will succeed or die. Inhibition of these pathways, either at the signal transduction level or by blockade by specific antagonists or antibodies of the survival factors involved would eliminate metastatic cells or induce a quiescence state and tumor dormancy for prolonged periods. In either case, this effect will reduce or inhibit metastasis, and will surely increase the survival of patients in advanced stages of cancer.

Acknowledgements

We thank Gloria Solarz for excellent technical assistance. This work was supported in part by grants from the University of Buenos Aires, from ANPCYT (PICT 00712 and 05-6114), from CONICET (PIP 4199) and from the National Health Ministry Grant R. Carrillo – A. Oñativia. A. Adam is a recipient of a postgraduate fellowship from CONICET.

References

1. Liotta LA, Stetler-Stevenson WG: Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res 51: 5054–5059, 1991
2. Park CC, Bissell MJ, Barcellos-Hoff MH: The influence of the microenvironment on the malignant phenotype. Mol Med Today 6: 324–329, 2000
3. Chambers AF, MacDonald IC, Schmidt EE, Koop S, Morris V, Khokha R, Groom AC: Steps in tumor metastasis: new concetps from intravital videomicroscopy. Cancer Met Rev 14: 279–301, 1995

4. McDonnell TJ, Meyn RE, Robertson LE: Implications of apoptotic cell death regulation in cancer therapy. Sem Cancer Biol 6: 53–60, 1995
5. Gottlieb TM, Oren M: *p53* in growth control and neoplasia. Biochim Biophys Acta 1287: 77–102, 1996
6. Strasser A, Huang DCS, Vaux DL: The role of the *bcl-2/ced-9* gene family in cancer and general implications of defects in cell death control for tumorigenesis and resistance to chemotherapy. Biochim Biophys Acta 1333: F151–F178, 1997
7. Gutérrez MI, Cherney B, Hussain A, Mostowski H, Tosato G, Magrath I, Bhatia K: Bax is frequently compromised in Burkitt's lymphomas with irreversible resistance to Fas-induced apoptosis. Cancer Res 59: 696–703, 1999
8. Friesen C, Herr I, Krammer PH, Debatin K-M: Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat Med 2: 574–577, 1996
9. Bal de Kier Joffé E, Alonso DF, Puricelli L: Soluble factors released by the target organ enhance the urokinase-type plasminogen activator activity of metastatic tumor cells. Clin Expl Metastasis 9: 51–56, 1991
10. Staroselsky AN, Radinsky R, Fidler IJ, Pathak S, Chernajovsky Y, Frost P: The use of molecular genetic markers to demonstrate the effect of organ environment on clonal dominance in human renal cell carcinoma grown in nude mice. Int J Cancer 51: 130–138, 1992
11. Puricelli L, Gómez DE, Vidal MC, Eiján AM, Spinelli O, Alonso DF, Lustig ES, Bal de Kier Joffé E: Effect of host-organ environment on the *in vivo* and *in vitro* behaviour of a murine mammary adenocarcinoma. Tumor Biol 15: 284–293, 1994
12. Radinsky R: Modulation of tumor cell gene expression and phenotype by the organ-specific metastatic environment. Cancer Met Rev 14: 323–338, 1995
13. Walker A, Taylor ST, Hickman JA, Dive C: Germinal center-derived signals act with *bcl-2* to decrease apoptosis and increase clonogenicity of drug-treated human B lymphoma cells. Cancer Res 57: 1939–1945, 1997
14. Schmidt C, Laporte A, de Baetselier P: Differential responsiveness of murine T lymphomas to local growth and invasion factors may determine metastasis formation in the ovaries. Inv Metast 17: 53–61, 1997
15. Shain KH, Landowski TH, Dalton WS: The tumor microenvironment as a determinant of cancer cell survival: a possible mechanism for de novo drug resistance. Curr Op Oncol 12: 557–563, 2000
16. Dong Z, Radinsky R, Fan D, Tsan R, Bucana CD, Wilmanns C, Fidler IJ: Organ-specific modulation of steady-state *mdr* gene expression and drug resistance in murine colon cancer cells. J Natl Cancer Inst 86: 913–920, 1994
17. Nicholson GL: Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. Cancer Met Rev 12: 242–325, 1993
18. Kataoka S, Naito M, Fujita N, Ishii H, Ishii S, Yamori T, Nakajima M, Tsuruo T: Control of apoptosis and growth of malignant T lymphoma cells by lymph node stromal cells. Exp Cell Res 207: 271–176, 1993
19. Gross A, McDonnell JM, Korsmeyer SJ: Bcl-2 family members and the mitochondria in apoptosis. Genes Dev 13: 1899–1911, 1999
20. Reed JC: Balancing cell life and death: bax, apoptosis, and breast cancer. J Clin Invest 97: 2403–2404, 1996
21. Wong CW, Lee A, Shientag L, Yu J, Dong Y, Kao G, Al-Mehdi AB, Bernhard E, Muschel RJ: Apoptosis: an early event in metastatic inefficiency. Cancer Res 61: 333–338, 2001
22. Liu R, Page C, Beidler DR, Wicha MS, Núñez G: Overexpression of Bcl-x_L promotes chemotherapy resistance of mammary tumors in a syngeneic mouse model. Am J Pathol 155: 1861–1867, 1999
23. Davel LE, Puricelli LI, Vidal MC, De Lorenzo MS, Lustig ES, Bal de Kier Joffé E: Soluble factors from the target organ enhance tumor cell angiogenesis: role of laminin SIKVAV sequence. Oncol Reports 6: 907–911, 1999
24. Urtreger A, Ladeda V, Puricelli L, Rivelli A, Vidal MC, Lustig ES, Bal de Kier Joffé E: Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastatic phenotype in two murine mammary cell lines. Int J Oncol 11: 489–496, 1997
25. Bal de Kier Joffé E, Puricelli L, Vidal MC, Lustig ES: Characterization of two murine mammary adenocarcinoma tumors with different metastatic ability. J Exp Clin Cancer Res 2: 151–160, 1983
26. Alonso DF, Farías EF, Urtreger A, Ladeda V, Vidal MC, Bal de Kier Joffé E: Characterization of F3II, a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a mouse adenocarcinoma. J Surgical Oncol 62: 288–297, 1996
27. Nagata S: Apoptosis by death factor. Cell 80: 355–365, 1997
28. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P: Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science 265: 528–530, 1994
29. Ogasawara J, Suda T, Nagata S: Selective apoptosis of CD4+ CD8+ thymocytes by the anti-Fas antibody. J Exp Med 181: 485–491, 1995
30. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS: Cell adhesion mediated drug resistance (CAM-DR): Role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 93: 1658–1667, 1999
31. Aoudjit F, Potworoski EF, St-Pierre Y: The metastatic characteristics of murine lymphoma cell lines *in vivo* are manifested after target organ invasion. Blood 91: 623–629, 1998
32. Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel M-T: Sensitization of cancer cells treated with cytotoxic drugs to Fas-mediated cytotoxicity. J Natl Cancer Inst 89: 783–789, 1997
33. Bennet M, Macdonald K, Chan S-W, Luzio JP, Simari R, Weissberg P: Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science 282: 290–293, 1998
34. Le-Niculescu H, Bonfoco E, Kasuya Y, Claret F-X, Green DR, Karin M: Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas Ligand induction and cell death. Mol Cell Biol 19: 751–763, 1999
35. Resnicoff M, Abraham D, Yutanawiboonchai W, Rotman HL, Kajstura J, Rubin R, Zoltick P, Baserga R: The insulin-like growth factor I receptor protects tumor cells from apoptosis *in vivo*. Cancer Res 55: 2463–2469, 1995
36. Dunn SE, Hardman RA, Kari FW, Barret JC: Insulin-like growth factor 1 (IGF-1) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. Cancer Res 57: 2687–2693, 1997
37. Sleijfer S, Asschert JGW, Timmer-Bosscha H, Mulder NH: Enhanced sensitivity to tumor necrosis factor-α in doxorubicin-resistant tumor cell lines due to down-regulated *c-erbB2*. Int J Cancer, 77: 101–106, 1998

38. Dixit M, Yang J-L, Poirier MC, Price JO, Andrews PA, Arteaga C: Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA. J Natl Cancer Inst 89: 365–373, 1997
39. Guerra FK, Eiján AM, Puricelli L, Alonso DF, Bal de Kier Joffé E, Kornblihtt AR, Charreau EH, Elizalde PV: Varying paterns of expression of insulin-like growth factors I and II and their receptors in murine mammary adenocarcinomas of different metastasizing ability. Int J Cancer 65: 812–820, 1996
40. Nooter K, Boersma AWM, Oostrum RG, Burger H, Joschemsen AG, Stoter G: Constitutive expression of the *c-H-ras* oncogene inhibits doxorubicin-induced apoptosis and promotes cell survival in a rhabdomyosarcoma cell line. Brit J Cancer 71: 556–561, 1995
41. Lotem J, Sachs L: Cytokine suppression of protease activation in wild-type p53-dependent and p53-independent apoptosis. Proc Natl Acad Sci 94: 9349–9353, 1997
42. Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, Bast Jr. RC, Berchuck A: Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor β. Cancer Res 55: 944–948, 1995
43. Ashley DM, Kong FM, Bigner DD, Hale LP: Endogenous expression of transforming growth factor β1 inhibits growth and tumorigenicity and enhances Fas-mediated apoptosis in a murine high-grade glioma model. Cancer Res 58: 302–309, 1998
44. Chen R-H, Chang MC, Su Y-H, Tsai Y-T, Kuo M-L: Interleukin-6 inhibits transforming growth factor-β-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway and signal transducers and activators of transcription 3 pathways. J Biol Chem 274: 23013–23019, 1999
45. Cheng EH-Y, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM: Conversion of *bcl-2* to a Bax-like death effector by caspases. Science 278: 1966–1968, 1997
46. Clem R, Cheng EH-Y, Karp CL, Kirsch DG, Ueno K, Takahashi A, Kastan MB, Griffin DE, Earnshaw WC, Veliuona MA, Hardwick M: Modulation of cell death by Bcl-x_L through caspase interaction. Proc Natl Acad Sci 95: 554–559, 1998
47. Rehemtulla A, Hamilton AC, Taneja N, Fridman J, Juan TSC, Maybaum J, Chinnaiyan A: A caspase-resistant form of Bcl-x_L, but not wild type Bcl-x_L, promotes clonogenic survival after ionizing radiation. Neoplasia 1: 63–70, 1999
48. Blagosklonny MK, Giannakakou P, El-Deiry WS, Kingston DGI, Higgs PI, Neckers L, Fojo T: Raf-1/Bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 57: 130–135, 1997
49. Kyprianou N, King ED, Bradbury D, Rhee JG: Bcl-2 overexpression delays radiation-induced apoptosis without affecting the clonogenic survival of human prostate cancer cells. Int J Cancer 70: 341–348, 1997
50. Wang C-Y, Mayo MW, Korneluk RG, Goeddel DV, Baldwin Jr. AS: NF-κB antiapoptosis: induction of Traf1 and Traf2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281: 1680–1683, 1998
51. Kulik G, Weber MJ: Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. Mol Cell Biol 18: 6711–6718, 1998
52. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: Akt phosphorylation of Bad couples survival signals to the cell-intrinsic death machinery. Cell 91: 231–241, 1997

Address for offprints and correspondence: Elisa Bal de Kier Joffé, Research Area, Institute of Oncology 'Angel H. Roffo', Av. San Martín 5481, (C1417DTB) Buenos Aires, Argentina; *Tel.:* (5411) 4504-7884; *Fax:* (5411) 4580-2811;
E-mail: elisabal@fmed.uba.ar