Int. J. Cancer: **100,** 14–21 (2002)

Publication of the International Union Against Cancer

DIFFERENTIAL PRODUCTION OF ANGIOSTATIN BY CONCOMITANT ANTITUMORAL RESISTANCE-INDUCING CANCER CELLS

M. Mercedes BINDA[1], Pablo MATAR[2], Alejandro D. GONZÁLEZ[1], Viviana R. ROZADOS[2], Silvia I. GERVASONI[2], O. Graciela SCHAROVSKY[2] and R. Daniel BONFIL[1]*

[1]*Laboratory of Fundación de Investigación del Cáncer at CEFYBO, Buenos Aires, Argentina*
[2]*Instituto de Genética Experimental, Facultad de Ciencias Médicas, UNR, Rosario, Argentina*

The phenomenon by which tumor-bearing hosts are capable of inhibiting secondary tumor implants or metastases, known as concomitant antitumoral resistance (CAR), is presumably due to antiangiogenesis at places distant from the primary tumor. Although angiostatin, a potent inhibitor of angiogenesis, has been reported to be one of the factors responsible for suppressing the growth of secondary tumors in mice bearing previous tumors, it has not been definitively proven yet. With the aim of investigating whether CAR-inducing cancer cells display a differential angiostatin production and to support the role ascribed to that molecule concerning the inhibition of secondary tumor implants, 5 tumor models with different CAR-inducing capacities were studied herein. One of the 2 human lung cancer cell lines analyzed revealed a strong CAR against secondary s.c. tumor implants in nude mice, and 2 of 3 of the murine mammary tumors used exhibited inhibitory effect on secondary s.c. and i.v. tumor inoculations in syngeneic hosts. Since angiostatin is a proteolytic fragment from plasminogen, we examined by Western blot the ability of all conditioned media collected from the tumor cells studied to convert plasminogen to angiostatin. An association between *in vivo* generation of CAR and *in vitro* conversion of plasminogen into angiostatin was found. Since different enzymatic mechanisms were described to explain the generation of angiostatin, we also studied gelatinase and urokinase-type plasminogen activator secretion in conditioned media by zymography. The conversion of plasminogen into angiostatin by conditioned media was mainly inhibited by broad-spectrum serine proteinase inhibitors, suggesting a possible role for 1 or more enzymes of that group in the process. These findings suggest the existence of a differential angiostatin generation by CAR-inducing cancer cells, providing additional support to previous data obtained by other authors.

Key words: *angiostatin; concomitant antitumoral resistance; serine proteinases; metalloproteinases; angiogenesis, metastasis*

Based on clinical observations, at least 4 common patterns of metastases are observed in cancer patients, namely that of *(i)* rapid growth of metastases after surgical extirpation of a primary tumor; *(ii)* presence of metastases at the time of primary tumor diagnosis; *(iii)* metastases with occult primary tumor; and *(iv)* appearance of metastases many years after removal of the primary tumor.[1] The first of the cases mentioned above, observed not only in clinical[2–5] but also in experimental[6–12] situations, suggests that some primary tumors are capable of inhibiting the growth of secondary tumors or metastases. This phenomenon was described at the beginning of 20th century by Ehrlich[13] and was designated as concomitant immunity by Bashford,[14] assuming that it could be explained by immunologic means. Many years passed by until the phenomenon could also be recognized with tumors of nondetectable immunogenicity or in immunodeficient mice,[6,7] and the term CAR[3] was proposed.[8] The paradoxical inhibitory effect exerted by some primary neoplasms on the growth of secondary tumors at distant places is also observed on metastasis development. In fact, we observed an inverse correlation between metastatic capacity and CAR.[9]

Angiogenesis, the formation of new blood vessels from the existing vascular bed, is pivotal to primary tumor growth and metastasis. Folkman's pioneering work introduced the concept that all solid tumors depend on angiogenesis to grow.[15,16] The corroboration of his hypothesis is well supported by several investigations and opened new avenues for novel therapeutic strategies based on the use of antiangiogenic factors. One of the main advantages of this type of treatment is supported by the assumption that they would not generate resistance mechanisms, due to the genetic stability of endothelial cells.[17–19] Some of the antiangiogenic factors are secreted in their active form, such as thrombospondin,[19] whereas others are converted into antiangiogenic by proteolytic action on inactive extracellular molecules, as is the case of angiostatin and endostatin.[10,20]

Since others and we have previously observed that antiangiogenesis and apoptosis could act as mediators of CAR,[10,21–23] it is of great importance to study the role of different natural inhibitors of angiogenesis in such phenomenon.

Angiostatin, an internal fragment of plasminogen including kringles 1–4, is a potent angiogenesis inhibitor that has been found in the circulation of tumor-bearing mice, which disappears after surgical tumor removal.[10] Although the mechanisms by which it is generated *in vivo* remain unknown, different authors have shown that angiostatin can be generated *in vitro* by the proteolytic cleavage of plasminogen by different serine-, metallo- and aspartic proteases secreted by tumor cells themselves or tumor-infiltrating macrophages.[24–31]

Here, we studied the ability of 3 murine mammary adenocarcinomas and 2 human lung cancer cell lines to produce angiostatin, in relation to their capacity to induce CAR. An association between both phenomena, as well as the involvement of serine proteinases in the generation of the angiostatic factor, was found.

MATERIAL AND METHODS

Cell culture

Human lung cancer-derived cell lines Calu-6 and H460, obtained from ATCC (Rockville, MD), and M3MC, M234p and

Abbreviations: ATCC, American Type Culture Collection; CAR, concomitant antitumoral resistance; CM, conditioned medium; FBS, fetal bovine serum; MMP, matrix metalloproteinase; PSA, prostate-specific antigen; SBTI, soybean trypsin inhibitor; TIMP, tissue inhibitor of metalloproteinases; tPA, tissue plasminogen activator; TVI, tumor volume inhibition; uPA, urokinase-type plasminogen activator.

Dr. Bonfil's current address is: Departments of Urology and Pathology, Wayne State University School of Medicine, Detroit, MI, USA.

*Correspondence to: Departments of Urology and Pathology, Wayne State University School of Medicine, 540 E. Canfield, Room 9105, Detroit, MI 48201, USA. Fax: +313-577-0057. E-mail: fundic@velocom.com.as

Received 23 October 2001; Revised 27 November 2001; Accepted 6 March 2002

DOI 10.1002/ijc.10425

M234m, mammary adenocarcinomas spontaneously arisen in BALB/c mice, were used throughout. Tumor cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Gen, Buenos Aires, Argentina), except for M234m that used MEM medium (Sigma) with 20% FBS. CMs were generated by 48 hr exposure of confluent monolayers to their respective FBS-free media.

Concomitant antitumoral resistance assay

Three-month-old N:NIH (S) nude mice (Animal Care Facilities of the School of Veterinary Medicine, University of La Plata, La Plata, Argentina) were inoculated s.c. in their left flanks with 2×10^6 Calu-6 or H460 cells, as previously reported.[21] In the case of M3MC, M234p and M234m, 1×10^6 cells were inoculated s.c. in 3-month-old female BALB/c mice (Animal Care Facilities of the School of Veterinary Medicine, University of La Plata and School of Medical Sciences, University of Rosario, Rosario, Argentina). When tumors grew to a median volume of 600 mm^3, a secondary inoculation of the respective cell type at the same initial dose was carried out in the right flank. The phenomenon of CAR was evidenced as delayed, lower or no growth of the secondary tumor, with respect to control mice (with no previous tumor inoculation in left flank). Percentage of secondary TVI was calculated as $(1 - E/C) \times 100$, where E is the median volume of secondary tumors in tumor-bearing mice and C is that of control mice injected with tumor cells in the second instance. Other experiments in which the nonspecificity of CAR was analyzed were performed as above, except that the tumor cells of the secondary inoculum were different from those of the primary one. The assay for CAR against experimental metastases was carried out with M3MC and M234p. In that case, the secondary tumor inoculation was carried out i.v. with 4×10^4 and 10^6 cells, respectively. The number of metastatic nodules observed in lungs 21 days later was used to evaluate the CAR generation. Metastasis inhibition was calculated in a similar way to TVI, except that E represents the number of lung nodules in tumor-bearing mice and C that of control mice. The animals were subjected to a 12 hr light/12 hr dark cycle and maintained on pellets and water *ad libitum.* Nude mice were housed in sterile polycarbonate cages with microbarrier tops (Nalge, Rochester, NY) and maintained in aseptic conditions inside animal storage cabinets with sterile air laminar flow and controlled environment (Flufrance, Wissous, France).

Angiostatin detection

Sera obtained from mice bearing tumors derived from each one of the cancer cells under study were purified with lysine-sepharose (Pharmacia, Uppsala, Sweden). Briefly, 200 μl of each serum were added to 50 μl lysine-sepharose slurry, incubated on a roller bank for 60 min at room temperature and finally eluted with 50 μl of 0.2 M ϵ-amino-caproic acid.[32] Purified samples were analyzed for angiostatin by Western blot using a rabbit antibody raised against a synthetic peptide whose sequence is derived from the amino terminus (residues 1–19) of mouse angiostatin protein (Affinity Bioreagents, Golden, CO). Briefly, samples were subjected to SDS-PAGE under reducing conditions and then electrotransferred to 0.45 nitrocelullose membrane (Bio-Rad, Hercules, CA). After blocking the membrane for 30 min (2% skim milk in TBS, pH 7.5), it was probed with the rabbit antibody to angiostatin (8 μg/ml) for 1 hr at room temperature. This antibody does not work under nonreducing conditions. The membrane was washed, then incubated for 30 min with a 1:60,000 dilution of a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma) and finally developed using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ) and exposed to X-ray film.

To investigate the *in vitro* generation of angiostatin, 100 μl of cell-number-standardized samples of the different CMs were incubated with 2.8 μg of human plasminogen (Sigma) for 48 hr at 37°C. Control groups consisted of FBS-free culture media instead of CMs. Aprotinin (100 μM), leupeptin (5 μM), soybean trypsin inhibitor (100 μg/ml), $EDTA.Na_2$ (5 mM) (all purchased from Sigma), TIMP-1 and TIMP-2 (Calbiochem, San Diego, CA) at concentrations 0.01–1 μg/ml, α2-antiplasmin (Sigma) 0.01–10 μg/ml and neutralizing antibody against uPA (American Diagnostica, Bethesda, MD), 0.01–10 μg/ml, were used for enzyme inhibition studies. In all cases the inhibitors were incubated with CMs for 1 hr at 37°C before exposure to plasminogen. Aliquots were withdrawn from the reaction mixture, subjected to nonreducing SDS-PAGE and transferred to nitrocellulose. Detection of plasminogen conversion was evidenced by Western blot analysis using a 1:1,000 dilution of a monoclonal antibody specific for kringles 1–3 domains of human plasminogen (VAP 250L; Enzyme Research Laboratories, South Bend, IN). To visualize binding of the first antibody, a rabbit anti-mouse Fc secondary antibody conjugated to alkaline phosphatase (1:10,000) and nitroblue tetrazolium as substrate were used. In all cases, commercially available human angiostatin (Angiogenesis Research Industries, Chicago, IL) was run as a positive control.

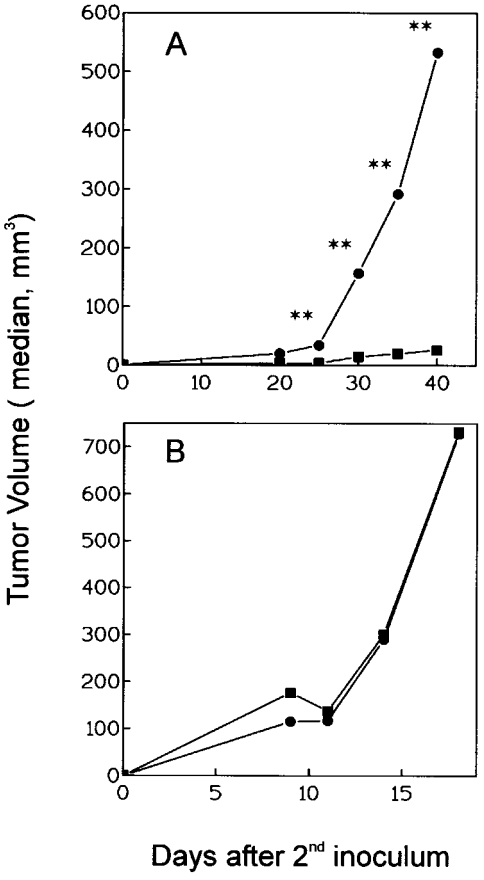

FIGURE 1 – Concomitant antitumoral resistance induced by Calu-6 *(a)* and H460 *(b)* human lung cancer cell lines in nude mice. Median tumor volume in right flank *vs.* time is shown: ■, mice with primary tumor grown in left flank; ●, control mice without previous tumor. Experiments were conducted as described in Material and Methods. Dimensions of tumors in right flank (2nd tumors) were calculated at the indicated intervals according to the equation $V = 0.4\ (ab^2)$, where V = volume, a = largest diameter and b= smallest diameter. The data shown represent the median values ($n = 5$). p according to Mann-Whitney U-test: ** < 0.01.

Zymographic analysis

Gelatinase (MMP-2 and MMP-9) and uPA activities were analyzed in CMs by standard techniques using 7.5% and 11% SDS-PAGE with gelatin or casein/plasminogen, respectively.[33]

Statistical analysis

The nonparametric Mann-Whitney *U*-test was performed on data employing the statistical software package GraphPad In Stat™. Differences were considered significant when $p < 0.05$.

RESULTS

Concomitant antitumoral resistance

To study the capacity of the neoplastic cells here used to induce CAR, mice bearing 600 mm^3 median volume s.c. tumors were contralaterally s.c. challenged with the same tumor cells. Controls consisted of mice that only received the latter tumor inoculation. CAR was evidenced as delayed, lower or no growth of the secondary tumor with respect to control mice. As for human lung cancer cell lines transplanted in nude mice, Calu-6 revealed a strong CAR (Fig. 1*a*), while H460 did not (Fig. 1*b*). The results obtained with Calu-6 confirmed those previously reported.[21] With respect to BALB/c mouse mammary tumors studied, M3MC and M234p exhibited a very strong inhibition of secondary tumor implants (Fig. 2*a,b*, respectively). TVI for M3MC varied from 95.49–96.53% between 15–30 days after secondary tumor inoculation. In the case of M234p, TVI was 100% from day 18 on, since median secondary tumor volume in tumor-bearing mice was 0. In the experimental group, only 1/6 of M234p tumor-bearing mice developed a secondary tumor growth, while 6/6 of the tumor implants carried out at the same time in control mice grew. On the contrary, M234m exhibited no differences in the growth of tumor implants carried out in the second instance (Fig. 2*c*).

The nonspecificity of CAR was assayed in mice bearing CAR-inducing tumors secondarily challenged s.c. with those tumor cell types that did not induce the phenomenon. As shown in Table I, the human lung cancer cell line Calu-6 was able to inhibit in a significant way the growth of secondary contralateral implants of H460 carried out in nude mice. Similarly, M234p induced CAR against M234m in BALB/c mice (Table I). Previous results demonstrated the ability of M3 primary tumors to exert CAR against i.v. inocula of the metastatic MM3 tumor cells, inhibiting significantly the number of lung metastases in mice with respect to control mice with no primary tumor.[9]

Concomitant antimetastatic resistance

To verify whether the capacity to induce CAR against s.c. secondary implants is also effective against experimental lung metastases, M3MC and M234p were i.v. inoculated in tumor-bearing and control naive mice. The presence of s.c. primary tumors in both models markedly reduced the outgrowth of lung metastatic nodules (Fig. 3), revealing metastasis inhibitions of 83% and 98.65% for M3MC and M234p, respectively.

Analysis of angiostatin in tumor sera and conditioned media

The presence of angiostatin in sera from tumor-bearing mice was investigated by Western blot, as described in Material and Methods. The angiogenesis inhibitor protein was not detected in either case, although plasminogen was observed in all sera (data not shown). As an alternative, we analyzed the *in vitro* capacity of tumor cells to generate angiostatin from plasminogen. Conditioned media obtained from Calu-6, M3MC and M234p, the tumor cells that manifested the capacity of generating *in vivo* CAR, were able to convert plasminogen into angiostatin (Fig. 4). This molecule is usually detected as a doublet at approximately 50 kDa, as confirmed by the human angiostatin run as a control. A band corresponding to the approximately 65 kDa heavy chain of plasmin was also detected in all the cases. As a consequence of the enzymatic conversion into plasmin and angiostatin, a decrease in density of plasminogen band at approximately 92 kDa was observed (Fig. 4).

TABLE I – *IN VIVO* INHIBITORY EFFECT OF CAR-INDUCING PRIMARY TUMORS ON CONTRALATERAL IMPLANTS OF NONINDUCING CAR TUMOR CELLS[1]

Primary tumor inoculum	Contralateral tumor inoculum	Tumor volume of contralateral tumor[2] (median [range], mm^3)	TVI[3]
Calu-6	H460	28.4 [0–50]**	94.7
None (control)	H460	538.5 [324–680]	
M234p	M234m	47.8 [0–101]*	81.9
None (control)	M234m	263.6 [48–454]	

[1]Primary tumors were generated by injecting s.c. 2×10^6 Calu-6 human lung cancer cells or 1×10^6 M234p murine mammary cancer cells in nude and BALB/c mice, respectively. When the tumors reached a median volume of approximately 600 mm^3, the animals were contralaterally challenged s.c. with 2×10^6 H460 human lung cancer cells (in the case of mice bearing Calu-6 primary tumors) or with 10^6 M234m murine mammary cancer cells (in the case of mice bearing M234p primary tumors). The tumor cells used as secondary implants are not capable of inducing CAR when used as primary tumors (see Figures 1 and 2).–[2]Tumor volumes measured 20 and 17 days after contralateral s.c. inoculation of H460 and M234m, respectively.–[3]Tumor volume inhibition was calculated as $(1 - E/C) \times 100$, where E is the median volume of secondary tumors in tumor-bearing mice and C is that of control mice injected with tumor cells in the second instance.–* $p < 0.05$, ** $p < 0.005$, Mann-Whitney *U*-test.

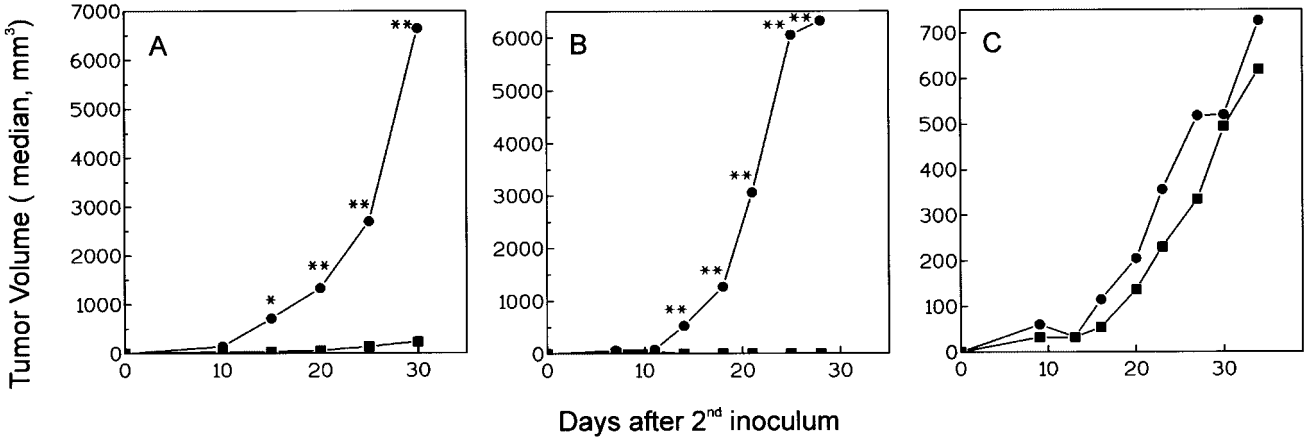

FIGURE 2 – Concomitant antitumoral resistance induced by M3MC *(a),* M234p *(b)* and M234m *(c)* murine mammary tumor cells in BALB/c mice. Median tumor volume in right flank (2nd inoculum) *vs.* time is shown: ■, mice with primary tumor grown in left flank; ●, control mice without previous tumor. Experiments were conducted as described in Material and Methods. Tumor dimensions were calculated as indicated in the legend for Figure 1. The data shown represent the median values ($n = 5–6$). *p* according to Mann-Whitney *U*-test: * < 0.02, ** < 0.01.

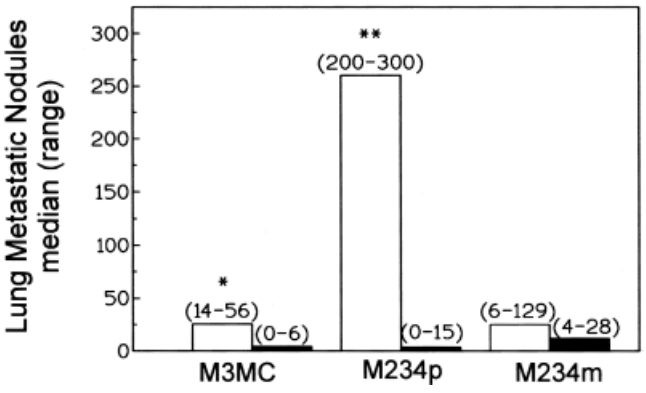

FIGURE 3 – Antimetastatic concomitant resistance. The median number of lung metastatic nodules generated by i.v. inoculation of M3MC, M234p and M234m tumor cells was evaluated in mice bearing their respective s.c. tumors (■) and in controls (□) without previous tumor, as described in Material and Methods. The data shown represent the median values ($n = 6–8$). p according to Mann-Whitney U-test: * < 0.01, ** < 0.002.

Conversely, non-CAR-inducing H460 and M234m cells left unmodified the plasminogen substrate, being unable to generate angiostatin from it (Fig. 4).

Effect of proteinase inhibitors on in vitro *generation of angiostatin*

CMs obtained from tumor cells that demonstrated the capacity to convert plasminogen into angiostatin were exposed to different enzyme inhibitors prior to their incubation with plasminogen, as explained in Material and Methods. The angiostatin conversion could not be abolished in either case by EDTA.Na_2, discarding the responsibility of metalloenzymes in the process (Table II). Although these results were confirmed with TIMPs, some inhibitory effect was observed with the highest doses of TIMP-1 and TIMP-2 when compared to untreated media (Fig. 5). On the other hand,

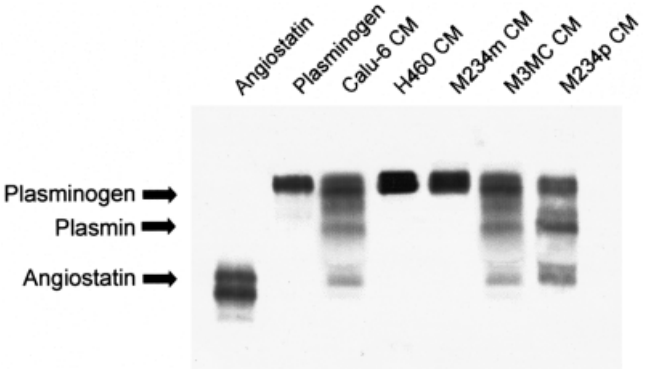

FIGURE 4 – Plasminogen conversion into angiostatin by conditioned media (CM) of the different tumor cells. One hundred microliters of serum-free CMs obtained from equal number of cells in all cases were exposed to human plasminogen (2.8 μg) for 48 hr at 37°C. Samples were then subjected to nonreducing SDS-PAGE and transferred to nitrocellulose. Detection of plasminogen conversion was evidenced by Western blot analysis using a monoclonal antibody (1:1,000) against kringles 1–3 domains of human plasminogen. To visualize binding of the first antibody, a rabbit anti-mouse Fc secondary antibody conjugated to alkaline phosphatase (1:10,000) and nitroblue tetrazolium as substrate were used. Purified angiostatin was used as a specific marker (lane 1). A negative control in which plasminogen was incubated with culture medium was also run (lane 2). Experiments were repeated at least 3 times with essentially identical results.

broad-spectrum serine and serine/cysteine protease inhibitors abrogated angiostatin generation from plasminogen at all doses assayed (Fig. 5). Inhibition of plasmin and uPA activities with α2-antiplasmin and neutralizing antibodies, respectively, suppressed Calu-6 and M234p angiostatin-converting capacity in a dose-dependent manner, but not that of M3MC, abolished by broad-spectrum inhibitors of serine proteases (Table II). Based on this result, the angiostatin-generating ability of M3MC could be ascribed to one or more alternative serine proteinases that remain to be identified.

Expression and activity of gelatinases and UPA in conditioned media

To study the cell production of proteolytic enzymes putatively involved in plasminogen conversion into angiostatin, we performed zymographic analyses of CMs derived from cells with angiostatin-converting capacity. While CMs from all the tumor cells capable of inducing CAR exhibited expression of uPA, neither H460 nor M234m, which were unable to generate CAR, did (Table III). In regard to gelatinolytic activities, only pro-MMP2 expression was observed in CMs from Calu-6, M234p and M234m. This result demonstrates a lack of association between the presence of this MMP and the *in vitro* capacity to generate angiostatin or the *in vivo* ability to induce CAR in the models used by us. Moreover, MMP2 was found in this cell-free system as a zymogen that, consequently, cannot digest plasminogen to generate angiostatin. Conditioned media obtained from H460 and M3MC cell lines did not express MMPs, as demonstrated by zymography.

DISCUSSION

The understanding of the events that drive a dormant tumor to an invasive and then metastatic neoplasm is of fundamental importance for future interventions in the therapy of cancer. Although no definitive conclusions to explain the conversion from one stage to the other could still be clearly drawn, many investigations ascribe an important role to angiogenesis on this aspect. When the delicate balance between positive and negative regulators responsible for tumor dormancy is broken, whether as a consequence of an increase in angiogenic factors, a decrease in angiogenic inhibitors or both, tumor progression would be triggered. On the contrary, an accumulation of antiangiogenic factors would result both in formation of focal necrosis at the primary tumor and suppression of angiogenesis in metastases at distal sites.[34]

In many occasions, primary tumor excision gives rise to uncontrolled growth of metastases that might be silent in distant organs for many years. This phenomenon, initially known as concomitant immunity, was then found to occur with tumors of nondetectable immunogenicity or in immunosuppressed hosts and, as a consequence, we changed the word immunity for resistance, and the concept of concomitant antitumoral resistance emerged.[8] The responsibility of systemic factors that act directly on tumor cell proliferation and/or increasing apoptosis in the generation of CAR cannot be discarded.[22,35,36] However, no experiments have been carried out yet to demonstrate that the phenomenon can be blocked with the use of specific inhibitors for the factors putatively involved in the process. Furthermore, the finding of impaired angiogenesis at places distant from the primary tumor in several experimental models[10,21,22] does not support the hypothesis of CAR mediated by tumoristatic factors. Unless they acted by diffusion on avascular secondary tumors, their direct delivery to tumor cells would depend on the presence of preexisting capillaries at the site. In this context, the finding of endogenous generation of angiostatin and other several angiogenesis inhibitors, mainly by tumor cells, posed the question about the relationship between their production and the induction of CAR.

Here we extended previous studies carried out in our laboratory in which Calu-6, a human lung carcinoma cell line showing strong CAR, prevented neovessels from forming at places that are distant

TABLE II – EFFECT OF BROAD-SPECTRUM AND SPECIFIC ENZYME INHIBITORS ON ANGIOSTATIN GENERATION FROM PLASMINOGEN BY CONDITIONED MEDIA[1]

Protease inhibitor	Specificity	Concentration used	Angiostatin converting capacity		
			Calu-6	M3MC	M234p
–	–	–	+++	+++	+++
Aprotinin	Inhibits serine proteases	100 μM	–	–	–
Leupeptin	Inhibits serine and cysteine proteases	5 μM	–	–/+	–
Soybean trypsin inhibitor	Inhibits trypsin and inhibits plasmin	100 μg/ml	–	–	–
EDTA-Na_2	Inhibits metalloenzymes	5 mM	+++	+++	+++
TIMP-1	Forms complex with latent and active MMPs, preferentially MMP-9	0.01 μg/ml	+++	+++	+++
		0.1 μg/ml	+++	+++	+++
		1 μg/ml	++	+++	++
TIMP-2	Forms complex with latent and active MMPs, preferentially MMP-2	0.01 μg/ml	+++	+++	+++
		0.1 μg/ml	+++	+++	+++
		1 μg/ml	++	+++	++
α2-antiplasmin	Physiologic inhibitor of free plasmin and also prevents the formation of plasmin from plasminogen by uPA	0.01 μg/ml	++	+++	+++
		0.1 μg/ml	+	+++	+++
		1 μg/ml	–	+++	–
		10 μg/ml	–	+++	–
Neutralizing anti-uPA antibody	Neutralizes uPA activity	0.01 μg/ml	++	+++	+
		0.1 μg/ml	+	+++	–
		1 μg/ml	–	+++	–
		10 μg/ml	–	+++	–

[1]The conditioned media obtained from tumor cells capable of generating angiostatin *in vitro* and CAR *in vivo* were incubated with various proteinase inhibitors 1 hr before their exposure to plasminogen for 48 hr. Aliquots were withdrawn from the reaction mixture and analyzed by Western blot to evidence angiostatin conversion from plasminogen or the inhibition of that process.

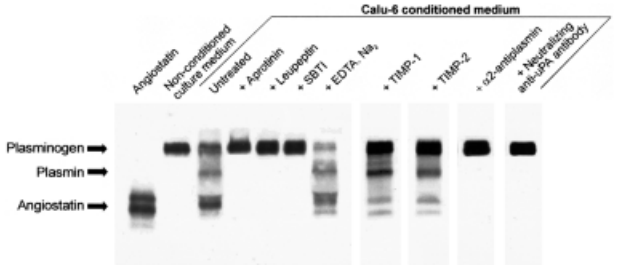

FIGURE 5 – Effect of broad-spectrum and specific enzyme inhibitors on angiostatin generation from plasminogen by Calu-6 conditioned media. The inhibitors were incubated with conditioned medium aliquots derived from the same number of Calu-6 cells for 1 hr at 37°C. Human plasminogen (2.8 μg) was then exposed to all the samples (including nonconditioned culture medium and untreated Calu-6 conditioned medium) for 48 hr at 37°C. Aliquots were withdrawn from the reaction mixture and analyzed by SDS-PAGE under nonreducing conditions and Western blot, using a monoclonal antibody to the kringles 1–3 domains of human plasminogen. Results obtained by preincubation of conditioned medium to highest concentration of each of the inhibitors used are shown here. Similar effects were found for M234p conditioned media treated with all the enzyme inhibitors assayed, while some differences were detected for some inhibitors in the case of M3MC (see Table II). Immunoblots in which the dose-response effect of specific enzyme inhibitors was analyzed are shown by representative lanes in which each highest concentration was tested.

from the primary tumor, with the consequent enhancement of cell apoptosis in secondary tumor implants.[21] One more human lung cancer cell line (H460) and 3 murine mammary cancer cells (M3MC, M234m and M234p) were used here to investigate their ability to induce CAR in nude and syngeneic BALB/c mice, respectively. We observed that 3 of the 5 tumor cell types analyzed, namely Calu-6, M3MC and M234p, displayed strong CAR, inhibiting secondary s.c. tumor implants and/or experimental lung metastases produced by i.v. inoculations of their respective cells. Moreover, secondary implants of other non-CAR-inducing tumor cell types were also inhibited by CAR-inducing primary tumors.

The presence of angiostatin in sera from tumor-bearing mice previously assayed for CAR was analyzed by Western blot after lysine-sepharose purification. Surprisingly, despite several attempts, we did not detect the antiangiogenic factor in either serum, whereas the plasminogen found in those samples was immunodetected in all cases. This could be due to the absence of angiostatin in sera or to the fact that biologically active levels of angiostatin are below the sensitivity threshold for the technique. Recently, other authors reported the inability to detect circulating angiostatin in SCID mice bearing primary tumors derived from human pancreatic cancer cells, which also exhibited a growth-inhibitory effect on secondary tumor implants.[11] Nonpublished results obtained by us demonstrate that angiostatin can be easily detected in ascites from cancer patients and mice, while the protein is absent or in very low amount in their respective sera. This suggests that angiostatin levels are high enough to be detected by Western blot only in those situations in which high tumor cell density is present.

As an alternative, we decided to investigate the *in vitro* capacity of all tumor cells under study to convert plasminogen into angiostatin, even though tumor environment probably influences *in vivo* production of angiostatin.[37] In fact, macrophage metalloelastase proved to have angiostatin-converting activity.[25] We found that the ability to convert plasminogen to angiostatin *in vitro* was only exhibited by those cells capable of inducing CAR. On the other hand, conditioned media obtained from H460 and M234m cells failed to produce proteolysis of plasminogen to generate angiostatin.

Several proteases have been described as responsible for plasminogen conversion into angiostatin, including uPA and plasmin in the presence of sulfhydryl donors,[24] macrophage-derived metalloelastase/MMP-12,[25] matrylisin/MMP-7 and gelatinase B/MMP-9,[26] plasmin,[27] stromelysin-1/MMP-3,[28] gelatinase A/MMP-2,[29] the serine protease prostate-specific antigen[30] and cathepsin D.[31] The conversion of plasminogen into angiostatin could take place in 2 steps if plasmin acts as an intermediary molecule that functions both as substrate and enzyme (Fig. 6*a*), or directly from plasminogen in 1 step

(Fig. 6*b*). In the first case, the Arg^{561}-Val^{562} peptide bond in native [Glu]-plasminogen is cleaved by uPA or tPA, yielding the 2-chain disulfide-linked enzyme plasmin. Angiostatin results as a consequence of plasmin autoproteolysis; its amino-terminal preactivation peptide is released by hydrolysis of the Lys^{76}-Lys^{77} peptide bond and the kringle domain 4 separated from kringle domain 5 by cleavage at Val^{441}-Val^{442}.[24] Reduction of plasmin disulfide bonds linking heavy and light (serine protease module) chains by free sulfhydryl donors has also been reported.[27] In the 1-step conversion process (Fig. 6*b*), a cleavage between amino acid residues Lys^{77}-Lys^{78} located at the amino-terminal region of plasminogen can occur by intervention of MMP-7,[26] MMP-9[26] or PSA,[30] between Glu^{59}-Asn^{60} when MMP-3 acts,[28] or between Leu^{74}-Phe^{75} by action of cathepsin D.[31] An additional cleavage occurs at the amino acid residues Pro^{446}-Pro^{447} in the linker region between the kringle domain 4 and kringle domain 5 by MMP-9,[26] or in the same region between Pro^{447}-Val^{448} by MMP-3[28] or MMP-7,[26] between Glu^{439}-Ala^{440} by PSA action,[30] or between Leu^{451}-Pro^{452} due to cathepsin D.[31] The cleavage sites for other proteinases involved in plasminogen conversion to angiostatin have not been clearly elucidated yet.

Here we intended to define the nature of the proteases involved in the *in vitro* generation of angiostatin by Calu-6, M3MC and M234p, cells that were capable of converting plasminogen into angiostatin, as demonstrated by Western blot. Their CMs were incubated with different enzyme inhibitors, followed by exposure to plasminogen and finally Western blot to detect the resulting product. The use of EDTA.Na_2 did not abolish angiostatin generation, discarding the involvement of metalloproteinases in the process. Similar results were obtained with TIMP-1 and TIMP-2, although the highest doses used revealed some inhibitory effect with respect to untreated media. Conversely, preincubation of CMs with 3 serine proteases inhibitors (one of them also an inhibitor of cysteine proteases) abrogated angiostatin conversion from plasminogen. The use of α2-antiplasmin and neutralizing anti-uPA antibody also reverted the capacity to generate angiostatin in Calu-6 and M234p, but could not do it in M3MC. These data indicate that, except for M3MC which seems to generate angiostatin by a still not identified serine protease, other than plasmin, the plasminogen-angiostatin-converting activity of Calu-6 and M234p cells would mainly depend on the action of uPA and plasmin. Furthermore, zymographic studies revealed uPA expression only in cells that were able to generate angiostatin, while MMP-2 was evident in 3 cases independently to their plasminogen-angiostatin-converting capacity and only in its latent form.

TABLE III – GELATINASE AND UPA EXPRESSION REVEALED BY ZYMOGRAPHIC STUDIES OF CONDITIONED MEDIA FROM THE DIFFERENT CANCER CELLS[1]

Enzyme	Enzymatic activity demonstrated in zymograms by the different tumor cells				
	Calu-6	H460	M3MC	M234p	M234m
ProMMP-9	−	−	−	−	−
MMP-9	−	−	−	−	−
ProMMP-2	+	−	−	+	+
MMP-2	−	−	−	−	−
UPA	+	−	+	+	−

[1]Substrate gel electrophoresis was performed on nonconcentrated cell-number-standardized conditioned media by standard techniques. Gelatinase or uPA activities, revealed as zones of clearance, were indicated as positive (+) or negative (−).

Other studies, as well as the present, reveal a constantly increasing list of proteolytic enzymes that are able to produce angiostatin[24–31] endowed with antitumoral and/or antimetastatic activity in several experimental models.[38–45] These findings put under consideration not only the already known complexity of the processes of tumor progression and metastatic growth control but also the apparent contradictory action of some proteolytic enzymes secreted by tumor cells or tumor-associated host cells. These proteinases can act in both supporting tumor angiogenesis, invasion and dissemination, and inducing the generation of factors that directly or indirectly inhibit them. Hence, certain patterns of homeostatic regulation would take place in cancer, where molecules that promote a process can also inhibit it. It is obvious that such "regulation" is not equilibrated in cancer as in health and, therefore, the knowledge of this delicate and complex mechanism could suggest new pathways for the proposal of therapies leading to normality.

Besides, an important warning can be drawn from these considerations. TIMP-2 expression, for example, not always would act as an inhibitor of MMPs but as an enhancer of pro-MMP-2 activation.[46] In the same direction, the presence of different proteolytic enzymes in tumors or in serum should not necessarily be

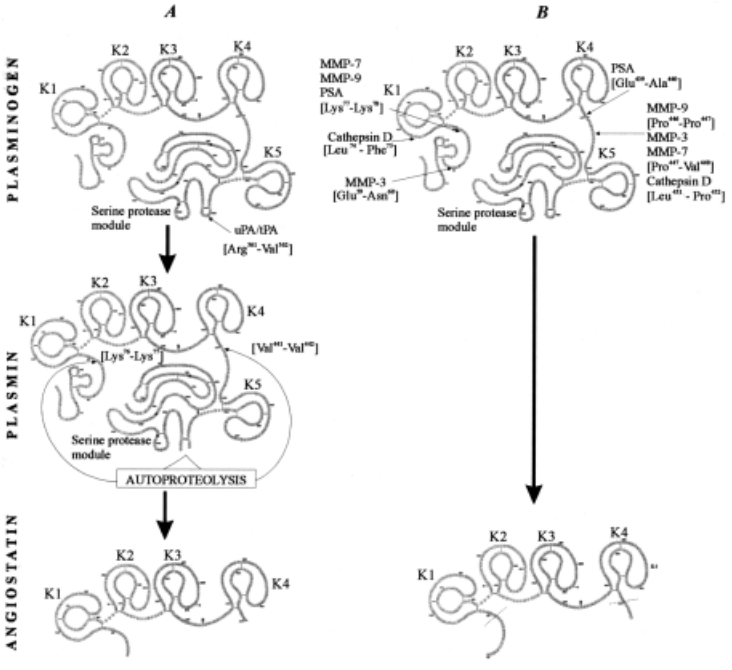

FIGURE 6 – Potential mechanisms by which angiostatin generation could take place. *(a)* In a two-step process, uPA (or tPA) generates the 2-chain disulfide-linked enzyme plasmin. Then, plasmin autoproteolysis gives rise to angiostatin. *(b)* A one-step conversion from plasminogen to angiostatin could be accomplished by a variety of MMPs, cathepsin D and the kallikrein-like serine protease PSA. Peptide bond where cleavage has been previously identified is shown in brackets for each proteinase. Dashed lines indicate alternative N-termini and/or C-termini in angiostatin, depending on cleavage sites of the proteinases involved.

taken as an indicator of progression. It could also suggest the generation of angiostatic factors and the development of CAR with the resulting halt in metastasis growth.

Our results, based in 5 different tumor models, reveal a differential *in vitro* production of angiostatin by CAR-inducing cancer cells and non-CAR-inducing cancer cells. Whether this finding should be considered the actual explanation of the phenomenon of CAR remains to be investigated. Unfortunately, a causative relation could not be verified by us, owing to the nonexistence of specific inhibitors of angiostatin that could help to clarify the CAR phenomenon. Since angiostatin is an internal fragment of plasminogen, all anti-angiostatin antibodies will recognize the precursor molecule as well as plasmin, making them useless for *in vivo* blocking studies. The data obtained by us do not exclude the possibility that, in some cases, CAR may be explained by mechanisms that do not rely on the production of angiostatin. For that reason, we are now studying the presence of other angiogenic inhibitors and inducers in different tumor models in which the CAR capacity will be analyzed. This type of study could help to understand the phenomenon that puzzled surgeons for so many years, that of rapid growth of visceral metastases occurring in many occasions after surgical resection of primary tumors.

ACKNOWLEDGEMENTS

The technical help of Ms. N. Rizzo and the secretarial assistance of Ms. C.V. Martínez are gratefully acknowledged. This work was supported by a grant from Fundación A. J. Roemmers, Buenos Aires, Argentina (to R.D.B.) and fellowships from Fundación de Investigación del Cáncer, FUNDIC, Buenos Aires, Argentina (to M.M.B.) and from Consejo de Investigaciones, Universidad Nacional de Rosario (to P.M.). O.G.S. is an investigator of Consejo de Investigaciones, Universidad Nacional de Rosario. R.D.B. is a member of the Research Career from CONICET (National Council of Scientific and Technical Research, Argentina).

REFERENCES

1. Folkman J. Tumor angiogenesis. In: Bast RC, Kufe DW, Pollock RE, Weichselbaum RR, Holland JF, Frei III E. Holland-Frei cancer medicine, 5th ed. Lewinston, NY: B.C. Decker, 2000. 132–52.
2. Brunschwig A, Southam CM, Levin AG. Host resistance to cancer. Clinical experiments by homotransplants, autotransplants and admixture of autologous leucocytes. Ann Surg 1965;162:416–25.
3. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). Cancer 1974; 33:1027–33.
4. Lange PH, Hekmat K, Bosl G, Kennedy BJ, Fraley EE. Accelerated growth of testicular cancer after cytoreductive surgery. Cancer 1980; 45:1498–506.
5. Demicheli R, Abbatista A, Miceli R, Valagussa P, Bonadonna. Time distribution of the recurrence risk for breast cancer patients undergoing mastectomy: further support about the concept of tumor dormancy. Breast Cancer Res Treat 1996;41:177–85.
6. Gorelik E. Concomitant tumor immunity and the resistance to a second tumor challenge. Adv Cancer Res 1983;39:71–120.
7. Ruggiero RA, Bustuoabad OD, Bonfil RD, Meiss RP, Pasqualini CD. "Concomitant immunity" in murine tumours of non-detectable immunogenicity. Br J Cancer 1985;51:37–48.
8. Meiss RP, Bonfil RD, Ruggiero RA, Pasqualini CD. Histologic aspects of concomitant resistance induced by nonimmunogenic murine tumors. J Natl Cancer Inst 1986;76:1163–75.
9. Bonfil RD, Ruggiero RA, Bustuoabad OD, Meiss RP, Pasqualini CD. Role of concomitant resistance in the development of murine lung metastases. Int J Cancer 1988;41:415–22.
10. O'Reilly MS, Holmgrem L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman K. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315–28.
11. Kisker O, Onizuka S, Banyard J, Komiyama T, Becker CM, Achilles EG, Barnes CM, O'Reilly MS, Folkman J, Pirie-Shepherd SR. Generation of multiple angiogenesis inhibitors by human pancreatic cancer. Cancer Res 2001;61:7298–304.
12. Li T-S, Kaneda Y, Ueda K, Hamano K, Zempo N, Esato K. The influence of tumour resection on angiostatin levels and tumour growth—an experimental study in tumour-bearing mice. Eur J Cancer 2001;37:2283–8.
13. Ehrlich P, Apolant H. Beobachtungen über maligne mäusentumoren. Berl Klin Wochenschr 1905;42:871–4.
14. Bashford EF, Murray JA, Cramer W. The natural and induced resistance of mice to the growth of cancer. Proc R Soc Lond Biol Sci 1907;79:164–87.
15. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–6.
16. Gimbrone MA Jr, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 1972;136:261–76.
17. Kerbel RS. Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. Bioessays 1991;13:31–6.
18. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997;390:404–7.
19. Rastinejad F, Polverini P, Bouck NP. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 1989;56:345–55.
20. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
21. Bonfil RD, Bustuoabad OD, Binda MM. Antiangiogenesis and apoptosis as mediators of concomitant tumor resistance induced by Calu-6, a human lung carcinoma cell line, in nude mice. Oncol Res 1998;10:15–21.
22. Di Gianni PD, Bonfil RD, Genovese J, Dran G, Meiss RP, Bustuoabad OD. Abrogation of neovascularization in tumor bearing mice: a putative explanation of concomitant resistance. J Exp Clin Cancer Res 1995;14:189–95.
23. Chen C, Parangi S, Tolentino MJ, Folkman J. Strategy to discover circulating angiogenesis inhibitors generated by human tumors. Cancer Res 1995;55:4230–3.
24. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, Castellino FJ, Enghild J, Kwaan HC, Lee F, Kramer RA, Volpert O, Bouck N. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci USA 1997;94: 10868–72.
25. Dong Z, Kumar R, Yang X, Fidler IJ. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell 1997;88:801–10.
26. Patterson BC, Sang QA. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J Biol Chem 1997;272:28823–5.
27. Stathakis P, Fitzgerald M, Matthias LJ, Chesterman CN, Hogg PJ. Generation of angiostatin by reduction and proteolysis of plasmin. J Biol Chem 1977;272:20641–5.
28. Lijnen HR, Uguwu F, Bini A, Collen D. Generation of angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). Biochemistry1998;37:4699–702.
29. O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, Folkman J. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J Biol Chem 1999;274:29568–71.
30. Heidtmann HH, Nettelbeck DM, Mingels A, Jäger R, Welker H-G, Kontermann RE. Generation of angiostatin-like fragments from plasminogen by prostate-specific antigen. Br J Cancer 1999;81:1269–73.
31. Morikawa W, Yamamoto K, Ishikawa S, Takemoto S, Ono M, Fukushi J-i, Naito S, Nozaki C, Iwanaga S, Kuwano M. Angiostatin generation by cathepsinD secreted by human prostate carcinoma cells. J Biol Chem 2000;275:38912–20.
32. Westphal JR, Van't Hullenaar R, Peek R, Willems RW, Crickard K, Askaa J, Clemmensen L, Ruiter DJ, De Waal RM. Angiogenic balance in human melanoma: expression of VEGF, bFGF, IL-8, PDGF and angiostatin in relation to vascular density of xenografts in vivo. Int J Cancer 2000;86:768–76.
33. Gude RP, Binda MM, Löpez Presas H, Klein-Szanto AJP, Bonfil RD. Studies on the mechanisms responsible for inhibition of experimental metastasis of B16-F10 murine melanoma by pentoxifylline. J Biomed Sci 1999;6:133–41.
34. Ramanujan S, Koenig GC, Padera TP, Stoll BR, Jain RJ. Local imbalance of proangiogenic and antiangiogenic factors: a potential mechanism of focal necrosis and dormancy of tumors. Cancer Res 2000;60:1442–8.
35. Ruggiero RA, Bustuoabad OD, Cramer P, Bonfil RD, Pasqualini CD. Correlation between seric antitumor activity and concomitant resistance in mice bearing nonimmunogenic tumors. Cancer Res 1990;50: 7159–65.
36. Franco M, Bustuoabad OD, di Gianni PD, Meiss RP, Vanzulli S, Buggiano V, Pasqualini CD, Ruggiero RA. Two different types of

concomitant resistance induced by murine tumors: morphological aspects and intrinsic mechanisms. Oncol Rep 2000;7:1053–63.
37. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 1994;79:185–8.
38. Lannutti BJ, Gately ST, Quevedo ME, Soff GA, Paller AS. Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. Cancer Res 1997;57:5277–80.
39. Sim BK, O'Reilly MS, Liang H, Fortier AH, He W, Madsen JW, Lapcevich R, Nacy CA. A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. Cancer Res 1997;57:1329–34.
40. Wu Z, O'Reilly MS, Folkman J, Shing Y. Suppression of tumor growth with recombinant murine angiostatin. Biochem Biophys Res Commun 1997;236:651–4.
41. Dong Z, Yoneda J, Kumar R, Fidler IJ. Angiostatin-mediated suppression of cancer metastases by primary neoplasms engineered to produce granulocyte/macrophage colony-stimulating factor. J Exp Med 1998;188:755–63.
42. Cao Y, O'Reilly MS, Marshall B, Flynn E, Ji RW, Folkman J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. J Clin Invest 1998;101:1055–63.
43. Kirsch M, Strasser J, Allende R, Bello L, Zhang J, Black PM. Angiostatin suppresses malignant glioma growth in vivo. Cancer Res 1999;58:4654–9.
44. Drixler TA, Rinkes IH, Ritchie ED, van Vroonhoven TJ, Gebbink MF, Voest EE. Continuous administration of angiostatin inhibits accelerated growth of colorectal liver metastases after partial hepatectomy. Cancer Res 2000;60:1761–5.
45. Sacco MG, Caniatti M, Cato EM, Frattini A, Chiesa G, Ceruti R, Adorni F, Zecca L, Scanziani E, Vezzoni P. Liposome-delivered angiostatin strongly inhibits tumor growth and metastatization in a transgenic model of spontaneous breast cancer. Cancer Res 2000;60:2660–5.
46. Hernandez-Barrantes S, Toth M, Bernardo MM, Yurkova M, Gervasi DC, Raz Y, Sang QA, Fridman R. Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation. J Biol Chem 2000;275:12080–9.