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Targeting different angiogenic pathways with combination of curcumin, leflunomide and perindopril inhibits diethylnitrosamine-induced hepatocellular carcinoma in mice

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ABSTRACT

No effective chemopreventive agent has been approved against hepatocellular carcinoma (HCC) to date. Since HCC is one of the hypervascular solid tumors, blocking angiogenesis represents an intriguing approach to HCC chemoprevention. The aim of the current study was to examine the combined effect of the anti-angiogenic agents: leflunomide; a disease modifying antirheumatic drug, perindopril; an angiotensin converting enzyme inhibitor (ACEI) and curcumin; the active principle of turmeric, on diethylnitrosamine (DEN)-induced HCC in mice. Eight weeks following DEN administration, there was a significant rise in immunohistochemical staining of CD31-positive endothelial cells and consequently hepatic microvessel density (MVD) as compared to normal liver. DEN treatment was associated with elevation in hepatic vascular endothelial growth factor (VEGF) level as compared to normal controls ($P<0.05$, 3842 ± 72 pg/ml and 2520.8 ± 97 pg/ml, respectively). Similarly, increased hepatic expression of hypoxia inducible growth factor-1α (HIF-1α) was observed in 100% of the DEN-treated animals compared to 0% in their normal counterparts. Treatment with leflunomide, perindopril or curcumin alone abrogated the DEN-induced increased MVD as well as the elevated expression of VEGF, while only curcumin inhibited HIF-1α hepatic expression. Combination of these agents showed further inhibitory action on neovascularization and synergistic attenuation of hepatic VEGF (1954.27 ± 115 pg/ml) when compared to each single agent. Histopathological examination revealed a more beneficial chemopreventive activity in the combination group compared to each monotherapy. In conclusion, the combination treatment of leflunomide, perindopril and curcumin targeting different angiogenic pathways, resulted in synergistic inhibition of angiogenesis and consequently more effective chemoprevention of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and third most common cause of cancer mortality (El-Serag, 2011). In view of severity of the disease and the limited treatment options (Yamane and Weber, 2009), a critical need exists for novel chemopreventive strategies which reduce the incidence of HCC. Angiogenesis, a complex and critical process that leads to the formation of new blood vessels from pre-existing ones, is essential for the growth and maintenance of solid tumors, especially HCC which represents a malignancy characterized by distinct hypervascularization (Tanaka and Arii, 2006). Further, angiogenesis plays a pivotal role in early stages of murine hepatocarcinogenesis (Park et al., 2000). Therefore, inhibiting angiogenesis before it starts, that is, preventing the angiogenic switch in a concept named "angioprevention" has the potential to block nascent tumors into clinically quiescent foci (Bhat and Singh, 2008). Several anti-angiogenic agents have already been employed in clinical practice, including sorafenib and bevacizumab (Kerbel, 2008). However, there are serious concerns in employing such agents in chemoprevention of HCC due to severe adverse effects and cost performance on long term administration (Verheul and Pinedo, 2007). An alternative approach is to find clinically available compounds that exhibit anti-angiogenic activity for which the safety of long term administration has been proven.

Perindopril, an angiotensin converting enzyme inhibitor (ACEI), is widely used as an antihypertensive agent and has been suggested to decrease the risk of cancer (Lever et al., 1998). Studies have reported that perindopril possesses a strong anti-angiogenic activity and inhibits murine hepatocarcinogenesis (Yoshiji et al., 2005, 2009).

Leflunomide, an inhibitor of pyrimidine synthesis, has been approved for the therapy of rheumatoid arthritis. This agent was

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also developed as a small molecule inhibitor of tyrosine kinases (Shawver et al., 1997) to be used as an antitumor and antiangiogenic agent.

Curcumin, a major constituent of the yellow spice turmeric, has been studied as a chemopreventive agent against HCC (Sreepriya and Bali, 2005). However, mechanisms by which curcumin exerts its effects are not settled. Inhibition of tumor formation by curcumin has been attributed to its anti-initiation and antipromotion effects (Huang et al., 1992; Sikora et al., 1997). Further, curcumin is a highly pleiotropic molecule that interacts physically with numerous molecular targets, including transcription factors, cytokines, protein kinases, redox status, growth factors and enzymes that have been linked to angiogenesis and tumorigenesis (Shehzad and Lee, 2010).

Emerging evidence indicates that a single anti-angiogenic molecule may not suffice to combat the wide array of angiogenic factors produced by cancer cells and several pathways served in the angiogenic process. Thus, the objective of the current study was to examine the combined effect of curcumin, leflunomide and perindopril on the incidence of diethylnitrosamine (DEN)-induced HCC. We also investigated the combined effect of these agents on angiogenesis and hepatic expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1α, key factors relevant to the regulation of angiogenic pathways, during DEN-induced hepatocarcinogenesis compared to single agent, in an attempt to achieve better chemopreventive management of HCC.

2. Material and methods

2.1. Chemicals

DEN and curcumin powder (95% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Leflunomide and perindopril were generously supplied by EVA Pharm Co. (Cairo, Egypt) and Servier Co. (France), respectively. Mouse platelet endothelial cell adhesion molecule (PECAM-1, also referred as CD31) antibody, mouse HIF-1α antibody and ABC staining system were purchased from Thermo Fisher Scientific Fremont (CA 94538, USA). All other chemicals and reagents used were purchased from Chematech Co (Alexandria, Egypt).

2.2. Animals

Male Albino mice, 6–8 weeks old were purchased from the Animal House of the Medical Research Institute, Alexandria University. They were housed in stainless-steel mesh cages in groups of eight, under controlled conditions of light illumination, relative humidity and temperature. Animals were allowed access to food and tap water ad libitum throughout the acclimatization and experimental periods. All animal procedures were performed according to approved protocols and in accordance with the standard recommendations for the proper care and use of laboratory animals.

2.3. Experimental design

Mice were randomly divided into six groups ($n=8$ in each group) except the DEN group which comprised of 12 animals. While the negative control group served as the vehicle control, mice in the other groups received an intraperitoneal (i.p.) injection of 75 mg/kg of DEN weekly for 3 weeks, then 100 mg/kg weekly for another 3 weeks. Mice in DEN group did not receive any additional treatment and served as positive control group. Four mice from the DEN group were sacrificed starting from the 6th week following administration of the hepatocarcinogen DEN, at weekly intervals till the end of the 8th week, to monitor the appearance of HCC by histopathological examination of livers. Animals in leflunomide group and perindopril group were treated with 10 mg/kg/day of leflunomide and 2 mg/kg/day of perindopril by oral gavage, respectively. Mice in curcumin group were treated with 100 mg/kg of curcumin 3 days a week intraperitoneally (for better absorption kinetics). The combination treatment of leflunomide, perindopril and curcumin group was designated as combination group. The hepatocarcinogen DEN was dissolved in phosphate buffer saline (PBS), while curcumin was dissolved in polyethylene glycol (PEG) 80%+deionized water 20%. All drug treatments were given 2 weeks prior to DEN administration and for eight consecutive weeks as shown in Fig. 1. After 10 weeks, animals of all groups were sacrificed by cervical dislocation. Livers were excised and cut into two sections. The first section was used for histopathological and immunohistochemical examinations, while the second was used for the preparation of liver homogenate to be used for enzyme linked immunosorbent assay (ELISA) analysis.

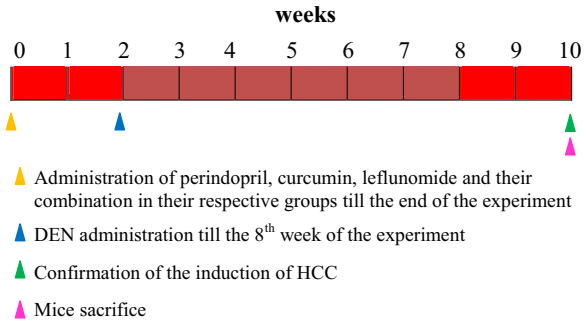

Fig. 1. The experimental design diagram.

2.4. Histopathological examination

Tissue specimens from mice livers were collected from all experimental groups and fixed in neutral buffered formalin 10%, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin. Sections 4–5 μm in thickness were stained using conventional H & E. Tumor grade was scored using the modified nuclear grading scheme outlined by Edmondson and Steiner (1954), with tumor grade categorized as well, moderately and poorly differentiated.

2.5. Immunohistochemical examinations

Further sections on coated slides were performed for immunohistochemical staining using the avidin biotin peroxidase complex method (Hsu et al., 1981). Briefly, after inhibition of endogenous peroxidases with 3% hydrogen peroxide, slides were treated by microwave in 10 mM citrate buffer for 15 min. After washing the slides in tris buffered saline (TBS), they were incubated with 10% normal goat serum at 23 °C for 30 min. Slides were then incubated at 4 °C overnight with the following primary antibodies: 200 μg/ml mouse HIF-1α & ready to use CD31. The incubation was followed by addition of biotin-conjugated goat anti-mouse, anti-rabbit IgG diluted 1:200 for 20 min. A final 45 min incubation with streptavidin-conjugated horseradish peroxidase diluted 1:200 in TBS was performed. The reaction product was developed for 5 min with diaminobenzidine tetrahydrochloride (DAB) and sections were lightly counterstained with Mayer's Hematoxylin.

Microvessels labeled with CD31 antibody were quantified by the counting procedure of Weidner et al. (1991). Briefly, the 3 most vascular areas (ie, hot spots defined as any brown-stained transversely sectioned tubes with a single layer of endothelial cells), with the highest numbers of microvessel profiles were chosen subjectively from each liver section under a low-power lens (× 100), and their microvessel numbers were counted under a