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Blockage of angiotensin II type I receptor decreases the synthesis of growth factors and induces apoptosis in C6 cultured cells and C6 rat glioma

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Angiotensin II (Ang II) is a main effector peptide in the renin–angiotensin system and participates in the regulation of vascular tone. It also has a role in the expression of growth factors that induce neovascularisation which is closely associated to the growth of malignant gliomas. We have shown that the selective blockage of the AT_1 receptor of angiotensin inhibites tumour growth, cell proliferation and angiogenesis of C6 rat glioma. The aim of this study was to study the effects of the blockage of AT_1 receptor on the synthesis of growth factors, and in the genesis of apoptosis in cultured C6 glioma cells and in rats with C6 glioma. Administration of losartan at doses of 40 or 80 mg kg^{-1} to rats with C6 glioma significantly decreased tumoral volume and production of platelet-derived growth factor, vascular endothelial growth factor and basic fibroblast growth factor. It also induced apoptosis in a dose-dependent manner. Administration of Ang II increased cell proliferation of cultured C6 cells which decreased by the administration of losartan. Our results suggest that the selective blockage of AT_1 diminishes tumoral growth through inhibition of growth factors and promotion of apoptosis.

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The renin angiotensin aldosterone system (RAAS) has an important role in the regulation of blood pressure and fluid electrolyte balance. Angiotensin II (Ang II), a major participant in the RAAS, was initially described as a vasoconstrictor, but recent studies have revealed that it also participates in cell growth, cell differentiation and apoptosis (Stoll *et al*, 1995; Escobar *et al*, 2004), and has a role in cell migration and conformation of the extracellular matrix (Coker *et al*, 2001). Some reports indicate that Ang II induce neovascularisation (Fernandez *et al*, 1985; Le Noble *et al*, 1991; Andrade *et al*, 1996) due to stimulation of growth factors, such as platelet-derived growth factor (PDGF) (Khachigian *et al*, 2000; Cook *et al*, 2002), transforming growth factor beta β (TGFβ) (Kagami *et al*, 1994; Ohta *et al*, 1994; Hamaguchi *et al*, 1999; Weigert *et al*, 2002), insulin-like growth factor 1 (IGF-1) (Brink *et al*, 1999; Haddad *et al*, 2003), basic fibroblast growth factor (bFGF) (Peng *et al*, 2001), vascular endothelial growth factor (VEGF) (Otani *et al*, 1998; Tamarat *et al*, 2002) and angiopoietin 2 (Otani *et al*, 2001). Angiotensin II also induces the expression of proto-oncogenes in smooth vascular muscle cells, including *c-fos*, *c-jun*, *c-myc*, *erg-1*, *VL-30*, and the activator of the protein 1 complex (Cook *et al*, 2002), Interestingly, many of these effects are inhibited by the blockage of the AT_1 receptor (Fujiyama *et al*, 2001). Angiotensin II has a dual and paradoxical intervention in apoptosis through its receptors AT_1 functioning as antiapoptotic and AT_2 functioning as proapoptotic (Stoll *et al*, 1995).

Experimentally, the RAAS has been associated to proliferation of tumours; Ang II receptors have been found on the cell surface and cytoplasm of human tumours such as breast cancer (Guerra *et al*, 1993; Inwang *et al*, 1997; Berry *et al*, 2000; Muscella *et al*, 2002), hepatic carcinoma (Yoshiji *et al*, 2002), renal carcinoma (Hii *et al*, 1998; Miyajima *et al*, 2002), melanoma (Egami *et al*, 2003), colorectal carcinoma, squamous cell carcinoma (Takeda and Kondo, 2001), pancreas cancer (Fujimoto *et al*, 2001) and sarcomas (Volpert *et al*, 1996; Fujita *et al*, 2002).

Glioma is the most frequent primary tumour of the brain. Malignant gliomas are still associated with poor prognosis, the mean survival time of patients with glioblastoma multiforme (GBM) is 1 year, and it has not changed significantly for the last three decades (Lopez-Gonzalez and Sotelo, 2000). Glioblastoma multiforme is accompanied by extensive angiogenesis which is essential for tumoral growth and invasiveness; it also produces a vast amount of growth factors such as PDGF, VEGF, HGF and FGF (Strugar *et al*, 1995; Arrieta *et al*, 1998, 2002; Moriyama *et al*, 1999; Schmidt *et al*, 1999). In brain parenchyma there is a local RAAS, some neurons, glial cells and glioma cells express renin, angiotensinogen and receptors for Ang II (AT_1 and AT_2) (Ganong, 1984, Ariza *et al*, 1988; Fogarty *et al*, 2002; Juillerat-Jeanneret *et al*, 2004). We have previously shown that the blockage of AT_1 receptors in rats with C6 glioma inhibited tumour growth, cell proliferation and angiogenesis (Rivera *et al*, 2001). The aim of this

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study was to determine *in vitro* (in cultured C6 glioma cells) and *in vivo* (in rats with C6 glioma) the effects of the blockage of the AT_1 receptor on the synthesis of growth factors and its relation with cell proliferation and apoptosis.

MATERIALS AND METHODS

Glioma induction

C6 glioma cells (American Tissue Culture Collection Rockville, MD, USA) were cultured under sterile conditions at 37°C in a humid environment with 5% CO_2 in Dulbecco's modified Eagle's medium (DME) (Sigma chemical company, Saint Louis Missouri, USA) supplemented with 10% foetal bovine serum (GIBCO, New York, USA). After the cultures became confluent, the cells were washed with saline solution and harvested; 10^7 C6 cells from this source were inoculated intraperitoneally in a male Wistar rat; 2 weeks later, a large tumour was obtained (Guevara and Sotelo, 1999), it was mechanically dispersed in saline solution (1 : 1) at 4°C, 10^7 cells from this source were subcutaneously injected in the left thigh of 40-day-old Wistar rats (Arrieta *et al*, 2001). All animals developed a noticeable tumour within 2 weeks. All animals used in this study were handled in accordance to the guidelines of the coordinating committee on cancer research for the welfare of animals with experimental neoplasms.

Blockage of AT_1 *in vivo*

When the tumour had reached a diameter of 1.5 cm, the rats were randomly assigned either to the control group ($n = 40$), to the losartan 40 mg Kg^{-1} group (L40) ($n = 40$), or to the losartan 80 mg Kg^{-1} group (L80) ($n = 40$). Losartan (Merck-Sharp & Dohme, Mexico) was given orally, once a day, for 30 days. At the end of the experiment, 20 rats from each group were anaesthetised and perfused by intracardiac route with 10% formalin in saline solution for histological study. Before perfusion, the animals were bled by intracardiac puncture to analyse haematological and chemical parameters in blood; the body weight of animals from all groups remained similar throughout the study. The tumour was dissected and its volume was determined by water displacement. The tumours from nonperfused animals were kept at −70°C until analysis.

Histological analysis and apoptosis *in vivo*

For microscopic study, the tumour was embedded in paraffin: 5 μm sections were stained with haematoxylin and eosin. Apoptosis was detected by DNA fragmentation using TdT incorporation of nucleotides on 3′ ends of DNA (TUNEL technique). Briefly, 5 μm of glioma C6 tumour sections was dewaxed and immersed in 3% H_2O_2 to block endogenous peroxidase; after washing with distilled water, proteinase K (20 μg ml^{-1}) was applied to the specimens for 15 min at room temperature. Detection of apoptosis *in situ* was made by ApopTag Peroxidase (Oncor, Gaithersburg, MD, USA) that detects fragmented DNA and was performed according to the manufacturer's conditions. Sections were counterstained with methyl-green. Positive control sections were prepared by nicking DNA with DNase and negative control sections were prepared by substituting with distilled water for working strength TdT enzyme. The proportion of apoptotic cells was expressed as apoptotic rate, which represented the number of apoptotic cells among 1000 glioma C6 nucleated cells, excluding segmented neutrophils due to their short lifespan.

Determination of growth factors *in vivo*

In all, 10 samples from each experimental group were unfrozen, weighted, and homogenised at 4°C (1 : 1) in saline solution; 70 μg ml^{-1} of phenylmethylsulphonyl fluoride (PMSF) was added for inhibition of proteases, the lysate was centrifuged at 6500 g for 20 min and kept at −70°C until analysis. Tissue contents of bFGF, VEGF, PDGF and HGF were measured by enzyme-linked immunosorbent assay (ELISA) (R&D system, Minneapolis, MN, USA). Each sample was assayed by duplicate and reported as means ± s.d.

Treatment of C6 cells in culture

We separated C6 glioma cells in four groups: control cells (without treatment); cultures treated with Ang II (10^{-7} M); cultures treated with Losartan (10^{-5} M) (Stroth *et al*, 2000) and cultures treated with both Ang II and Losartan (L – A) at the same doses. All cultures were kept in a serum-free medium at 37°C, in humidified environment with 5% CO_2. The culture medium used for cells incubated for long periods was changed every 24 h containing either Ang II, losartan or Ang ll plus losartan at the mentioned doses.

Cell viability of C6 glioma in culture

For quantification of cell viability, 6.5×10^5 glioma cells were cultured in microtitre plates (96 wells) with 100 μl culture medium and incubated for 24 h in a humidified atmosphere; 10 μl of tetrazolium salts (MTT) was added to each well and left for 4 h; these salts were cleaved into a coloured formazan product by metabolically active cells; afterwards 100 μl of solubilisation solution was added, the plates were allowed to stand overnight inside the incubator. After checking for complete solubilisation of the purple formazan crystals, the absorbance was measured at 570 nm, the reference wavelength was 650 nm. We obtained the percentage of viable cells from three assays, the control was used as reference (100%). Two replicates were perfomed using the same dosages.

Apoptotic rate *in vitro* by flow cytometry

For quantification of apoptosis by flow cytometry, 10^6 C^6 glioma cells were trypsinised and transferred to 1.5 ml Eppendorf tubes and cultured with DME culture medium either alone (controls) or with Losartan 10^{-5} M, or with Ang II 10^{-7} M, or with the mixture of losartan/Ang II (LA). Cultured cells were maintained at 37°C with 5% CO_2 during 6, 12, 24, 48 or 96 h. The culture medium of those experimental groups which needed a long time of incubation was changed every day. At the end of the incubation period, the cells were centrifuged at 2000 g, the supernatant was discarded. Induced apoptosis was detected by flow-cytometric analysis of the permeabilised, propidium-iodide-stained cells (Telford *et al*, 1992). Samples (10^6 cells ml^{-1}) were washed once in PBS and the pellets were re-suspended in 80% ethanol at 4°C for 60 min. To detect apoptosis by flow cytometry, fixed cells were centrifuged, re-suspended in 1 ml PBS, and kept at 37°C for 20 min before staining with a solution of 0.1% Triton X-100, 0.1 mM $EDTA(Na)_2$, 5 U ml^{-1} RNase and 20 mg ml^{-1} propidium iodide in PBS. Samples were stored in the dark at room temperature and analysed with a Facscalibur Registered Trademark (Becton Dickinson & Co., San Jose, CA, USA). Using the cell quest software (San Jose CA, USA), cell number (10^4) was detected. Cell percentages in the different phases of the cell cycle were estimated according to the Fox's method (Lacombe *et al*, 1988). The assays were made by triplicate.

Apoptotic rate *in vitro* by ELISA

For quantification of apoptosis by ELISA, 10^5 C^6 glioma cells were trypsinised and transferred to 1.5 ml Eppendorf tubes and cultured with DME culture medium either alone (controls) or with Losartan

10^{-5} M, or with Ang II 10^{-7} M, or with the mixture of LA. Cultured cells were maintained at 37°C with 5% CO_2 during 6, 12, 24, 48 or 96 h. The culture medium of those experimental groups which needed a long time of incubation was changed every day. At the end of the incubation period, the cells were centrifuged at 2000**g**, the supernatant was discarded and the cells were re-suspended in 1 ml of culture medium with 10% DMSO and kept in liquid nitrogen until processed. At that time, the vials were warmed at 37°C and the cells were washed with culture medium by centrifugation at 2000**g**. The cells were then re-suspended with incubation buffer (Cell Death detection ELISA Kit, Boehringer Mannheim catalogue: 1544675) at 4°C during 30 min. The cellular lysate was centrifuged at 20 000**g** for 10 min and 400 μl of the supernatant were removed (cytoplasmic fraction) and diluted 1 : 10 with incubation buffer, absorbance was measured at 405 nm, the substrate solution was used as blank. The assays were made by triplicate; 10^6 C6 cells in hypertonic buffer and 10^6 C6 cells with camptotensin were used as positive controls.

Statistical analysis

Values were expressed as means $\pm$ s.d.; for the quantification of apoptotic rate, the kappa value was used as the interobserver variability value. Statistical analysis was made using SPSS v10 software. Comparisons between groups were made by ANOVA and Tukey tests. Statistical significance was set at a *P*-value of 0.05.

RESULTS

Effect of the blockage of AT_1 receptor on tumoral growth and apoptotic rate *in vivo*

All animals survived until the end of the experiment. In all controls, the tumour grew to a very large size (over 6 cm diameter); there was no case of spontaneous involution. When compared to controls (mean volume: 55 ± 10 cm^3) a significant decrease in tumour volume was seen in animals treated with L40 (30 ± 7 cm^3) and with L80 (19 ± 9 cm^3) ($P < 0.05$ and 0.01, respectively). Results were similar to those from our previous report (Rivera *et al*, 2001). The apoptotic rate was higher in the L80 group (84.2 ± 4.1) than in controls (23 ± 8) or in the L40 group (13.6 ± 4.3) ($P < 0.01$); no differences were seen when L40 group was compared with controls (Figure 1). Comparisons of haematological and chemical blood parameters measured at the end of the study showed no differences between groups.

Growth factors in neoplastic tissue

Mean contents of PDGF, bFGF and VEGF were higher in tumours from control animals (PDGF = 1,195 $\pm$ 261, bFGF = 893 $\pm$ 4, and VEGF = 154 $\pm$ 7 pg mg^{-1} of tissue) than in tumours of animals treated with L40 (PDGF = 609 $\pm$ 84, $P = 0.05$; bFGF = 857 $\pm$ 12,

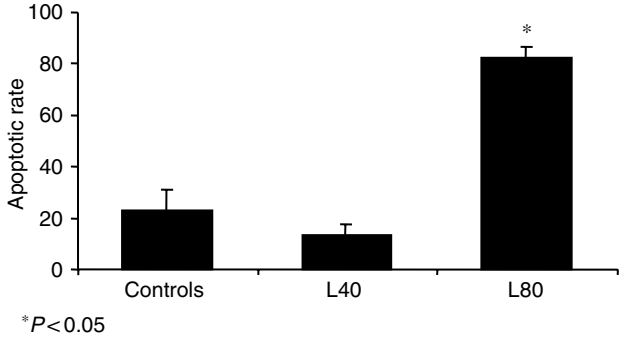

Figure 1 Effect of losartan administration at doses of 40 and 80 mg Kg^{-1} on the apoptotic rate in tissue sections of C6 glioma (TUNEL stain). A significant increase of the apoptosis was observed with losartan treatment at 80 mg Kg^{-1}.

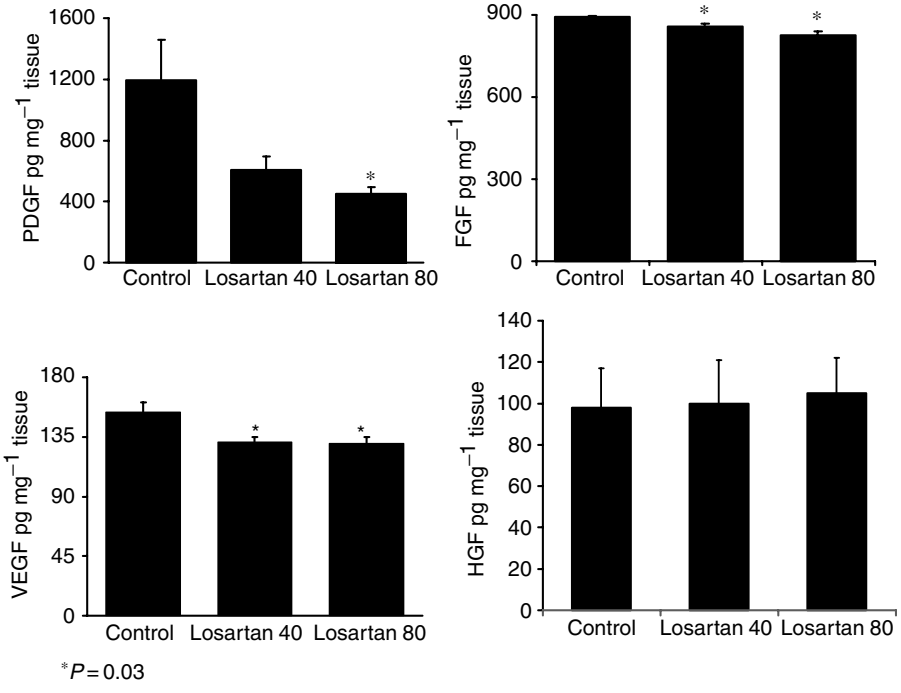

Figure 2 Effect of the blockage of AT_1 receptor with losartan on the contents of PDGF, FGF, VEGF and HGF in C6 rat glioma. A significant reduction in the concentration of PDGF, FGF and VEGF was obtained with losartan treatment.

$P=0.035$; and VEGF $=131\pm4\,pg\,mg^{-1}$, $P=0.011$) and those treated with L80 (PDGF $=451\pm45$, $P=0.018$; bFGF $=827\pm14$, $P=0.001$; and VEGF $=105\pm19\,pg\,mg^{-1}$, $P=0.013$). However, no significant differences were seen on HGF contents between controls ($97\pm20\,pg\,mg^{-1}$) and L40-($100\pm29\,pg\,mg^{-1}$) or L80-treated rats ($105\pm19\,pg\,mg^{-1}$) ($P=0.65$) (Figure 2).

Effects of Ang II, losartan and LA on cell cultures

The percentage of viable cells was significantly smaller in cultures treated with L–A at 6 and 12 h as compared with controls ($P=0.01$ and 0.05, respectively); however, no differences were seen later, at 24, 48 and 96 h. The percentage of viable cells was higher in cells cultured with Ang II at 48 and 96 h as compared to controls ($P=0.04$ for both determinations). Treatment with Losartan had no effect on cell viability (Figure 3).

Apoptosis rate *in vitro* by flow cytometry

A significative increase of apoptosis was observed in cells treated with L–A particularly at 6 h as compared to controls ($P=0.05$) and with the losartan group ($P=0.012$), and at 12 h as compared with the groups Ang II ($P=0.007$) and losartan ($P=0.001$), which was maintained at 24 h with losartan ($P=0.007$). No differences were found at 48, 72 and 96 h (Figure 4A).

Apoptosis rate *in vitro* by ELISA

Cells cultured with Ang II showed increased apoptotic rate at 6 h when compared with losartan ($P=0.012$) and at 12 h when compared to controls ($P=0.012$). When compared with controls, apoptosis was increased in cells treated with L–A ($P=0.007$), with losartan ($P=0.003$) and with Ang II ($P=0.011$) at 6 h, as well as the cells treated with L–A at 12 h ($P=0.05$). No differences were found at 24, 48, 72 and 96 h (Figure 4B).

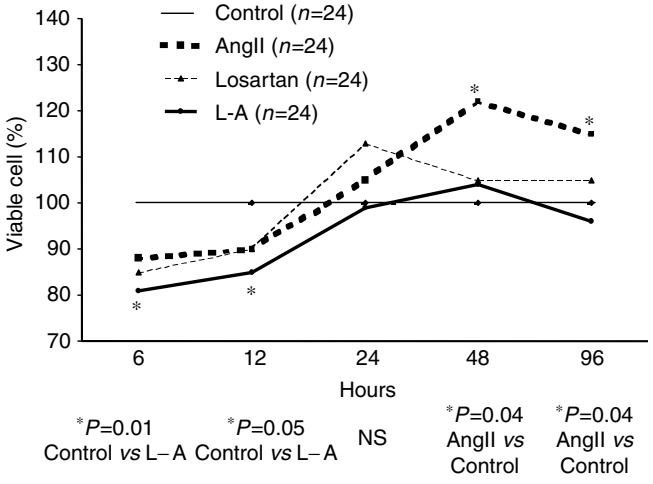

Figure 3 Effects of the administration of Ang II (10^{-7} M), losartan (10^{-5} M), or Ang II plus losartan on cultured C6 cells. During the initial 12 h of treatment, there was a significant reduction of viability in the cells treated with Ang II plus losartan; in contrast, after 48 h, a significant increase of viability was observed in the cells treated with Ang II alone.

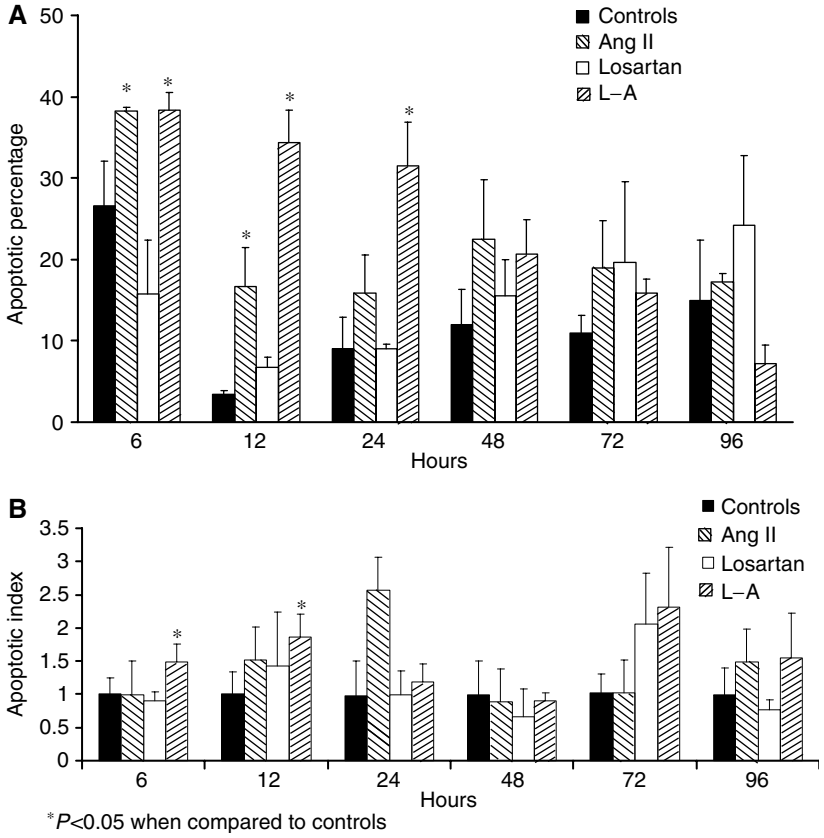

Figure 4 Effects of Ang II (10^{-7} M), losartan (10^{-5} M) and Ang II plus losartan on apoptosis in cultured glioma C6 cells measured either by flow cytometry (**A**) or by ELISA (**B**). In both determinations the cells treated with losartan plus Ang II showed an increase of apoptosis during the initial hours after treatment.

DISCUSSION

Malignant gliomas contain large quantities of VEGF, PDGF, bFGF and HGF, as well as their receptors (Stefanik *et al*, 1991; Westermark *et al*, 1995; Arrieta *et al*, 2002; Steiner *et al*, 2003); their concentrations are related to vascular density, radioresistance, cell proliferation, degree of malignancy and patient's survival (Westermark *et al*, 1995; Gorski *et al*, 1999). Angiotensin II stimulates the production of these growth factors (Peng *et al*, 2001; Tamarat *et al*, 2002); this effect can be blocked by AT_1 antagonists but not by AT_2 antagonists (Tamarat *et al*, 2002). Normal and neoplasic astrocytes including C6 glioma cells express AT_1 and AT_2 receptors for Ang II (Rivera *et al*, 2001; Fogarty *et al*, 2002). We have previously shown in experimentally induced C6 glioma in rats that the selective blockage of AT_1 receptor reduces cell proliferation, angiogenesis and tumour growth (Rivera *et al*, 2001). However, the participative mechanisms were unclear. In this study, we found that the blockage of AT_1 decreases the synthesis of the growth factors VEGF, PDGF and bFGF, coincident with the reduction of tumour size, cell proliferation and vascular density. The effect of losartan in the synthesis of growth factors is more intense in PDGF, which is inhibited with the blockage of AT1 in several experimental models; nonetheless, VEGF and FGF syntheses are also statistically reduced. However, the reduction of angiogenesis previously reported might be a consequence of VEGF and FGF inhibition due to a blockage of PDGF's stimuli, this would explain why the effect is not dose-dependent on losartan. Although HGF is also overexpressed in malignant gliomas and related to the degree of malignancy (Arrieta *et al*, 2002) and Ang II also participates in its synthesis (Matsumoto *et al*, 2003), in this study no effect on HGF contents was observed after blockage of the AT_1 receptor.

Additionally, our results *in vitro* show that the reduction on cell viability within the first h of Ang II plus losartan administration was also associated with the induction of apoptosis but only during the first 12 h, whereas the administration of Ang II alone stimulates cell proliferation during 24 h after exposure. Increase of apoptosis in glioma C6 *in vivo* was seen only at high doses of losartan ($80\,mg\,Kg^{-1}$); this effect was also obtained in cultured C6 cells with the simultaneous administration of losartan and Ang II; it seems that the selective blockage of AT_1 may lead to disequilibrium of AT_1/AT_2 relation that promotes AT_2 receptor stimulation which, in turn, could increase its proapoptotic effects. It is also likely that, in this condition, the presence of Ang II promotes apoptosis, as this effect is prevented by deletion of the AT_2 receptor gene (Yamada *et al*, 1996). Our findings are in agreement with studies in PC12W cells and neurons from newborn rats, showing that apoptosis is increased either by the selective stimulation of AT_2 receptor or by the blockage of AT_1 receptor simultaneous to the administration of Ang ll (Stoll *et al*, 1995; Yamada *et al*, 1996; Goldenberg *et al*, 2001; Suzuki *et al*, 2002).

Subucutaneous C6 glioma, in contrast to brain glioma, allowed us to measure tumour growth for a longer time and to determine a potential therapeutic effect when the drug is administered for long periods. However, similar results are to be expected in brain glioma as the blood-brain barrier is interrupted in brain tumours, facilitating the entrance of drugs into the tumour.

Angiogenesis and apoptosis are primordial features of malignant tumours, constituting attractive therapeutic targets; drugs that inhibit angiogenesis or promote apoptosis could be associated to cytotoxic agents to improve antitumoral therapy. The fact that multifunctional hormonal systems, such as the renin–angiotensin–aldosterone system, influence tumour growth and angiogenesis provides interesting pathways to the study of cancer. The antineoplastic activity obtained by the selective blockage of AT_1 in malignant glioma seems to be mediated by two different mechanisms, inhibition of the synthesis of growth factors and promotion of apoptosis, providing a potential therapeutic adjuvant for malignant gliomas.

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