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ORIGINAL ARTICLE

Inhibition of angiotensin II receptor 1 limits tumor-associated angiogenesis and attenuates growth of murine melanoma

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Abstract
Purpose We evaluated the involvement of angiotensin II (AngII)-dependent pathways in melanoma growth, through the pharmacological blockage of AT1 receptor by the anti-hypertensive drug losartan (LOS).
Results We showed immunolabeling for both AngII and the AT1 receptor within the human melanoma microenvironment. Like human melanomas, we showed that murine melanomas also express the AT1 receptor. Growth of murine melanoma, both locally and at distant sites, was limited in mice treated with LOS. The reduction in tumor growth was accompanied by a twofold decrease in tumor-associated microvessel density and by a decrease in CD31 mRNA levels. While no differences were found in the VEGF expression levels in tumors from treated animals, reduction in the expression of the VEGFR1 (Flt-1) at the mRNA and protein levels was observed. We also showed downregulation of mRNA levels of both Flt-4 and its ligand, VEGF-C.
Conclusions Together, these results show that blockage of AT1 receptor signaling may be a promising anti-tumor strategy, interfering with angiogenesis by decreasing the expression of angiogenic factor receptors.

Keywords Melanoma · Angiogenesis · AT1 receptors · Angiotensin II · Losartan

Abbreviations

AngII	Angiotensin II
LOS	Losartan
MVD	Microvascular density
RAS	Renin–angiotensin system

Introduction

Angiotensin II (AngII), a multifunctional bioactive peptide in the renin–angiotensin system (RAS), has an important role in the regulation of cardiovascular and renal homeostasis [1]. Initially described as a vasoconstrictor peptide [2], it also promotes proliferation, migration, and growth factor synthesis in several types of vascular cells [3, 4], including smooth muscle cells and pericytes [5–8]. Moreover, AngII can induce the proliferation of endothelial cells, favoring angiogenesis induced by ischemia or associated with tumors [9]. These cellular effects are mainly mediated by angiotensin II receptor type 1 (AT1) [10]. Recent studies showed that inhibition of the angiotensin-converting enzyme (ACE) or blockage of AT1-dependent pathways attenuates VEGF secretion by mesothelial cells, suggesting that AngII modulates VEGF expression [11, 12]. Elements

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of the RAS signaling pathways may be effective targets not only for the treatment of hypertension but also for controlling angiogenesis, a critical process in both inflamed and neoplastic tissue microenvironments [13]. For example, AT1 inhibitors, such as losartan potassium (LOS) and candesartan cilexetil [10], have been widely used in clinical practice as anti-hypertensive agents [14].

Recently, some of us have shown local production of AngII and activation of AngII-dependent pathways in chronically inflamed tissues leading to progressive experimental nephropathies and that high doses of losartan exerted a potent anti-inflammatory role, leading to kidney protection [15, 16]. Sustained inflammation plays an important role in cancer initiation, promotion, and progression [13]. Accordingly, chronic administration of AT1 antagonists can block tumor-associated angiogenesis and tumor growth [17, 18]. Different approaches now highlight novel roles for elements of the RAS. Epidemiological studies published in 1998 [19] first suggested that inhibition of angiotensin-converting enzymes might help preventing cancer. While this notion is still in debate, as pointed out by Deshayes and Nahmias [20], a recent randomized clinical trial showed evidence for skin cancer prevention by the association of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers [21]. A retrospective study on survival of patients with advanced non-small-cell lung cancer undergoing platinum-based chemotherapy showed that addition of inhibitors of RAS prolonged survival of the treated patients [22]. Neither epidemiological nor clinical data are available for many of the more lethal tumors, such as metastatic melanomas.

As clinical results for the treatment of metastatic melanomas are still poor, identification of novel therapeutic targets for their treatment is necessary. In the present study, we showed that human melanomas express both AngII and AT1 receptors in microenvironmental cells, suggesting that AngII can be locally produced within primary tumors. Like human melanomas, murine melanomas also express the AT1 receptor. Further, we evaluated the effect of blockage of AT1-dependent pathways on tumor growth, tumor-associated angiogenesis, and expression of angiogenesis-related genes following a prevention protocol in B16F10 murine melanoma-bearing mice.

Experimental procedures

The experimental procedures followed in this study and listed below were approved by the local Ethics Committee, Comissão para Análise de Projetos de Pesquisa-Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (protocol no. 0061/07).

Human specimens

A collection of 12 paraffin-embedded metastatic melanoma specimens from the Hospital A. C. Camargo tumor bank was used for immunohistochemical analysis of both AT1 receptor and Ang II expression at the protein level, after approval of the local ethics committee.

Animals and melanoma cell line

Female syngeneic C57BL/6 mice (6–8 weeks old, 20 g of weight) were obtained from the Animal House Facility at the University of São Paulo Medical School. All mice were maintained at controlled room temperature (25°C) in a 12-h light/dark cycle. All animal experiments were performed in strict conformity with the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA).

B16F10 metastatic melanoma cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Cultilab, Campinas, BR) in incubators maintained at 37°C in a 5% CO_2 atmosphere. B16F10 cells were washed three times with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with 5 mM EDTA and harvested with 0.2% trypsin (Adolph Lutz Institute, São Paulo, BR). Cells were centrifuged at 300*g* at 25°C, the supernatant was aspirated, and the cells were resuspended in PBS. 5×10^5 cells in 100 μL of PBS were inoculated subcutaneously in the dorsal flank of C57BL/6 mice. The day of inoculation was defined as day 0. After day 11, tumor masses were daily measured using a caliper. At the day 14 (when the average tumor diameter in the control group exceeded 1 cm), all animals were killed. Tumors were then carefully dissected, weighed, and prepared for immunohistochemical analysis and RNA extraction.

Drugs

LOS was administered orally 10 days before the day of tumor implantation in the drinking water. Animals were given LOS, beginning with doses of 75 mg/kg/day, which were doubled every 3 days until reaching the maximal tolerated dose of 300 mg/kg/day (established in pilot experiments) which was then maintained throughout the rest of the experiment.

Morphometry and immunohistochemistry

Human melanoma specimens, tumors, or lungs excised from either control or LOS-treated animals were fixed and embedded in paraffin for histopathological analysis. Routine histopathologic techniques followed by hematoxylin and eosin staining were performed in all experiments.

Assessment of tumor areas in the lungs from both control and LOS-treated animals was performed by morphometry, using a point graticule to determine the relative area occupied by tumors in the lung parenchyma. Images were collected at lower magnification (4× objective, using a Nikon Eclypse E600 microscope), at least 20 independent fields, reaching 200 independent tumor cell clusters that were counted for each experimental condition.

Immunohistochemical analysis from tumors was performed by incubation with anti-CD34 polyclonal antibody, anti-VEGFR1 and VEGFR2 mouse monoclonal antibodies, anti-AT1 antibody (Santa Cruz, CA, USA), or anti-angiotensin II antibody (Santa Cruz, CA, USA) overnight at 4°C, thoroughly rinsed with PBS and incubated with either peroxidase- or alkaline phosphatase-conjugated anti-rabbit IgG for 30 min at room temperature, as indicated. For peroxidase development, slides were rinsed with PBS, and incubated with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA), and counterstained with hematoxylin. For alkaline phosphatase development, slides were rinsed with Tris–buffered saline and incubated with Fast Red™ (Dako), according to the manufacturer's instructions, and counterstained with hematoxylin [23]. Microvascular density and density of positive structures for either anti-VEGFR1 or VEGFR2 antibodies were determined by morphometry as previously described [24]. Histopathological analyses were performed blindly by at least two independent observers (either ALM and RC or CMLM, SN, and RC).

mRNA expression

Tumors from control and LOS-treated animals were excised and total RNA was isolated using TRIzol® (Invitrogen, Carlsbad, CA, USA) reagent following the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) for AT1 receptor was performed using Superscript II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and 1 μg of total RNA was used.

Ribonuclease protection assay (RPA) for angiogenesis-associated mRNA

A panel of angiogenesis-associated mRNA molecules was evaluated using a multiprobe protection assay system (RiboQuant, Becton–Dickinson, BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions. Radiolabeled probes were synthesized from DNA templates containing a T7 RNA polymerase promoter (BD Biosciences, San Jose, CA, USA), transcribed in the presence of 100 μCi [α-^{32}P] dUTP (GE Healthcare, Little Chalfont, UK) to radioactive probes of defined sizes. Probes were hybridized with 10 μg of total RNA, then samples were treated with RNase A and T1 to digest single-stranded RNA. Intact double-stranded RNA hybrids were resolved on 5% polyacrylamide and 7 M urea gels at 50 W for 3 h. The dried gel was exposed to X-ray films (Kodak X-AR) for 16–48 h using intensifying screens. The probes used come as a kit, mAngio-1 (catalog number 551418), which allows for the analysis of the following murine angiogenesis-related molecules: CD31 (platelet endothelial cell adhesion molecule-1), VEGF-C, endoglin (CD105), VEGF, angiopoietin-1; Flt-1 (VEGF receptor, VEGFR1), Flt-4 (VEGF-C receptor), Tie (angiopoietin-2 receptor), Tie-2 (angiopoietin-1 receptor), thrombin receptor; and the housekeeping genes L32 (ribosomal protein) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Quantification of mRNA levels was done by densitometry and the ratio between specific gene expression and L32 gene expression was calculated.

Experimental metastasis assay

A total of 5×10^5 B16F10 melanoma cells in 100 μL of medium were injected into the lateral tail vein of 6- to 8-week-old female C57BL/6 mice ($n = 19$ for control and $n = 8$ for treated group, divided into two independent experiments). LOS was orally administered as described above. Mice in each group were weighed every 2 days. The day of tumor inoculation was defined as day 0. On day 14, all animals were killed; their lungs were removed and fixed in PBS-buffered formaldehyde (3.7%). Metastatic foci that appeared as black spots on the lung surfaces were counted using a magnifying glass. Lungs were then routinely processed for histopathological analysis (hematoxylin and eosin staining). Relative tumor area was determined by morphometry, analyzing nodules present in the lung parenchyma, as described above.

Statistical analysis

Statistical analysis was performed using Student's t test or one-way ANOVA followed by either Bonferroni's test (parametric analysis) or Dunn's multiple comparison test (non-parametric analysis) using GraphPad Prism version 4.0 for Windows® (GraphPad® Software, San Diego, CA, USA), http://www.graphpad.com. A probability (P) value of less than 0.05 was considered statistically significant. The results represent mean ± SEM, as indicated.

Results

Presence of AT1 receptors and angiotensin II in human melanoma tissues

We have found by immunohistochemical analysis the presence of AT1 receptors and local accumulation of

AngII in 12 out of 12 clinical specimens obtained from the Hospital A. C. Camargo tumor bank. The staining pattern showed that vascular structures, and not tumor cells, expressed the angiotensin receptor (Fig. 1a, b) Both negative and positive controls for AT1 receptor staining in human samples are shown in Supplemental Fig. 1. AngII was also found within the stroma of human melanoma (Fig. 1c, d) showing that there may be a local production of this peptide. Therefore, both AT1 receptor and its ligand, AngII, are present within the microenvironment of human melanoma tissues.

Angiotensin II antagonists limited murine melanoma growth

AT1 receptor mRNA was also found in tumors derived from inoculation of B16F10 cells (Supplemental Fig. 2). We have then examined B16F10 melanoma engraftment in C57BL/6 mice treated with AngII receptor antagonists. Two weeks after the inoculation of melanoma cells, the volume of tumors in the control became 82 ± 39 mm^3 (mean ± SEM), $n = 8$, while tumor volume from LOS-treated animals was 39 ± 13 mm^3 (mean ± SEM), $n = 8$. Tumors were detectable in both groups at day 11, but their growth rates were significantly different, 165 ± 8 mm^3/day (mean ± SEM) for control and 77 ± 27 mm^3/day (mean ± SEM) for LOS-treated animals (Fig. 2a). Accordingly, the weight of tumors in LOS-treated animals was about half of that in the control group (Fig. 2b).

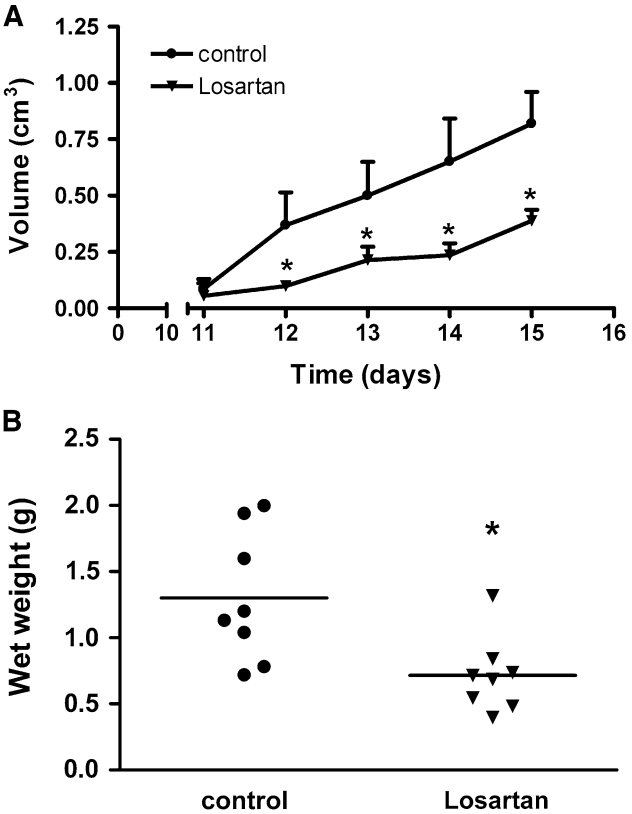

Fig. 2 Angiotensin II antagonists limited murine melanoma growth. **a** Tumor growth rates were significantly decreased in LOS-treated animals (*$P < 0.05$). **b** Animals were killed after 14 days of tumor implantation and the masses were carefully dissected and weighed (*$P < 0.05$). Results in **a** and **b** represent mean ± SEM

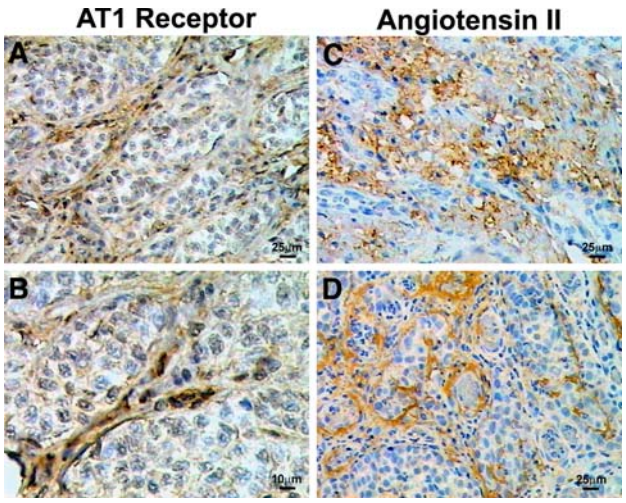

Fig. 1 Human melanoma tissues express AT1 receptors and angiotensin II. **a**, **b** Staining for the AT1 receptor was observed mainly in tumor-associated vessels. **c**, **d** Angiotensin II was found in melanoma and surrounding stromal cells. *Scale bar* 25 μm (**a**, **c**, and **d**) and 10 μm (**b**)

Microvascular density was decreased upon LOS treatment

We next investigated microvascular density (MVD) in the tumors, as a marker of tumor-associated angiogenesis. While in the control group, vessels were homogeneously distributed within tumors, $CD34^+$ vascular structures were scarce and tended to be smaller in tumors from LOS-treated mice (Fig. 3a–d). Values for MVD were 60 ± 9 vessels/mm^2 in the control group and 31 ± 6 vessels/mm^2 in the treated group ($P < 0.05$). No differences were found in the number of larger vessels in these two groups, as indicated in the right panel (Fig. 3e).

LOS modulates the expression of some angiogenesis-associated genes

In LOS-treated animals, there was a decrease in the expression of some angiogenesis-related molecules (Fig. 4a). Among them there were the receptors for angiogenic factors Flt-1, Flt-4, Tie, Tie-2, thrombin receptor, besides a decrease in CD31 expression, and VEGF-C, a lymphangiogenic factor. On the other hand, expression of angiogenic

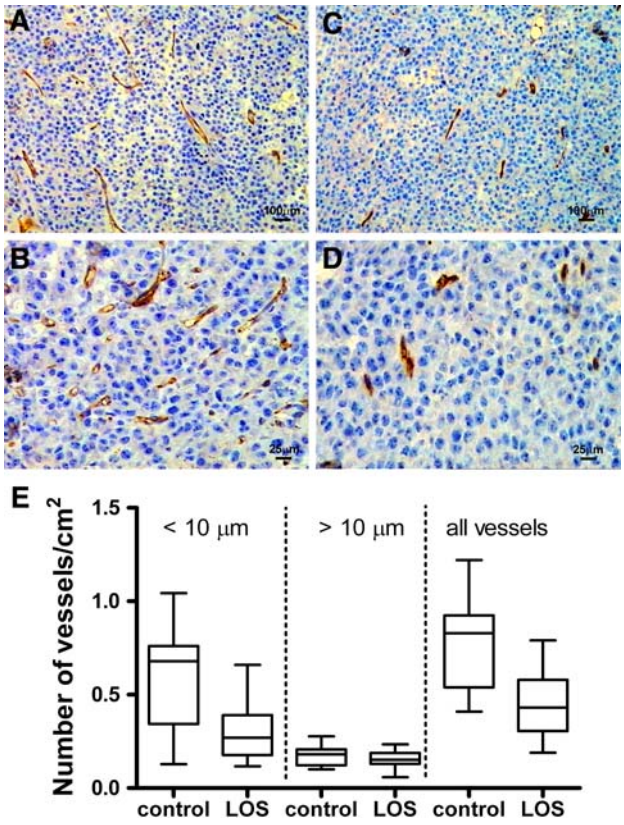

Fig. 3 Microvascular density decreases after losartan (LOS) treatment. Immunohistochemical pattern of vascularization of tumors from control (**a**, **b**) and LOS-treated (**c**, **d**) tumors stained with anti-CD34 antibodies. *Scale bars* 100 μm (**a**, **c**) and 25 μm (**b**, **d**). **e** Box plots (minimum, interquartile range and maximum) display the distribution of microvascular density within groups (*$P < 0.05$)

factors such as VEGF and angiopoietin-1, and the coreceptor endoglin, did not change upon treatment with LOS (Fig. 4b). LOS led to a global decrease in molecules expressed preferentially in endothelial cells, which may indicate that fewer endothelial cells exist in the tumor microenvironment of LOS-treated animals. Decrease of both Flt-4 and VEGF-C expression may indicate that LOS interferes with lymphangiogenesis. Data from RPA analyses, which showed a decreased expression on Flt-1 mRNA levels, were confirmed by immunohistochemistry for the VEGFR-1 receptor, as shown in Fig. 5. LOS led to a decrease in the density of intratumoral VEGFR1 positive vascular structures. No changes were observed in the density of infiltrating VEGFR1 positive mononuclear cells. Our observation is consistent with the notion that VEGFR1 positive cells associated with tumor angiogenesis reside in the perivascular walls of existing vessels [25]. We then extended our observations to VEGFR2 positive cells and vascular structures. VEGFR2 positive cells are recruited from the bone marrow, and together with VEGFR1 positive cells originate new vessels [25]. Treatment with LOS decreased density from both VEGFR2 positive vascular structures and VEGFR2 positive infiltrating mononuclear cells within tumors (Fig. 5). Our data indicate that RAS supports at least in part the neovascularization of engrafted melanoma.

LOS impairs the growth of metastatic lung nodules in mice

LOS treatment did not reduce the number of B16F10 pulmonary metastatic colonies neither at the maximal tolerated dose 300 mg/kg/day nor at lower doses as 150 mg/kg/day (Fig. 6a). However, metastatic foci in lung parenchyma were significantly smaller in the group treated with LOS (Fig. 6b, c). Median values for the relative tumor area for the control group were 10.8% (25th percentile, 4.6%; 75th percentile, 19.2%), while for LOS-treated group were 4.6% (25th percentile, 3.1%; 75th percentile, 17.7%). The difference was statistically significant ($P = 0.001$), thus indicating that the growth of pulmonary metastasis was impaired upon LOS treatment.

Discussion

Tumors have been compared to wounds that do not heal [26]. Tumor microenvironments are characterized by persistent inflammation, which in turn sustains angiogenesis, an essential step for tumor cell growth and survival. As shown in chronically inflamed renal tissues [15], both AT1 and AngII were expressed in human melanoma tissues. Interference with AT1 signaling by reduced levels of the ligand AngII or by inhibition of the receptor may interfere with angiogenesis and impair tumor growth. Since the publication of a retrospective cohort analysis of 5,207 patients in an anti-hypertensive trial, which revealed a decreased cancer incidence in patients on ACE inhibitor drugs [19], the notion that RAS inhibitors may be beneficial as an adjuvant to cancer treatment has been discussed (for examples, see Refs. 20–22). In the present study, we modeled the preventive use of LOS, which could be useful either in a scenario where patients are chronically treated with the anti-hypertensive drug or in a scenario where recurrent tumors arise. We showed that murine melanoma growth was limited upon treatment with the AT1 antagonist LOS, leading to a twofold decrease in the wet weight and tumor volume in the treated animals. The microvessel density within tumors from LOS-treated animals and CD31 mRNA level was diminished, evidencing a lower new vessel formation in tumors due to LOS administration. Transcript analysis of different genes associated with angiogenesis showed that LOS interferes with pathways involved in both tumor-associated formation of blood vessels and lymphangiogenesis. In the former, LOS targets were mainly angiogenic factor

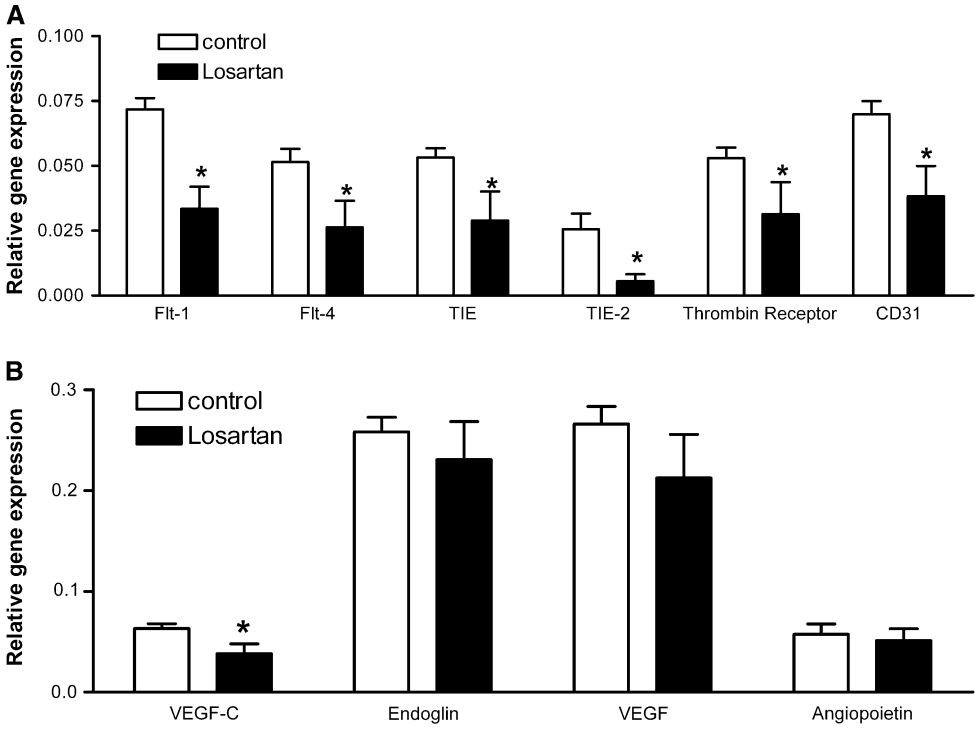

Fig. 4 Angiotensin II receptor antagonist modulates the expression of mRNA of angiogenesis-related genes in melanoma tissues. **a**, **b** Quantification of mRNA levels was done by densitometry and the ratio between specific gene and L32 gene expression was calculated. *Bars* represent mean ± SEM (control, $n = 7$; Losartan, $n = 8$; $^*P < 0.05$, Student's t test)

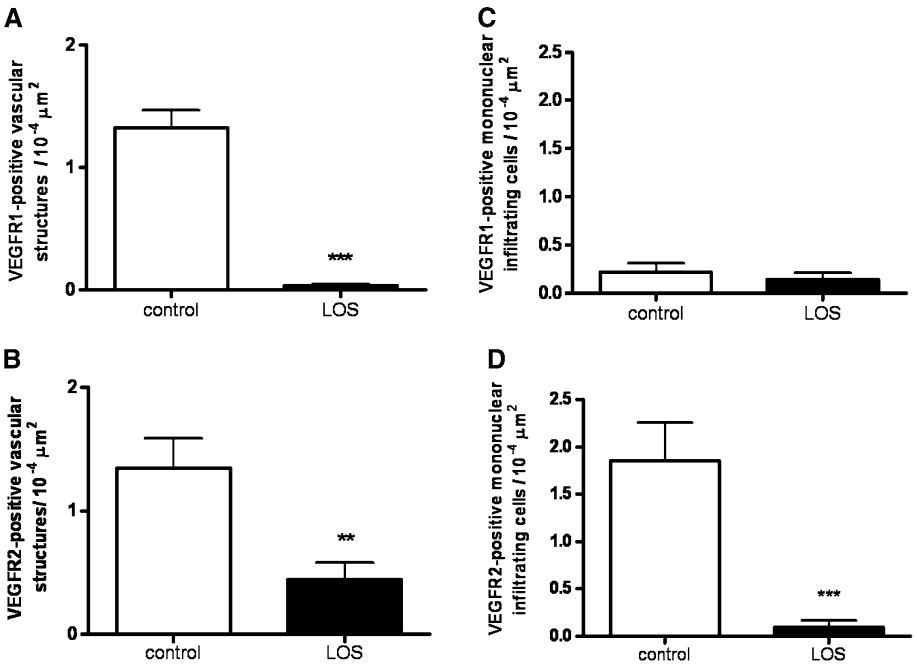

Fig. 5 LOS treatment led to a decrease in both VEGFR1- and VEGFR2-positive vascular structures and to a decrease of infiltrating VEGFR2-positive mononuclear cells within tumors. *Bar graphs* represent the density of either vascular structures (**a**, **b**) or infiltrating mononuclear cells (**c**, **d**) immunostained with antibodies against VEGFR1 (**a**, **c**) or VEGFR2 (**b**, **d**). VEGFR1 immunostaining validated the data on mRNA expression for *Flt-1*, which codes VEGFR1. $^{**}P < 0.01$ and $^{***}P < 0.001$; *LOS* losartan. *Bars* represent mean ± SD

receptors, such as VEGFR1 (Flt-1), VEGFR2 (Flk-1), and members of the Tie family, but not their ligands, such as VEGF and angiopoietin. As to lymphangiogenesis, the levels of both ligand (VEGF-C) and its receptor, Flt-4, were downregulated. Additionally, treatment of animals with LOS did not lead to a decreased number of lung metastatic foci. However, the metastatic foci growth was significantly decreased upon LOS treatment.

Ino et al. demonstrated a positive correlation between the expression of the AT1 receptor and angiogenesis by analyzing human ovarian carcinoma tissues. They showed an upregulation of the receptor, followed by an increase in MVD and VEGF expression in about 87.5% of the cases [27]. Arrieta et al. [28] showed that expression of AT1 and AT2 receptors in astrocytomas is associated with poor prognosis, which correlates the AT1 expression with tumor

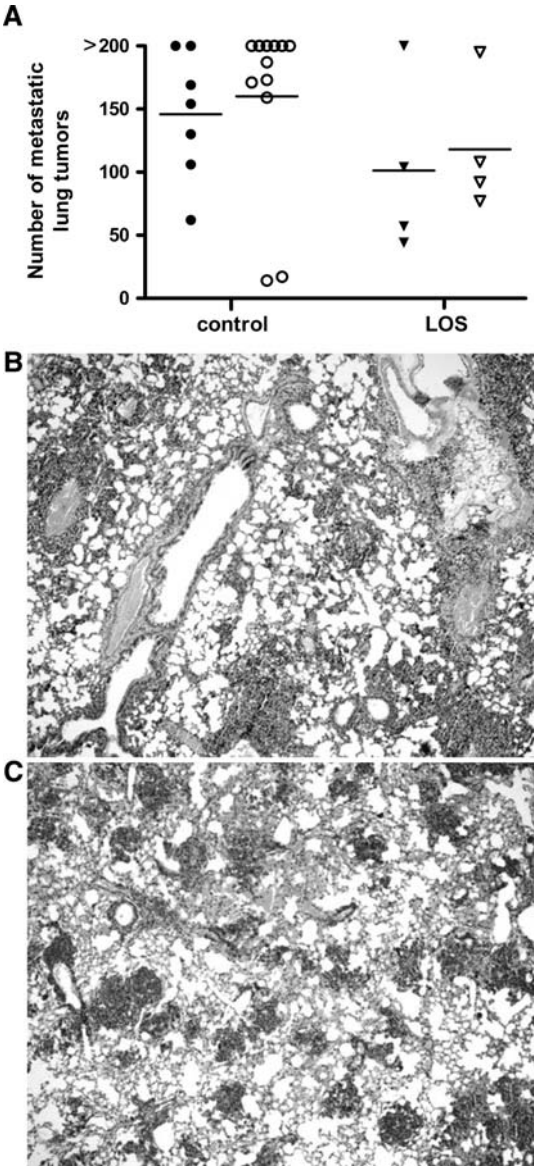

Fig. 6 Angiotensin II receptor blockage does not decrease the number of metastatic foci of B16F10 cells in the lungs of C57BL/6 mice, but impairs their growth. B16F10 melanoma cells were inoculated into the lateral vein of female mice. Metastatic lung foci on lung surfaces were counted (**a**), *black and white spots* represent two independent experiments. Tumors were processed for histopathological analysis (**b** control, **c** LOS-treated). Tumors were easily identified as dense clusters of cells within the lung parenchyma (low magnification). Relative tumor area was determined by morphometry. *LOS* losartan

development. Engraftment of tumor cells was impaired in AngII receptor type 1a (AT1a) knockout mice [12, 18, 29]. Our results are in accordance with those found by Shen et al. [30], who showed that melanoma growth in animals receiving either LOS or the angiotensin-converting enzyme inhibitor captopril was delayed in wild type C57BL/6 mice. The protocol for adapting animals to LOS treatment did not allow for performing therapeutic protocols, but it is well suited for preventive protocols. Regardless, the data shown herein indicated that LOS administration led to an anti-angiogenic tissue microenvironment. Despite its intrinsic limitation, this model opens a perspective for application in specific clinical situations, such as in cases where recurrences are more likely.

In our study, LOS modulated angiogenesis without interfering with VEGF expression level. Rather, the effect observed may reflect cellular insensitivity to VEGF, due to a decrease in the expression of VEGF receptors as Flt-1, also known as VEGFR1, VEGFR2, and Flt-4. FGF-2, another angiogenesis-associated growth factor, also activates new blood formation by inducing VEGF expression [31]. Moreover, thrombin receptor activation can modulate the expression of VEGF receptor, which is expressed in endothelial cells, macrophages, smooth muscle cells, fibroblasts, and platelets [32]. Thrombin receptor activation was shown to upregulate the expression of VEGF receptors in endothelial cells, sensitizing them to VEGF [33]. In the present study, the finding of downregulation of the thrombin receptor by LOS is consistent with the concept that angiogenesis may be regulated by varying the expression of VEGF receptors, thus changing cellular sensitivity to VEGF.

Other angiogenesis-related receptors have also been modulated in this model. The Tie family of receptors seems to be required for the angiogenic remodeling and vessel stabilization that follow the initial angiogenic actions of VEGFR1 (Flt-1) and VEGFR2 [34]. Tie and Tie-2 receptors are expressed mainly in endothelial cells, in their precursors and in hematopoietic progenitor cells [35]. The downregulation of all these receptors may explain the decrease in angiogenesis observed in this study. Signaling of Tie receptors depends on the balance of their ligands, members of the angiopoietin family, which may control vascular plasticity, angiogenesis, and vascular permeability [36]. We did not observe alterations in the mRNA levels of angiopoietin-1 within the tumor microenvironment upon LOS treatment. These results are in agreement with those reported by Otani and colleagues, who showed that while AngII controls expression of angiopoietin-2, it did not modulate angiopoietin-1 expression [37].

Besides angiogenesis, lymphangiogenesis is also critical to tumor progression. Activation of VEGFR-3 (Flt-4) by its ligand, VEGF-C, is necessary for lymphangiogenesis [38–40]. It is well known that upregulated expression of VEGF-C or VEGF-D in a variety of human tumors predicts metastasis to regional lymph nodes and poor prognosis [41]. Modulation of lymphangiogenesis by AT1 signaling is a potentially interesting phenomenon that warrants further investigation. Accordingly, Wang et al. recently showed that AT1 blockers, such as LOS, led to a decrease in gastric cancer growth. Besides that, they showed that VEGF-C

expression was diminished upon LOS treatment, as evaluated by immunohistochemistry [42].

Advanced malignant melanoma has a poor prognosis and conventional chemotherapies have showed high toxicity with low objective response against tumor growth. Despite the use of protocols involving multiple drugs, genetic instability of tumor cells can lead to selection of resistant clones. In this regard, anti-angiogenic agents which target normal microenvironmental cells show less toxicity and induce little or no drug resistance [43]. As the first trials directed toward inhibiting angiogenesis indicate, VEGF signaling pathways are indeed promising targets for therapy. Most trials targeted VEGF and not its receptors. As we have shown here, LOS was effective in downregulating the expression of VEGF receptors (Flt-1, Flk-1, and Flt-4). The safety in the clinical use of LOS and other AT1 receptor antagonists has been extensively evaluated, as they are routinely used as anti-hypertensive drugs. Based on our results, we could anticipate that the combination of both anti-VEGF strategies [44] and blockage of AT1 receptor signaling will prove useful in the control of tumor-associated angiogenesis. Moreover, as the trials with the humanized anti-VEGF antibody (bevacizumab) are indicating, hypertension is one of the most serious complications of anti-angiogenic therapies [45–47]. Within this context, regimens exploiting both the anti-hypertensive and anti-angiogenic properties of AT1 blockers may prove beneficial to cancer patients [44, 48, 49]. Finally, as we had shown here, RAS may constitute a novel target for the treatment of metastatic melanoma in an adjuvant setting.

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Conflict of interest statement The authors declare no conflict of interest.

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