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Losartan improves the distribution and efficacy of doxorubicin in CT26 tumor

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Abstract. – OBJECTIVE: The effectiveness of chemotherapeutic agents is impaired by limited delivery of chemotherapeutic agents to the tumor cells. Improving drug penetration in tumor tissues is very important. We tested whether losartan, a selective antagonist against type 1 angiotensin II receptors (AT1R) with noted antifibrotic activity, can enhance the penetration and efficacy of doxorubicin.

MATERIALS AND METHODS: BALB/C mice, which implanted with CT26 tumor cells, were divided into four groups: control, doxorubicin alone, losartan alone and doxorubicin + losartan combination groups. At day 0, the losartan alone and doxorubicin + losartan combination groups received losartan; and at day 8, the doxorubicin alone and doxorubicin + losartan combination groups received doxorubicin i.v. Tumor growth and intratumoral distribution of doxorubicin were evaluated. The mechanism underlying the enhanced anti-tumor effect of the combination of doxorubicin and losartan was investigated by immunohistochemical analysis.

RESULTS: Treatment with losartan alone did not suppress tumor growth; In contrast, treatment with doxorubicin alone decreased tumor growth; losartan and doxorubicin were administered in combination, had a synergistic effect that the tumor growth was much more inhibited. The decreased proliferation as indicated by down-regulation of Ki67, and increased apoptosis as indicated by TUNEL and caspase-3 staining. The expression of tumor suppressor gene P53 increased in doxorubicin + losartan combination groups.

CONCLUSIONS: Losartan can increase the therapeutic effectiveness of doxorubicin, yielding more great antitumor benefit. This study provided a rationale for initiating clinical trials using losartan in combination with chemotherapeutic agents to increase their therapeutic effectiveness.

Key Words:
Losartan, Doxorubicin, CT26 colon carcinoma, Drug resistance.

Introduction

The limitations of clinical chemotherapy have been ascribed primarily to the intrinsic tumor characteristics, such as tumor cells gene mutations, gene amplification, or epigenetic changes. However, substantial data suggests that tumor microenvironment contribute to drug resistance in cancer therapy[1-3]. The prerequisite of the anticancer drug to kill a high proportion of cancer cells in a solid tumor is that the chemotherapeutic agents must penetrate and distribute within tumors, and achieve a potentially lethal concentration in the tumor tissue. Namely that the anticancer agents must be distributed throughout the tumor vasculature, cross vessel walls, and traverse the tumor tissue. Drug distribution in tumors is determined by its concentration gradient in the tumor tissue. Current study indicated that the composition and organization of the extracellular matrix affect drug penetration[4]. The extracellular matrix, its main ingredient is collagen, also can affect the sensitivity of the tumor cells to apoptosis and their response to chemotherapy[5,6].

Losartan[7], an angiotensin II receptor (type AT1) antagonist, is approved to control hypertension in patients. In addition to its antihypertensive properties, losartan can reduce the expression and activation of transforming growth factor-β1 (TGF-β1)[8,9], also can inhibit the growth of pancreatic tumor and has synergistic inhibitory effect when used in combination with gemcitabine[10]. There is a growing body of data indicated that losartan as one antifibrotic agent has been shown to reduce the appearance of renal and cardiac fibrosis[11,12]. Losartan can inhibit the collagen deposition and maturation; and decreased microvessel density in tumors grown in SPARC+/+ mice[13]; and improve delivery of therapeutic nanoparticles by inhibiting the collagen synthesis[14]. It is gener-

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ally accepted that the collagen in tumors is mostly produced by cancer-associated fibroblasts.

Doxorubicin (DOX), an inhibitor of DNA topoisomerase II, is one of the most commonly used antitumor drugs, which acts by introducing double-strand breaks on DNA in proliferating cells to trigger apoptosis[15]. But doxorubicin show very poor distribution in solid tumors. The use of doxorubicin in cancer therapy has been limited by its serious side effects especially cardiotoxicity[16]; and the development of cellular resistance. Lack of p53 function is also associated with doxorubicin resistance[17]. Study indicated that the modification of the tumor extracellular matrix might facilitate the penetration of drugs into tumor tissue[13,14]. Therefore, it is more attainable to use a conventional therapeutic dose of doxorubicin in combination with the new drug which targeting the tumor extracellular matrix. Therefore, the aim of the present study was to investigate the anticancer properties of the combination of doxorubicin and losartan as a treatment strategy in CT26 colon carcinoma mice model, and, if such properties were found, to determine the underling mechanism.

Materials and Methods

Animals and Cell Culture

BALB/C mice, 5 to 7 weeks old, were purchased from the HFK bioscience Co., LTD (Beijing, China). All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. All studies involving mice were approved by the Institute's Animal Care and Use Committee. CT26 colon carcinoma (CT26; ATCC) cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

Tumor Regression Activity in vivo

To generate tumors, BALB/C mice (n=10 per group) were subcutaneously implanted in the right hind flank with 150 ml of a single-cell suspension containing 5×10^5 CT26 tumor cells. The mice implanted with CT26 tumor cells were divided into four groups as follows: (1) untreated controls; (2) mice treated with doxorubicin alone (ZheJiang Hisun Pharmaceutical Co., LTD); (3) mice treated with losartan alone (Merck, Shanghai, China); (4) mice treated with a combination of doxorubicin + losartan. At day 0, the losartan alone and doxorubicin + losartan combination groups received 150 μl of losartan intraperitoneal injection (20 mg/kg/day), which was repeated every day for the duration of the experiment. At day 8, the doxorubicin alone and doxorubicin + losartan combination groups received 100 μl of doxorubicin i.v, which was repeated 3 times, and interval 5 days. The dose of doxorubicin was 8 mg/kg. Tumor growth was evaluated by measuring the tumor diameters every five days, and tumor volume was calculated as length × width^2 × 0.52.

The Distribution of Doxorubicin Within the Tumor Tissue

The mice were sacrificed, and tumors were removed 24h latter when the third doxorubicin was given. The tumor tissue was embedded in Tissue-Tek OCT; and cut into 6 μm sections. Confocal fluorescence images were collected using x 20 objectives on a Leica sp5 confocal upright microscope system (Leica, Jena, Germany). Confocal images were exported to Leica LAS-AF Lite-2.6.0-7266 software.

In situ Apoptosis Detection by TUNEL Staining

The mice were sacrificed, and tumors were removed 24h latter when the third doxorubicin was given. Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin; and cut into 5 μm serial sections. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to study the DNA fragmentation or apoptosis detection. TUNEL assay was performed according to the manufacturer's guide (Roche, Mannheim, Germany). TUNEL positive cells were analyzed by microscopy (Leica) at 20 × magnification; and counted per view at 40 × magnification. Five representative fields were chosen for counting. Apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells in the field

Immunohistochemistry of Tumor Tissue

The detailed description of techniques is as described above (TUNEL staining). For immunohistochemical staining with Ki-67 (Merck, Shanghai, China), P53 and caspase-3 (Cell Signaling, Danvers, MA, USA) antibodies, sections were blocked with 3% peroxidase in methanol after fixation in cold acetone and washing in tris-buffered sline (TBS) supplemented with 0.1% (w/v) bovine serum albumine (BSA). Following another washing step, sections were incubated

overnight at 4°C in 1:100 dilutions of rabbit anti-mouse Ki-67, P53 or caspase-3 antibodies in TBS/0.1% BSA. Slides were washed in TBS/0.1% BSA supplemented with 0.1% (v/v) Tween-20 and subsequently in TBS/0.1% BSA before addition of horse-raddish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (DAKO, Carpinteria, CA, USA) for 45 min at room temperature. After washing, sections were stained with 3,3'diaminobenzidine (DAB). Color development was monitored microscopically and stopped in distilled water (dH_2O). Sections were then counterstained in hematoxylin, dehydrated and mounted as mentioned above. The Ki-67, P53 and caspase-3 positive cell analyzed by microscopy (Leica) at 20 × magnification; and counted per view at 40× magnification. Five representative fields were chosen for counting.

Satistical Analysis

SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The statistical significance of results in all of the experiments was determined by ANOVA and Student's *t*-test. Survival curves were compared using the log-rank test. $p < 0.05$ was deemed statistically significant.

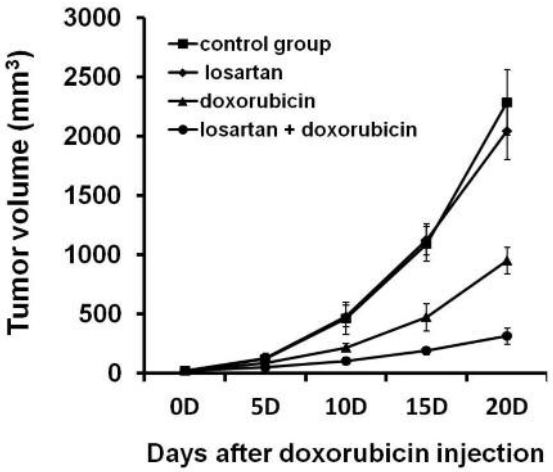

Figure 1. The anti-tumor activity vo. At day 0, the losartan alone and doxorubicin + losartan combination groups received losartan intraperitoneal injection (20 mg/kg/day), which was repeated every day for the duration of the experiment. At day 8, the doxorubicin alone and doxorubicin + losartan combination groups received 8mg/kg of doxorubicin i.v, which was repeated 3 times, and interval 5 days. The average tumor volumes are shown as a function of time after initiation of treatment. Note the enhanced efficacy of the combined treatment compared with either agent alone. $p < 0.01$, losartan + doxorubicin combination versus other groups; $p < 0.01$, doxorubicin alone versus control, losartan alone group

Results

Antitumor Activity in Subcutaneous Tumor Xenograft Model

To test whether losartan could improve the therapeutic effect of doxorubicin, their anticancer activity was tested using xenograft model of murine CT26 colon carcinoma. 7 days after tumor implantation and initiation of losartan treatment (20mg/kg/day), we treated mice with doxorubicin (8 mg/kg, i.v) every 5 days, which was repeated 3 times. The tumor growth was evaluated at the day 8, and interval 5 days. Losartan + doxorubicin combination treatment resulted in significantly slower tumor growth than the control, losartan, or doxorubicin alone group treatments. The growth delay was significant ($p < 0.001$) in CT26 tumors treated with losartan + doxorubicin combination compared with other groups (Figure 1). Losartan alone did not affect the growth of CT26 tumors.

Losartan Improved the Intratumoral Distribution of Doxorubicin

Based on current studies that losartan is an antifibrotic agent, and can inhibit the collagen deposition and maturation, and the collagen affect drug penetration[13,14]. We hypothesized that the losartan can improve the distribution of doxorubicin in the CT26 tumor tissue. We therefore measured the intratumoral distribution of doxorubicin with its natural orange-red fluorescence in the tumor tissue. We found that losartan can improve doxorubicin penetration and accumulation in the tumor tissue of losartan + doxorubicin combination group (Figure 2a). In compared, there was little doxorubicin accumulation in the tumor tissues of doxorubicin alone group (Figure 2b).

Immunohistochemical Evaluation of Antitumor Activity

The mechanism underlying the enhanced antitumor effect of the combination of doxorubicin and losartan was investigated by immunohistochemical analysis. The effect of tumor cells' proliferation was assessed by analyzing the expression of Ki67, a marker of the G1, S, M, and G2 phases of the cell cycle[18]. Strong immunostaining of Ki67 was observed in tumors from the control group, losartan alone, or doxorubicin alone

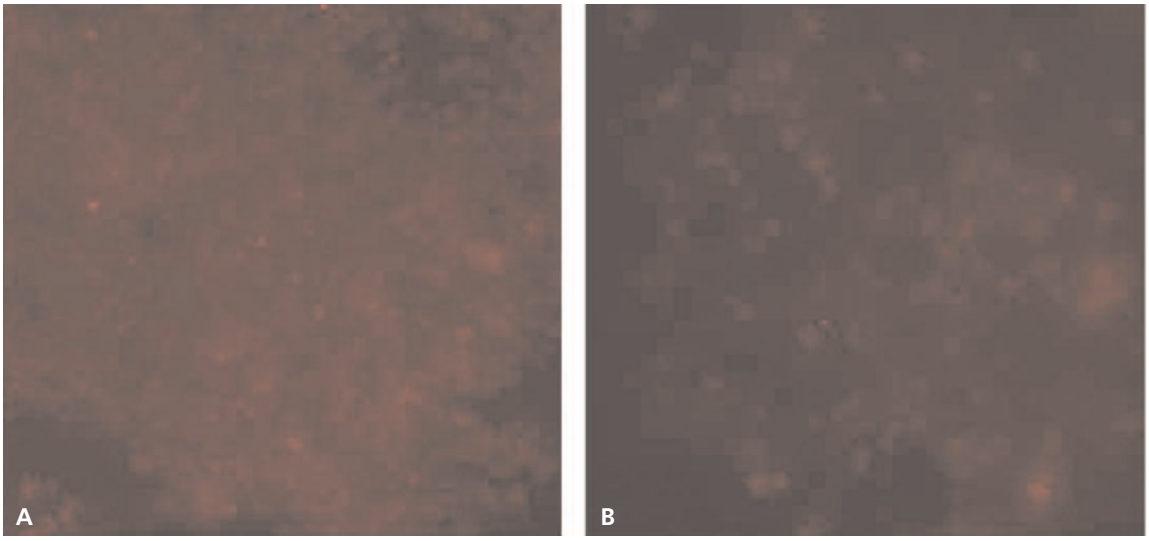

Figure 2. Losartan increases delivery of doxorubicin. Losartan significantly increased the distribution of i.v. injected doxorubicin. An analysis of the distribution pattern shows that there was a small amount of intratumoral doxorubicin in the tumor tissue ***(B)***, which was treated only with doxorubicin. In contrast, the tumor tissues which were treated with a combination of doxorubicin and losartan have a significant number of intratumoral doxorubicin ***(A)***.

group; whereas low-level staining was found in those from the doxorubicin + losartan combination treated group (Figure 3A). Quantification of the Ki67-positive spots showed that tumor sections from the groups treated with PBS or losartan alone contained 25% and 23% positive spots, respectively. The Percentage of Ki67-positive cells decreased to 10.2% in the group treated with doxorubicin alone and, more importantly, to 3.5% in the group treated with the doxorubicin + losartan combination (Figure 3B).

To analyze the degree to which doxorubicin and losartan combination induced apoptosis *in vivo*, we performed a TUNEL and immunohistochemical staining of caspase-3. Qualitative microscopic examination of TUNEL-stained sections revealed very few positive spots in tumors treated with phosphate buffered saline (PBS) or losartan (Figure 3A). In the doxorubicin alone treated group, TUNEL-positive nuclei were marginally detected. In contrast, the number of TUNEL-positive apoptotic cells was significantly higher in the doxorubicin + losartan combination treated tumor tissue than in PBS-, losartan alone-, or doxorubicin alone-treated tumor tissue. Quantification of the TUNEL-positive spots showed that tumor sections from the groups treated with PBS or losartan alone contained 3% and 4% positive spots, respectively. The Percentage of TUNEL-positive cells increased to 18% in the group treated with doxorubicin alone and, more importantly, to 38% in the group treated with the doxorubicin + losartan combination (Figure 3B). This demonstrated that the doxorubicin + losartan combination can induce more tumor cells to apoptosis than the observed in the other groups.

Consistent with this result, administration of the doxorubicin + losartan combination strongly reduced the degree of staining with antibody against the apoptotic markers caspase-3; whereas a high number of caspase-3 positive cells were observed in the groups treated with PBS, losartan alone, or doxorubicin alone group (Figure 3A). Quantification of the caspase-3 positive spots showed that tumor sections from the group treated with PBS or losartan alone contained 2% and 3.5% positive spots, respectively. The percentage of caspase-3 positive tumor cells increased to 19% in the group treated with doxorubicin alone and, more importantly, to 40% in the group treated with the doxorubicin and losartan combination (Figure 3B), demonstrating that the doxorubicin and losartan combination can induce more tumor cells to apoptosis than the observed in the other groups. Together, these findings suggest that the enhanced antitumor effect of doxorubicin and losartan combination resulted from inhibition of cell proliferation and promotion of apoptosis in the tumor.

Most of the anticancer drugs are believed to kill cancer cells by inducing apoptosis. The tumor suppressor protein p53 plays a critical role in DNA damage-induced apoptosis. Therefore, the possibility that the combination-treated of doxorubicin and losartan up-regulates the expression of tumor suppressor gene, leading subsequently to cell apoptosis, was examined. The high number of P53 positive cells were observed in the groups treated with the doxorubicin and losartan combination (Figure 3A). Quantification of the P53 positive spots showed that tumor sections from the groups treated with PBS or losartan alone contained 2% and 1.5% positive spots, respectively. The Percentage of P53 positive cells increased to 13% in the group treated with doxorubicin alone and, more importantly, to 22% in the group treated with the doxorubicin and losartan combination (Figure 3B), demonstrating that the doxorubicin and losartan combination can induce more P53 to express than the observed in the other groups.

Discussion

The doxorubicin, a topoisomerase II inhibitor, is one of the most effective and widely used chemotherapeutic agents. However, one of the remarkably limitations to the clinical application of doxorubicin is the acquired or intrinsic drug resistance and toxicities. Chemo-resistance is a major problem in the treatment of malignant tumours; hence, it will be of potential value to discover combination protocols that can overcome resistance. Recent evidence indicates that an-

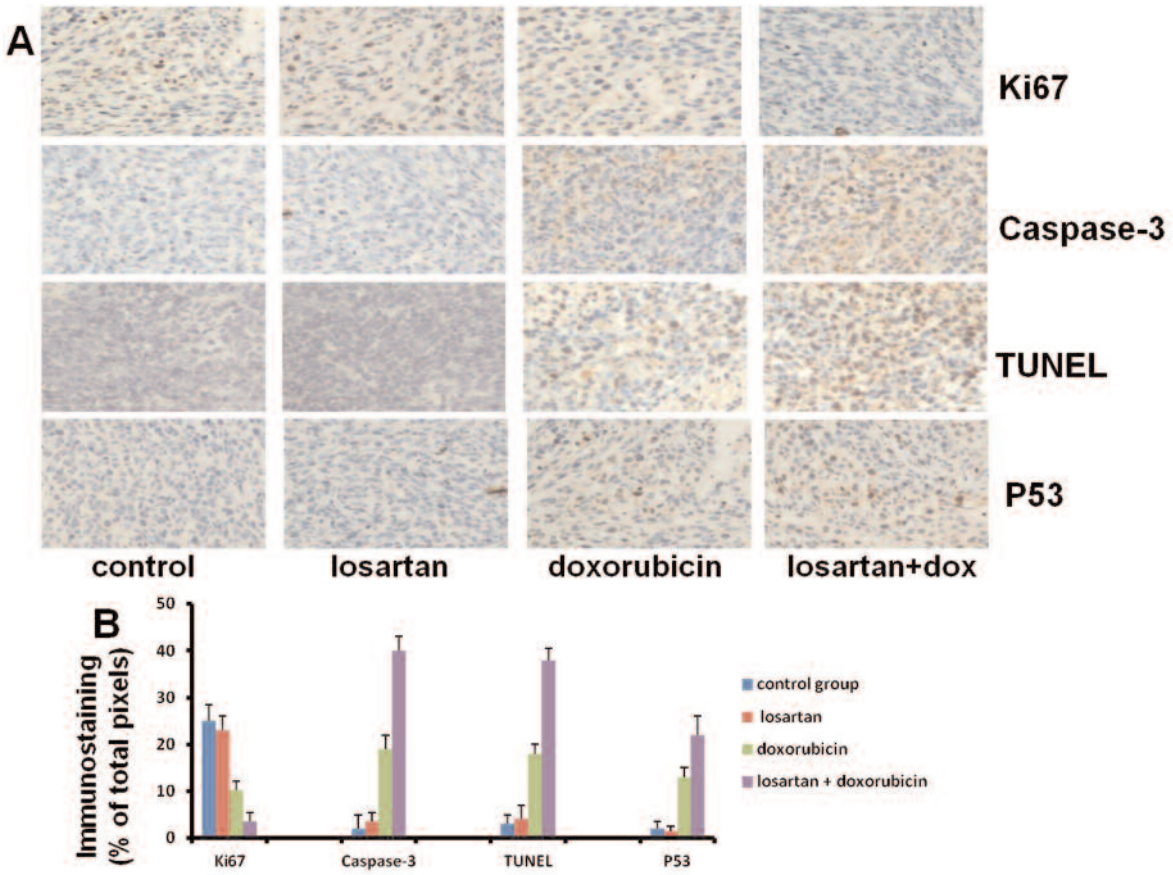

Figure 3. IHC analysis. The induction of apoptosis and inhibition of proliferation *in vivo* were evaluated by caspase-3, TUNEL, Ki67 and P53 staining. Tumor tissues were harvested at 24h after three injections of doxorubicin. ***A)*** Representative photographs of the tumor sections. Brown staining nuclei indicates Ki67, TUNEL or P53-positive cells; and brown staining cytoplasm indicates caspase-3 positive cells. ***B)*** Percentage of TUNEL, caspase-3, Ki67 and P53 positive cells was calculated. Quantitation of caspase-3, TUNEL, Ki67 and P53 staining from immunohistochemical analyses was shown. Results are the mean ± S.E. $p < 0.01$, losartan + doxorubicin combination versus other groups; $p < 0.01$, doxorubicin alone versus control and losartan alone group.

giotensin II can stimulate collagen production via both TGF-β1-dependent and -independent pathways[19]. As a result, losartan, an angiotensin II receptor antagonist can reduce the levels of collagen I and III and basement membrane collagen IV in various experimental models of fibrosis[20,21]; losartan also can inhibit collagen I production in tumors[14].

In the present study, we demonstrate that concomitant treatment of mice bearing CT26 colon carcinoma with losartan and a conventional therapeutic dose of doxorubicin results in a synergistic inhibition of tumor growth. Our finding strongly support the hypothesis that losartan can improve the intratumoral distribution of doxorubicin and enhanced the efficacy of doxorubicin. When used alone, losartan did not affect the growth of CT26 tumors. However, losartan can enhance the antitumor activity of doxorubicin when used in combination. The Ki67, caspase-3 and TUNEL data indicated that the losartan can improve the ability of doxorubicin to induce tumor cells apoptosis, and inhibit the tumor cells proliferation. Furthermore, our present findings also indicate that the synergistic effect of losartan and doxorubicin to inhibit tumor cell proliferation and induce tumor cells apoptosis may also be due to their ability to up-regulate the expression of tumor suppressor gene P53. P53 is a transcription factor that regulates apoptosis by inducing the transcription of specific genes. The tumor suppressor gene p53 plays a central role in the anticancer actions of DNA-damaging agents. Doxorubicin causes apoptosis by direct DNA damage, at least in part in a p53-dependent manner[22-25].

Conclusions

These data suggest that the losartan can increase the therapeutic effectiveness of doxorubicin, yielding a greater antitumor benefit. Furthermore, this combination strategy may provide a rationale for initiating clinical trials using losartan in combination with chemotherapeutic agents to increase their therapeutic effectiveness.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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