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VERAPAMIL INHIBITS TUMOR PROTEASE PRODUCTION, LOCAL INVASION AND METASTASIS DEVELOPMENT IN MURINE CARCINOMA CELLS

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The invasion and metastasis process involves degradation of the extracellular matrix mediated by tumor- and host-produced proteolytic enzymes. The main enzymes involved in this process are urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases (MMPs). Calcium is a main co-factor in the signaling pathways that regulate cell proliferation and protease production. We have studied here the effect of verapamil, a calcium channel blocker widely used to treat hypertensive diseases, on local tumor growth, spontaneous and experimental metastasis development, tumor-associated protease production and circulating MMP activity in tumor-bearing mice. BALB/c mice treated for 45 days with verapamil showed no toxic effects. Oral administration of verapamil to mice injected with F3II tumor cells, either pre-treated or not with verapamil, showed a significant decrease of local tumor invasion and both spontaneous and experimental metastasis development (51.3% inhibition of metastasis in both cases, $p < 0.01$). uPA and MMP-9 production by tumor cells *in vitro* was significantly inhibited by verapamil in a dose-dependent manner, showing a long-term inhibition after removal of the drug. Verapamil also exhibited a marked cytostatic effect on F3II cell proliferation *in vitro.* In addition, circulating MMP activity, usually enhanced in tumor-bearing mice, diminished significantly with all verapamil treatments. Our results suggest that modulation of the calcium-dependent signaling pathways that regulate tumor- or host-dependent production of proteases and tumor cell proliferation could contribute to the inhibition of metastasis development. Finally, we describe the inhibitory effects of a commonly used hypotensor in humans, verapamil, on the invasive and metastatic capacity of mammary tumor cells.
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The development of tumor metastasis is a complex process that, in order to be successful, requires multiple steps which depend on protein expression by host and tumor cells (Liotta and Stetler-Stevenson, 1991). The main events of this process include cellular invasion, intravasation, tumor thrombus circulation in blood or in the lymphatic system, tumor cell extravasation and target organ invasion (Mareel *et al.,* 1990). The proteins involved in these steps participate in many cellular processes, such as adhesion, migration and extracellular matrix (ECM) degradation. The main proteases associated with the ECM degradation and tumor invasion are urokinase-type plasminogen activator (uPA) (Ossowski *et al.,* 1991) and matrix metalloproteinases (MMPs) (Brown and Giavazzi, 1995). Many of the processes that occur during metastasis development, such as synthesis and secretion of proteases as well as adhesion and migration, are regulated by Ca^{2+}-dependent signaling pathways (Cole and Kohn, 1994).

Calcium channels are involved in the maintenance of intracellular Ca^{2+} homeostasis (Clapham, 1995). These channels can be blocked by specific compounds, such as verapamil, a phenylalkylamine that binds to type L voltage-dependent and -independent Ca^{2+} channels (Jamis *et al.,* 1987), or the carboxyamido-triazole CAI (Cole and Kohn, 1994; Wasilenko *et al.,* 1996). Verapamil and other compounds, such as nifedipine, diltiazem and nimodipine, have been used for a long time in humans as smooth muscle relaxants (Goodman *et al.,*, 1990). The protein kinase C (PKC)-dependent pathway, which can be calcium-dependent (Blobe *et al.,* 1994), participates in the control of uPA (Aguirre Ghiso *et al.,* 1997) and MMP production (Lauricella-Lefebvre *et al.,* 1993) in tumor cells. In addition, PKC is one of the most important members of the mitogenic signaling pathway, which has been delineated up to the mitogen-activated protein kinases (Burgering and Bos, 1995). Since calcium is a pleiotropic second messenger in the regulation of cellular functions (Cole and Kohn; 1994, Clapham, 1995), the blockade of Ca^{2+} ion exchange by verapamil could exert an inhibitory effect on one or more of the events associated with the metastatic process.

Verapamil has been used mainly in adjuvant chemotherapy, to reverse MDR-1 expression associated with the multiresistant drug phenotype in tumor cells (Blobe *et al.,* 1994). However, the effect of verapamil on the regulation of tumor invasion and metastasis, as well as on the regulation of tumor protease production, has not been explored. Since the calcium-dependent pathways are related to proliferation and protease production, the aim of our work was to analyze whether the blockade of calcium channels by verapamil could modulate *in vitro* production of uPA and MMPs. *In vivo,* we studied the effect of verapamil on local tumor growth and invasion, spontaneous and experimental metastasis development and circulating MMP activity.

MATERIAL AND METHODS

Animals

Inbred 2- to 4-month-old male BALB/c mice were obtained from the Animal Care Division of the University of Buenos Aires. Mice were bred with a 12-hr light–12-hr darkness cycle and a constant temperature of 20°C. Water and food were administered *ad libitum.*

Cells, reagents and culture conditions

The F3II cell line was established in our laboratory by clonal dilution from primary cultures of a BALB/c transplantable mammary adenocarcinoma (M3) (Alonso *et al.,* 1996*b*). This cell line, which constitutively produces high levels of uPA, is tumorigenic and exhibits extensive local invasiveness and high metastatic potential upon injection in syngeneic mice (Alonso *et al.,* 1996*b;* Aguirre Ghiso *et al.,* 1997). F3II cells from passages 30 to 50 were maintained in MEM 410–1500 (GIBCO, Grand Island, NY), supplemented with 5% heat-inactivated FBS (GEN, Buenos Aires, Argentina), 2 mM L-glutamine and 80 µg/ml gentamicin. Cells were routinely passaged using 0.25% trypsin (Sigma, St. Louis, MO) containing 0.02% EDTA (Sigma). In all experiments, cell harvesting was followed by washing and incubation in serum-free MEM for 2 hr at 37°C for recovery. The cell lines, periodically controlled, were free of *Mycoplasma.*

Preparation of conditioned media

Secreted uPA and MMP activities were investigated in conditioned media (CM). Briefly, semi-confluent F3II cells were exten-

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sively washed in PBS to eliminate serum traces. Serum-free MEM plus the tested drugs were added, and incubation was continued for different time lapses (see ''Results''). CM were individually harvested, the remaining monolayers trypsinized and hemocytometer cell counting or cell protein content determinations performed. CM samples, centrifuged (600 *g*), aliquoted and stored at –40°C, were used only once after thawing.

Effects of verapamil on F3II cell viability, growth and protease production

The possible cytotoxic effect of verapamil (1 to 250 µM) on F3II cells in culture was studied by morphological observation of cell monolayers for cytotoxicity, Trypan blue exclusion test and a metabolic titer assay that measures mitochondrial activity (MTS) (Promega Madison, WI).

To establish whether or not verapamil had any effect on uPA production, CM were prepared by incubating F3II cell monolayers for 20 hr with increasing amounts of verapamil (V-F3II, 0.1–100 µM; Sigma) or control treatment (MEM, C-F3II). To determine whether the effect on uPA production could persist after verapamil removal, verapamil-pre-treated F3II cell monolayers were washed with PBS and incubated for 5 or 21 hr with plain MEM. To determine whether verapamil had a direct action on uPA catalytic activity, purified human standard urokinase (0.2 to 100 IU/ml; Serono, Coinsins, Switzerland) and murine uPA from urine (5 to 20 IU/ml) were assayed in the presence or absence of verapamil (100 µM).

MMP activity was also tested in the CM of F3II cells, treated with or without verapamil, 50 or 100 µM, for 20 hr. CM were analyzed in SDS-polyacrylamide gels co-polymerized with 0.1% gelatin to reveal enzymatic activity.

To study the effects of verapamil on F3II cell proliferation, 2×10^5 cells were seeded in 35-mm plastic Petri dishes in the presence or absence of 100 µM verapamil in growth medium and quantitated over a 96-hr period with a hemocytometer. Every 24 hr, the medium was replaced for both control and verapamil-treated cells.

uPA Western blot analysis

Cell monolayers were cultured with MEM in the presence or absence of verapamil 50 µM for 20 hr. CM were collected, electrophoresed in a 9% SDS-PAGE gel and transferred to nitrocellulose. The filter was probed with or without rabbit IgG anti-murine uPA antibody (kindly provided by Dr. G. Høyel-Hansen) and revealed with a biotinylated IgG anti-rabbit alkaline phosphatase–conjugated polyclonal antibody (Alonso *et al.,* 1996*a*).

Zymography and radial caseinolytic u-PA assay

The type and activity of plasminogen activators present in the CM of F3II cells under different treatments were determined as follows. SDS-PAGE was performed as described by Laemmli (1970). Gels were washed with 2.5% Triton X-100 and incubated at the surface of a plasminogen-rich casein-agarose underlay, as described by Aguirre Ghiso *et al.* (1997). Non-specific protease activity was assayed on plasminogen-free casein-agarose plates and on plasminogen-casein plates containing 1 mM amiloride or 10 µg/ml of an anti-catalytic anti-murine uPA antibody, kindly provided by Dr. G. Høyel-Hansen (Aguirre Ghiso *et al.,* 1997). Secreted u-PA activity released by F3II tumor cells was quantified in CM with a radial caseinolytic assay in plasminogen-rich casein-agarose plates, as previously described (Saksela, 1981). Values were expressed as IU/10^6 cells/24 hr.

Tumorigenicity and spontaneous metastasis assays

Cell suspensions, prepared from 50 or 100 µM V-F3II or C-F3II subconfluent monolayers, were washed and incubated for 2 hr in medium for recovery.

To evaluate local tumor growth and spontaneous metastasizing ability, V-F3II or C-F3II cells ($2x10^5$ cells/0.2 ml MEM) were injected s.c. in the flank of normal syngeneic mice that had or had not received verapamil (V-BALB/c or C-BALB/c, respectively) in a dose of 171 µg/mouse/day in drinking water, 70 µM in total mouse blood volume calculated from oral intake, throughout the experiment. This dose was chosen as the equivalent to the maximum oral dose used in human patients (Goodman *et al.,* 1990). Tumor take and tumor latency period were determined for all groups. Growth kinetics were established, measuring the average tumor diameter 3 times per week with a caliper. The volume of water consumed by V-BALB/c and C-BALB/c animals was also measured. To study circulating MMP activity, blood samples were obtained from all experimental and control tumor-bearing mice by inserting a capillary tube into the lateral orbital sinus at the end of tumor growth. Complete autopsies with histo-pathological analysis were performed after day 35 of growth. Lungs were fixed with Bouin solution, and the number of metastatic lung nodules was quantified under a binocular stereoscopic magnifier. Also, normal mice treated with the same dose of verapamil were used to study toxicity, effects on weight gain, corporal temperature, histology of different organs and metalloprotease activity in the euglobulin fractions.

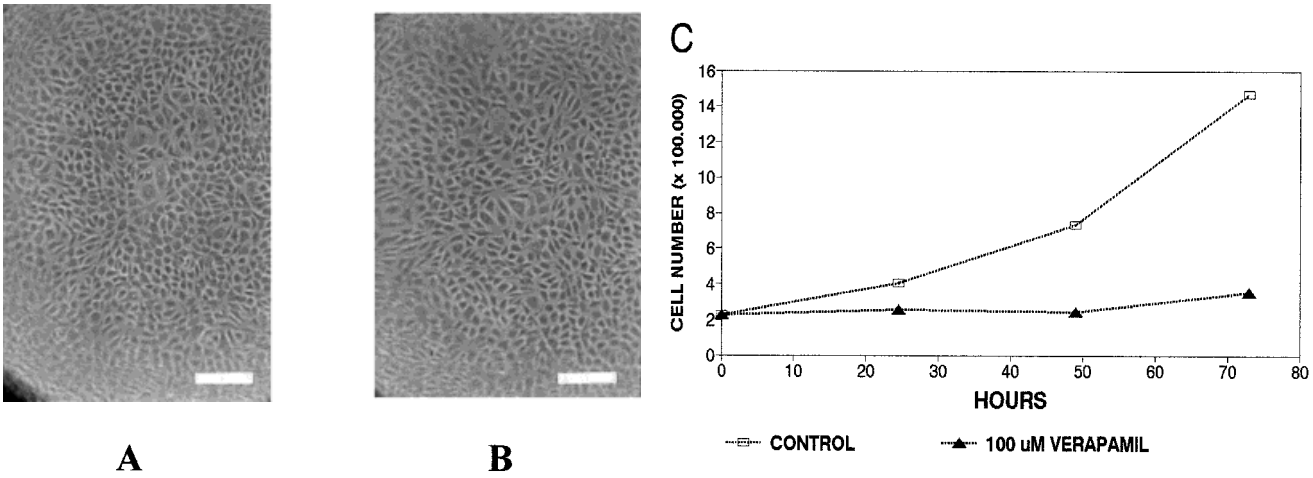

FIGURE 1 – Morphological characteristics of F3II cells in the presence of verapamil. *(a)* Control F3II cells. *(b)* F3II monolayers treated over 24 hr with 100 µM verapamil. No morphological cytotoxic effect was observed in a 50- to 100-µM verapamil dose range. Phase contrast. Scale bars: 100 µM. *(c)* Effects of verapamil (100 µM) on F3II cell proliferation.

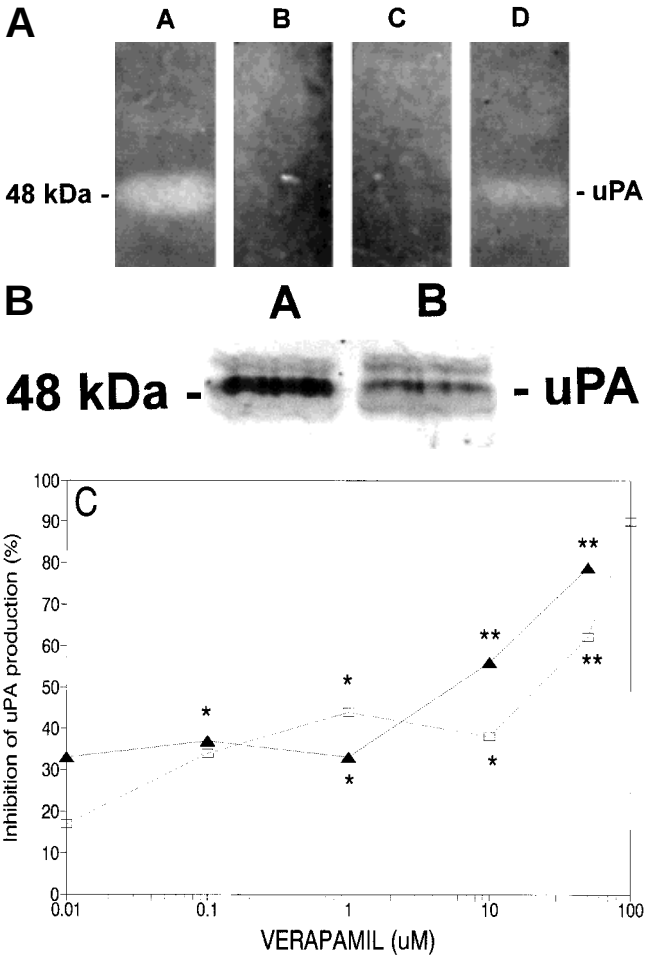

FIGURE 2 – Effects of verapamil on uPA production by F3II cells. *(a)* Zymography. F3II cells secreted a 48-kDa uPA activity (lane A), inhibited in the presence of specific anti-catalytic antibodies (lane B) or amiloride 1 mM (lane C). Verapamil 50 µM reduced uPA-secreted activity (lane D). *(b)* Western blot of CM. F3II control cells (lane A) and F3II cells treated with verapamil 50 µM (lane B). *(c)* Quantitation of the effect of verapamil on uPA-secreted activity by radial caseinolysis: verapamil treatment for 20 hr (empty squares) and after 5 hr of washout (filled triangles). uPA activity is expressed in IU/10^6 cells/24 hr for control and treated groups. *$p < 0.05$ and **$p < 0.001$, as determined by Student's *t*-test. SD was below 10%.

Experimental metastasis assay

V-F3II (pre-treated for 20 hr with 50 to 100 µM verapamil) or C-F3II cells were also injected at 2×10^5 cells/0.3 ml MEM into the lateral tail vein of normal mice that had or had not been treated with verapamil (171 µg/mouse/day in drinking water) 24 hr before. After cell injection, mice continued receiving or not verapamil in the drinking water throughout the assay. Complete autopsies with histo-pathological analysis were performed 21 days after injection, and lung subpleural metastatic nodules were counted as described above.

Preparation of the plasma euglobulin fraction

Euglobulins were prepared by mixing 0.1 ml of plasma with 0.9 ml of cold, de-ionized water, and pH was adjusted to 5.5 with 40 µl 1% v/v acetic acid. This mixture was incubated for 30 min at 4°C and centrifuged for 10 min at 25,00 *g*. Euglobulins were then dissolved in 0.5 ml PBS, pH 7.4, to a final dilution of 1:5 (Farías *et al.*, 1995).

Detection and quantitation of MMP activity in F3II cell CM and in the plasma euglobulin fraction

Gelatinolytic activity present in F3II cell CM or in the plasma euglobulin fraction of animals under the indicated treatments (see "Results") was determined in SDS-PAGE gels co-polymerized with 0.1% gelatin, as reported previously (Bonfil *et al.*, 1992). After running, gels were washed in 2% Triton X-100 and incubated for 72 hr in a 0.25 M Tris-HCl/1 M NaCl/25 mM $CaCl_2$ buffer (pH 7.4) for specific activity detection or in the same solution containing 40 mM EDTA to detect non-specific activity. Gels were fixed and stained with Coomassie blue. Activity bands were visualized by negative staining. Degradation bands were quantified with a Molecular Analyst TM/PC Densitometer (model GS-700; Bio-Rad, Hercules, CA) and analyzed with Image Analysis software.

Statistical analysis

Metastasis incidence was analyzed using χ^2 analysis. Differences in the number of metastatic nodules under different treatments were evaluated with the Kruskal-Wallis or Mann-Whitney non-parametric test. Differences in uPA activity were compared using Student's *t*-test. All tests were done with the Biostatistics software of McGraw-Hill (1991, version 1.0; New York, NY).

RESULTS

In vitro assays

Evaluation of cytotoxicity and cell proliferation

F3II cell monolayers cultured up to 80–90% confluence were assayed with different doses of verapamil in order to evaluate cytotoxicity. Doses up to 100 µM showed no morphological cytotoxic effects (Fig. 1) and 100% viability as assayed by Trypan blue exclusion test over 24 hr of treatment. Doses equal to or greater than 150 µM showed mild cytotoxicity with perinuclear vacuoles, and doses greater than 250 µM were highly cytotoxic, inducing a remarkable vacuolization and extensive cell death. To detect mild disruption of cellular metabolism, we also assayed the effects of verapamil (100 µM) on mitochondrial activity over 24 hr. No effect was observed, as measured by the MTS test (Fig. 1*c*).

Cell proliferation was profoundly affected by verapamil. Cells ceased to proliferate in the presence of 50 or 100 µM of the compound, remaining in this quiescent status for at least 72 hr (data not shown).

Effect of verapamil on uPA and MMP production

Zymography and Western blot indicated that these cells produce a 48-kDa uPA activity (Fig. 2*a,b*, lanes A), which could be

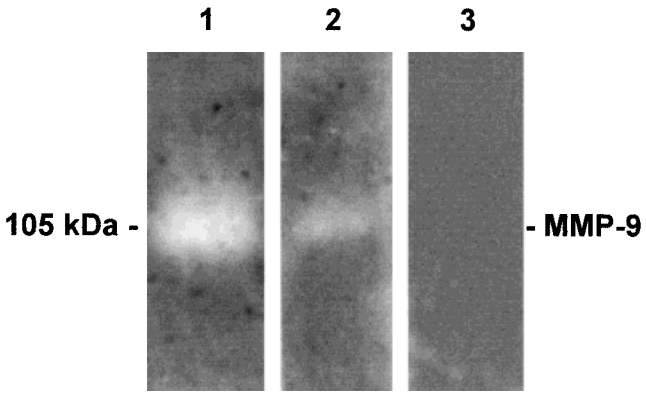

FIGURE 3 – Zymography showing the effect of verapamil on MMP-9 activity secreted by F3II cells. Control treatment (lane 1), 50 µM verapamil treatment for 20 hr (lane 2) and specificity control of MMP activity in the presence of 40 mM EDTA in the incubation buffer (lane 3). These results are representative of 3 independent experiments.

abolished with specific anti-catalytic antibodies (Fig. 2*a*, lane B) or with amiloride 1 mM (Fig. 2*a*, lane C), as previously reported (Aguirre Ghiso *et al.*, 1997).

Treatment with verapamil (0.1 to 100 μM) for 20 hr inhibited uPA activity secreted to the CM in a dose-dependent way, as quantified by radial caseinolysis (Fig. 2*c*). A significant inhibition was obtained at doses as low as 0.1 μM (IC_{50} 25 μM). We also observed that the dose-dependent uPA inhibition induced by verapamil remained even after removal of the compound for a period of 5 hr (Fig. 2*c*). In a further set of experiments, we observed that the 60% inhibition induced by 50 μM verapamil also persisted after 21-hr washout.

Zymograms and Western blot analysis confirmed the marked decrease of secreted uPA, in both activity and mass (Fig. 2*a*, lane D, *b*, lane B). Standard purified human and murine urine urokinase were not affected by the presence of 100 μM verapamil (data not shown), indicating that uPA decrease could not be attributed to a direct effect of compound on the secreted proteases.

We also observed that the activity of the 105-kDa MMP (gelatinase-B/ MMP-9) secreted to the CM by V-F3II cells (Fig. 3, lane 1) was inhibited (1 to 100 uM), as detected by zymography (Fig. 3, lane 2). The specificity of MMP activity was confirmed using EDTA in the incubation buffer (Fig. 3, lane 3). Furthermore, addition of PMSF (5 mM) and aprotinin, serine protease inhibitors, had no effect on the MMP activity present in F3II cell CM (data not shown).

In vivo assays

Effect of verapamil on local tumor growth and spontaneous metastasis development

Mice were treated for 45 days with verapamil (171 μg/mouse/day) in drinking water. Since gastro-intestinal absorption of verapamil is higher than 90% (Goodman *et al.*, 1990), blood concentration was equivalent to 70 μM, calculated directly from oral intake. No toxicity was observed, as indicated by general status (weight, corporal temperature and activity) and histo-pathological analysis of lungs, liver, spleen, kidneys and heart. In addition, no differences were found in the volume of drinking water consumed by V-BALB/c or C-BALB/c mice (5 ml/mouse/day). Histo-pathological analysis of the lungs showed enhancement of blood-vessel diameter in all verapamil-treated animals, confirming the relaxing effect on vascular smooth muscle (Fig. 4).

Treated (V-F3II) and control (C-F3II) cells were injected s.c. into the flank of normal V-BALB/c and C-BALB/c mice. No differences were found in either tumor take, latency period (8 ± 2 days) or tumor growth rate for V-BALB/c or C-BALB/c mice (Fig. 5*a*). However, an important reduction in tumor local invasiveness, also evidenced by skin integrity, was observed in verapamil-treated mice that received either V-F3II or C-F3II cells compared with C-BALB/c mice inoculated with non-treated C-F3II cells (Fig. 5*b*).

Moreover, verapamil caused a significant inhibition of spontaneous lung metastasis incidence and number in all groups. Interestingly, this inhibition was evident not only when V-F3II cells were inoculated in either control or verapamil-treated (V-BALB/c) mice but also when C-F3II cells were injected in mice receiving verapamil treatment (Table I). The distribution of metastases according to size indicated that the treated group did not show any nodule larger than 2 mm and did show a remarkable reduction in the number of metastases for each size analyzed (Table I).

Effect of verapamil on experimental metastasis development

V-F3II (100 μM) or C-F3II cells were injected into the lateral tail vein of C-BALB/c or V-BALB/c mice pre-treated for 20 hr. After cell injection, V-BALB/c mice continued receiving verapamil in the drinking water for 21 days. Both V-F3II cells in C-BALB/c mice and C-F3II cells in V-BALB/c mice developed a significantly lower number of experimental metastases than C-F3II cells inoculated into C-BALB/c mice (control) (Table II). When both cells and mice received verapamil treatment, a clear additive effect for the inhibition of experimental metastasis development was observed (Table II).

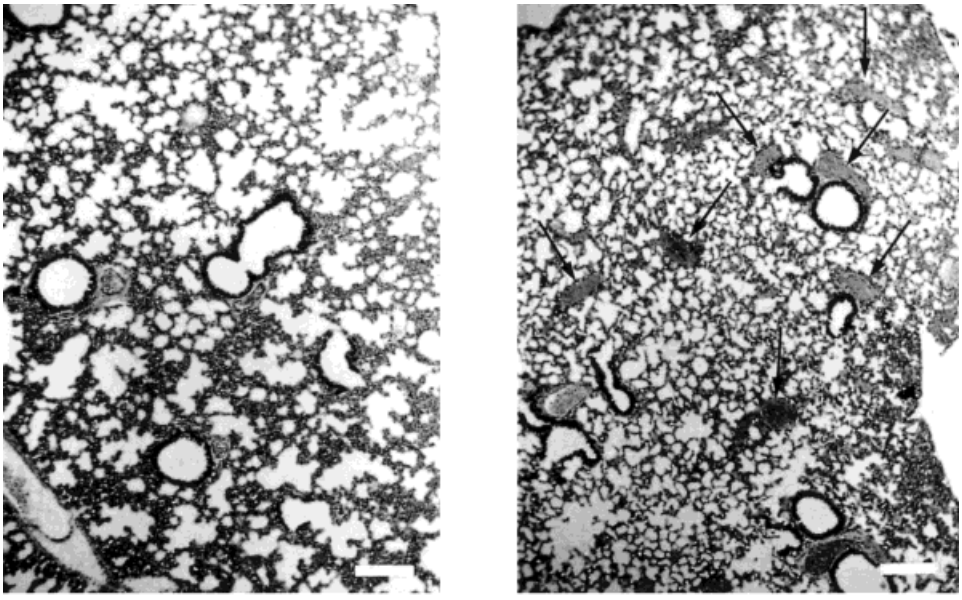

FIGURE 4 – Effect of verapamil on the lung vascular network. Histo-pathology of lungs of mice treated or not with 171 μg verapamil/mouse/day in drinking water. Control (C-BALB/c) and verapamil-treated (V-BALB/c) mice. Arrows show the increase of vascular diameter in V-BALB/c mice. Scale bars: 200 μM.

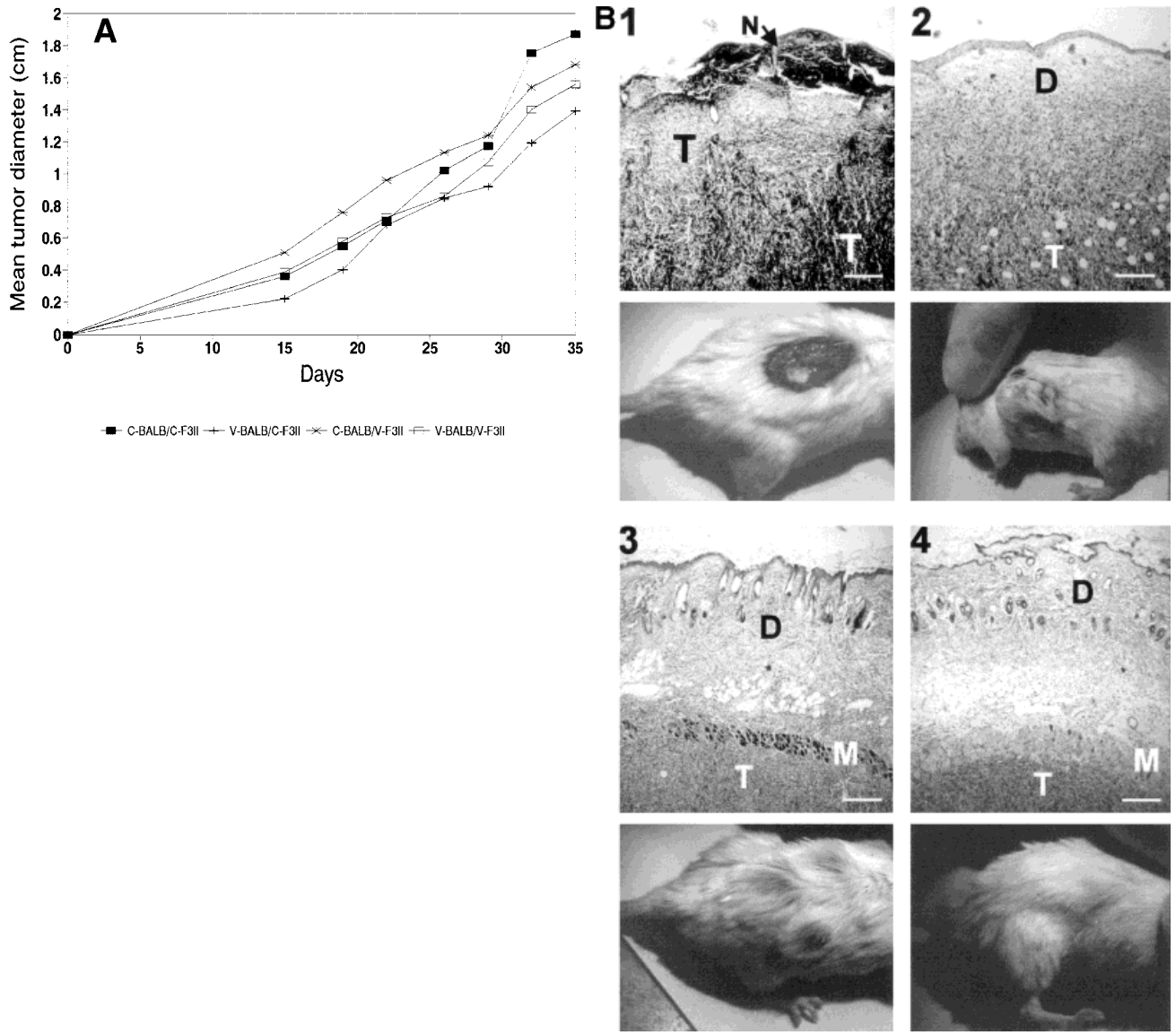

FIGURE 5 – Effect of verapamil on s.c. tumor growth. *(a)* F3II tumor growth rate. Animals were treated with 171 µg verapamil/mouse/day and F3II cells with 100 µM verapamil overnight. *(b)* Effect of verapamil on local invasiveness. (1) Control C-BALB/c/C-F3II, (2) V-BALB/c/C-F3II, (3) C-BALB/c/V-F3II and (4) V-BALB/c/V-F3II. In all panels (1–4), we show the macroscopic lesion (lower) together with its histological section (upper). T, tumor; N, skin necrosis; D, dermis; M, s.c. muscle. Two independent sets of experiments using 50 µM verapamil showed similar results. Group descriptions: C-BALB/c/C-F3II, non-treated mice inoculated with F3II cells without verapamil pre-treatment; V-BALB/c/C-F3II, mice treated with 171 µg/mouse/day in drinking water inoculated with F3II cells without verapamil treatment; C-BALB/c/V-F3II, non-treated mice inoculated with F3II cells pre-treated with 100 µM verapamil; V-BALB/c/V-F3II, mice treated with 171 µg/mouse/day in drinking water inoculated with F3II cells pre-treated with 100 µM verapamil.

Effect of verapamil on circulating MMP activity

Euglobulin fractions were prepared from peripheral blood of normal BALB/c mice treated with verapamil for 45 days. No variation in circulating MMP activity was detected between treated and non-treated control mice (data not shown). Normal mice displayed a prominent band of 60 kDa and a slight band of 105 kDa (Fig. 6*a*, lane A). In contrast, tumor-bearing mice showed a significant enhancement of the 105-kDa MMP activity as well as new gelatinolytic bands of 70, 130 and 200 kDa with no variation on the 60-kDa band (Fig. 6*a*, lane B), confirming our previous results (Farías *et al.*, 1995). Specific MMP activity was confirmed since no banding was observed when gels were incubated in the presence of 40 mM EDTA (Fig. 6*a*, lane F), and activity was not abolished by serine protease inhibitors PMSF and aprotinin (data not shown).

Incubation of F3II cells with 100 µM verapamil prior to s.c. inoculation was sufficient to induce a significant decrease in the 105- and 60-kDa MMP circulating activity, as analyzed in the euglobulin fraction by zymography (Fig. 6*a*, lane C) and densitometry (Fig. 6*b*). Similar results were obtained when verapamil treatment was given only to mice and to both mice and cells (Fig. 6*a*, lanes D and E, *b*).

DISCUSSION

Intracellular calcium homeostasis is critical for the proper operation of several cellular events, such as proliferation, protein synthesis, signal transduction and secretion (Cole and Kohn, 1994; Clapham 1995; Wasilenko *et al.*, 1996). Production of some

TABLE I – EFFECT OF VERAPAMIL ON THE SPONTANEOUS LUNG METASTATIC ABILITY OF F3II CELLS[1]

Spontaneous metastasis	n	Incidence of lung metastasis (%)	Number of lung nodules: median (range)	Distribution of total number of metastatic nodules according to size		
				0.5–1 mm	1–2 mm	>2 mm
C-BALB/c/C-F3II	10	100	6 (2–20)	40	15	7
V-BALB/c/C-F3II	12	67.7*	1 (0–4)*	7	6	0
C-BALB/c/V-F3II	12	69.7	2 (0–8)*	14	12	0
V-BALB/c/V-F3II	12	48.7**	1 (0–3)*	9	1	0

[1]A representative experiment of 3 independent ones with similar results is shown.–*$p < 0.05$ and **$p < 0.001$ *vs.* C-BALB/c/C-F3II, Kruskal-Wallis test. For group descriptions, see Figure 5.

TABLE II – EFFECT OF VERAPAMIL ON THE EXPERIMENTAL LUNG METASTATIC ABILITY OF F3II CELLS[1]

Experimental metastasis	n	Incidence of lung metastasis (%)	Number of lung nodules: median (range)	Distribution of total number of metastatic nodules according to size		
				0.5–1 mm	1–2 mm	>2 mm
C-BALB/c/C-F3II	11	100	9 (6–37)	136	39	12
V-BALB/c/C-F3II	12	87.7	6.5 (0–21)*	52	10	0
C-BALB/c/V-F3II	12	66.7	4.5 (0–14)*	63	12	0
V-BALB/c/V-F3II	12	48.7	3 (0–10)**	25	5	0

[1]A representative experiment of 2 independent ones with similar results is shown.–*$p < 0.05$ and **$p < 0.001$ *vs.* C-BALB/c/C-F3II, Kruskal-Wallis test. For group descriptions, see Figure 5.

proteases by tumor cells appears to be calcium-dependent (Wasilenko *et al.,* 1996).

In this report, we have studied the effect of a calcium channel blocker, verapamil, on different aspects of tumor biology *in vitro* and *in vivo.* This compound is a potent hypotensive drug, and it is widely employed to treat hypertension-associated diseases (Goodman *et al.,* 1990). Verapamil is also used in chemo-adjuvant therapy, to revert multidrug resistance by inhibition of MDR-1 pump expression (Blobe *et al.,* 1994). However, its effects on the invasive properties and metastatic ability of tumor cells (Yohem *et al.,* 1991) have not been extensively explored.

First, we analyzed the toxicity of verapamil on F3II cells *in vitro* as well as *in vivo* on BALB/c mice. The treatment of F3II cells with verapamil showed no cytotoxic effects up to doses of 125 µM, as measured by the MTS assay. Toxicity became evident when doses higher than 150 µM were used. BALB/c mice orally treated for 45 days with verapamil (171 µg/mouse/day) showed no toxic effects, as evidenced by general status and histo-pathological analysis of different organs. These studies prompted us to use the indicated doses for the possible *in vitro* and *in vivo* effects on tumor cell behavior.

uPA is a key protease, over-expression of which has been correlated with the invasive and metastatic phenotype in experimental tumor models and human malignancies (Laiho and Keski-Oja, 1989; Moller *et al.,* 1993; Aguirre Ghiso *et al.,* 1997). This enzyme bound to its receptor is responsible, upon conversion of plasminogen to plasmin, for the activation of a proteolytic cascade that involves its auto-activation and the activation of MMPs (Moller *et al.,* 1993). F3II cells constitutively produce uPA, and the uPA–uPA receptor complex has a predominant role in the invasive and metastatic properties of these cells (Alonso *et al.,* 1994, 1996*b*). We have previously shown that uPA over-production in tumor cells is regulated through a PLD- and PKC-dependent pathway (Aguirre Ghiso *et al.,* 1997), which, depending on the PKC isoform involved, requires intracellular or extracellular calcium in order to be functional (Blobe *et al.,* 1994; Alonso *et al.,* 1996*a*). This prompted us to establish whether verapamil could inhibit uPA production by F3II cells. Interestingly, verapamil inhibited uPA production in a dose-dependent manner. This result strongly suggests that the PKC-derived signal participating in the endogenous pathway controlling uPA expression in F3II cells is dependent on extracellular calcium and probably involves classical isoforms of PKC. Previously, we determined that F3II cells express at least 3 of the classical isoforms of PKC, namely, α, β and δ (data not shown), which are both calcium- and diacylglycerol-dependent. CAI, another calcium channel blocker, has been described to block calcium influx through receptor-gated channels also in the micromolar range (Cole and Kohn, 1994; Wasilenko *et al.,* 1996). However no reports exist regarding its effects on uPA expression.

To establish whether uPA inhibition was a transient or stable effect, cells treated for 20 hr with verapamil were washed and incubated in the absence of verapamil for periods up to 21 hr. Surprisingly, the inhibitory effect persisted at least for the 21 hr assayed, indicating that the inhibition of uPA by verapamil appears to be established as a long-lasting event. In this regard, long-term modulation by verapamil has been reported only for the down-regulation of MDR-1 mRNA expression in P388 leukemia cells, an effect spontaneously reversed after 72 hr (Tsuruo *et al.,* 1981). Also, CAI has inhibitory and reversible effects on tumor cell proliferation after a 3-day recovery period (Wasilenko *et al.,* 1996).

MMPs are critical proteases involved in the metastatic cascade (Liotta and Stetler-Stevenson, 1991). Their expression has been clearly demonstrated to be associated with tumor development during mammary gland evolution (Reich *et al.,* 1995). In the present work, we have also established that the constitutive production of a 105-kDa MMP by F3II cells could be inhibited by verapamil treatment. Consistent with this observation is the result reported by Cole and Kohn (1994), that MMP-2 expression is decreased by the calcium channel blocker CAI. Expression of MMPs also has been reported to depend on a TPA-response element (Lauricella-Lefebvre *et al.,* 1993), which involves PKC activation (Chambers *et al.,* 1992).

Since verapamil inhibited the secretion of uPA and MMPs, both critical proteases for tumor invasion and metastasis, we assayed its effect on tumor growth as well as on the development of spontaneous and experimental metastatic foci in the murine mammary tumor model. Although verapamil had presented a potent inhibitory effect on F3II cell proliferation *in vitro,* no significant effect on tumor growth or tumor latency period was observed in BALB/c mice with any kind of treatment. However, an evident decrease in the local invasive ability of F3II cells was observed in all groups that received verapamil. The effects were more striking for the group of mice inoculated with verapamil-treated cells and receiving verapamil orally, in which the tumor, although actively growing, remained limited and non-invasive. These results are in accordance with previous reports from our laboratory, in which the blockade of secreted and cell-associated

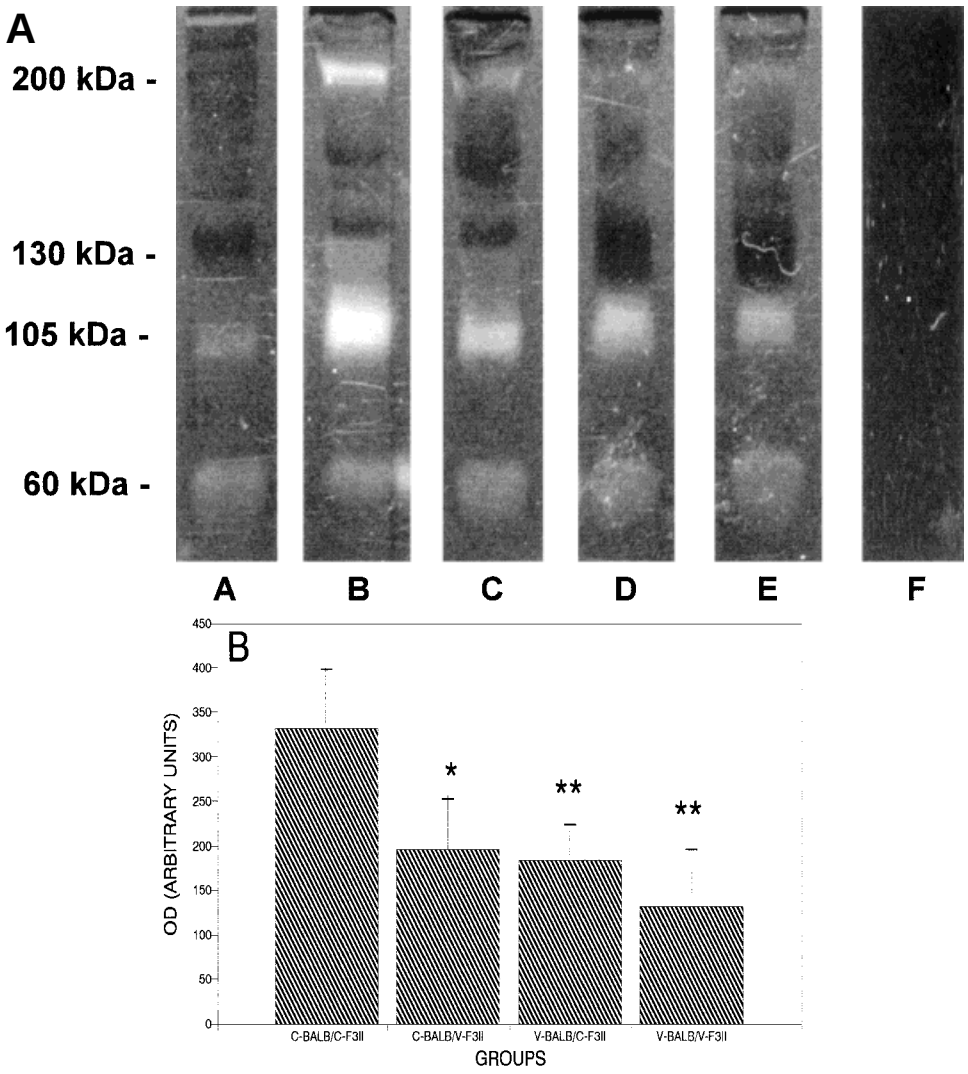

FIGURE 6 – Effect of verapamil on circulating MMP activity. *(a)* Circulating MMP activity in normal mice without tumor (lane A), C-BALB/c/C-F3II (lane B), C-BALB/c/V-F3II (lane C), V-BALB/c/C-F3II (lane D) and V-BALB/c/V-F3II (lane E). Specificity control for MMP activity (40 mM EDTA in the incubation buffer, lane F). *(b)* Densitometric analysis of total MMP activity in euglobulin fractions. Mean ± SD of at least 12 independent samples per group of a representative experiment of 3. $*p < 0.05$ and $**p < 0.001$, Student's *t*-test.

uPA activity by specific synthetic anti-catalytic compounds decreased the local invasiveness of s.c. F3II carcinoma (Alonso *et al.*, 1996*b*). Since verapamil significantly reduced uPA and MMP production, the impairment of invasion could be clearly associated with the reduced pericellular proteolysis.

Interestingly, both the incidence and the number of spontaneous metastases were significantly decreased by verapamil treatment, mainly in the V-F3II/V-BALB/c group. This indicates that verapamil, through blockade of the calcium-dependent signals in F3II cells, could reduce the ability of F3II cells to exit the primary tumor, intravasate, attach and extravasate in the target organ. The fact that the size of the metastatic nodules was also decreased in verapamil-treated mice suggests that the compound could also exert a cytostatic effect on the proliferative capacity of metastatic nodules, not observed for s.c. tumor growth but quite evident for *in vitro* growth of F3II cells. However, it is difficult to explain why a short pre-treatment of F3II cells with verapamil prior to injection reduced local invasion and spontaneous metastasis development without modulating local tumor growth. It is possible that verapamil treatment specifically shuts down some cellular functions related to invasion but not to proliferation and survival.

Impairment of the experimental metastatic ability of F3II cells by verapamil, in any treatment combination, indicates that this compound could have an astonishing inhibitory effect on the invasive processes associated with extravasation and settlement of new metastatic foci. The inhibitory effects were observed either when only the animals were treated or when only the cells received verapamil, with a clear enhancement in the combined treatment. The simple pre-treatment of F3II cells during 20 hr with a dose of 50 or 100 µM of verapamil was sufficient to exert a striking inhibition on experimental metastasis development. This result may be associated with the persistent inhibition of uPA production *in vitro* that was observed for at least 21 hr after verapamil withdrawal. Since most tumor cells die 2 to 4 hr after i.v. injection (Chambers *et al.*, 1992) if they do not attach and start to extravasate, it is probable that the impairment of uPA production for 21 hr is critical for the failure of this process. Evidence supports a new role for proteases in metastasis, suggesting that the enzymes are important in creating and maintaining an environment that supports the initiation and maintenance of growth of primary and metastatic tumors (Chambers and Matrisian, 1997). Probably, the inhibition of this enzyme in the host and tumor cells modulates in a

differential way primary tumor and metastatic nodule proliferation depending on the site of tumor growth.

Another interesting observation is that verapamil increased the diameter of lung blood vessels. Since the mechanical arrest of tumor cells in the lung microvasculature is a critical step of the metastatic cascade (Chambers *et al.,* 1992), an increase in capillary diameter could also cooperate with the inhibitory effects of verapamil. Such an increase of blood-vessel diameter could be a consequence of the relaxing effects verapamil can exert on the vascular smooth muscle (Goodman *et al.,* 1990).

Tumor cells not only can over-produce proteases but also induce the production of enzymes like MMP by the host cells, contributing to the invasive phenotype (Stetler-Stevenson *et al.,* 1993). It is thus probable that circulating MMP activity, derived either from host or from tumor cells, somehow contributes to the metastatic cascade. We have previously shown that MMP activity in the plasma euglobulin fraction of tumor-bearing mice was significantly enhanced with respect to control (Farías *et al.,* 1995). Similar results were observed in plasma from breast cancer patients compared with healthy women and patients with benign fibro-adenoma (Farías *et al.,* 1996). Here, we have shown a marked decrease of circulating MMP activity in the euglobulin fraction of tumor-bearing mice with any verapamil treatment, particularly in the V-F3II/V-BALB/c group. The fact that non-treated mice given verapamil-treated F3II cells (V-F3II/C-BALB/c group) also showed a reduction in circulating MMP activity suggests that F3II cells are probably the main contributors to the enhancement of plasma MMP activity along tumor progression and the target of verapamil.

In summary, verapamil, a commonly used hypotensive compound, has unexpected anti-invasive and anti-metastatic properties. The reduction of both protease production by tumor cells and circulating MMP activity, as well as other effects on the host, such as vessel relaxation, are probably some of the cellular mechanisms by which verapamil exerts its potent inhibitory effects on tumor progression. Our findings could give new insight into the development of anti-invasive or anti-metastatic compounds.

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