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New role of lupeol in reticence of angiogenesis, the cellular parameter of neoplastic progression in tumorigenesis models through altered gene expression

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ABSTRACT

There is a major unmet medical need for effective and well tolerated treatment options for cancer. The search now seeks to identify active biomolecules with multiple targets. Lupeol, an important dietary triterpenoid known as anticarcinogen by inducing apoptosis. But it is still more to reveal the potency of lupeol in the inhibition of neovascularization in cancer context. The study aimed to explore the efficacy of the lupeol in targeting angiogenesis. In this study, the inhibition of neovessel formation was assessed by preliminary antiangiogenesis assays like chorio allontoic membrane (CAM) and rat corneal micro pocket models. Further, validated for the micro vessel density (MVD) in histological sections of peritoneum, solid tumor and xenograft tumor by immunostaining with anti CD31 antibody. Antitumor potency was verified in ascites carcinoma, solid lymphoma and human nueroblastoma xenograft in CAM. Altered angiogenic gene expression by RT-PCR, ELISA and gelatin zymography. Lupeol significantly inhibits the neovessel formation in CAM and in the rat cornea. The similar effect was ascertained in mice and human xenograft tumor models with the regressed growth. Eventually reflecting on the differential transcription of angiogenic genes like MMP-2 & 9, HIF-1α, VEGFa and Flt-1 was noteworthy. It is now evident from our studies that, a new avenue of dietary triterpenoid lupeol by targeting angiogenesis, potentially inferring the multimode action in cancer prevention.

1. Introduction

The new challenge in conventions of anticancer therapy is to recognize the multi compartment nature of the tumor microenvironment which reflexes in the radically different approaches toward the discovery of new treatments. Resistance to apoptosis and progressing angiogenesis are such important parameters which are required for tumor growth, invasion and metastatic dissemination targeted during anticancer therapeutics [1]. The search now seeks to identify active biomolecules which would specifically target these two parameters and there by inhibiting the tumor growth [2]. Therefore, it is necessary to intensify our efforts for better understanding and development of novel treatment and preventive approaches for cancer.

Fruits and other plant derived products have gained considerable attention as they can reduce the risk of several cancer types [3]. Epidemiological and experimental studies provide evidence that some naturally occurring chemical agents in the human diet can reduce cancer risk [4]. Lupeol (Lup-20(29)-en-3b-ol) (Supplementary Fig. 1), a triterpene present in fruits such as mango, olive, strawberry, grapes, figs, etc., vegetables and in several medicinal plants, is used for treatment of number of ailments worldwide [5]. Recent reports showed that lupeol directly induces the apoptosis of tumor cells under *in-vitro* and *in-vivo* situation. These include its beneficial activity against inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity and hepatic toxicity [6–9]. Data emanating from molecular studies with various tumorigenic models suggest that lupeol modulate host systems potentially enabling more robust antitumor responses by aberration of Ras oncoprotein and induction of Fas receptors and its adaptor protein their by inducing apoptosis [10–14]. But

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it is still more to reveal the potency of lupeol in the inhibition of neovessel formation in various cancerous conditions and to unwrap the lupeol as a potent antiangiogenic bullet. The present study explores the efficacy of the lupeol to evaluate the angiopreventive effect.

2. Materials and methods

2.1. Chemicals and others

Lupeol, gelatin type-A porcine skin, TRI reagent, ECM gel, hydron polymer poly-hydroxyethyl-methacrylate (poly HEMA), primers, Anti-VEGF and anti mouse IgG antibodies were obtained from Sigma–Aldrich, USA. Superscript first strand synthesis and PCR supermix for RT PCR from Invitrogen, USA. Anti CD31antibody, Immunostaining kit from Leica Biosystems, Germany. All the other chemicals used were of analytical grade. Firtilized hen's eggs (Giriraja breed) were procured from local market in Shimoga, India. All the photographs were taken using Canon power shot Sx500 IS camera.

2.2. In-vivo and ex-vivo chorioallontoic membrane (CAM) assay

The recombinant $VEGF_{165}$ ($rVEGF_{165}$) induced *in-vivo* and *ex-vivo* CAM angiogenesis models was performed to study antiangiogenis effect of lupeol (10 μM) in fertilized eggs and change in the vascularisation pattern of with or without lupeol treated egg preparations were photographed as described previously [15].

2.3. Animals and ethics

Healthy Swiss Albino male mice weighing 25 ± 2.0 g and male Swiss albino whistler rat weighing 150 ± 5.0 g were grouped (n = 10) and housed in polyacrylic cages and maintained under standard conditions (25 ± 2 °C) with 12 ± 1 h dark/light cycle. All procedures for animal experimentation were approved by the Institutional Animal Ethics Committee, National College of Pharmacy, Shimoga, Karnataka, India (NCP/IAEC/CL/101/05/2012-13).

2.4. Rat corneal micropocket assay

The $rVEGF_{165}$ (1 μg/pellet) induced neovascularization in rat cornea was examined by treating with or without lupeol (10 μM/pellet), formulated in poly HEMA into rat corneas for treatment [16]. The number of blood vessels and length of the vessels were quantified [17].

2.5. Tumor models and treatment

Ehrlich ascites carcinoma (EAC) cells and Dalton's lymphoma ascites (DLA) were maintained separately *in-vivo* by intraperitoneal transplantation to develop ascites tumor [18]. Solid Dalton's lymphoma (DL) tumor induction was developed by injecting tumor cells into the thigh of the experimental animals subcutaneously. Lupeol (40 mg/kg body weight) was administered interaperitoneally (i.p) after onset of tumor in both EAC and solid DL tumor bearing mice on 4th and 10th day, respectively on every alternate day and verified for antitumor potency along with survivability analysis as described previously [15].

2.6. Peritoneal angiogenesis and immunohistopathology

Carcinoma induced peritoneal neovascularization was visualized and documented in with or without lupeol treated animals. Corresponding peritoneum tissue sections were processed for Hematoxyline & Eosin (H&E) staining and immunostain with anti CD31 antibodies as per the manufacturers recommendations [19].

2.7. CAM xenograft model

In-vivo CAM xenograft model to assess angiogenesis was performed with slight modification as described previously [16]. In brief, the plastic rings coated with ECM gel were placed on the growing CAM of 5th day of incubation of fertilized eggs under sterilized condition by making window in the egg shell. The rings were then adsorbed with 5×10^6 B16F10 cell suspension along with $rVEGF_{165}$ (10 ng/ml) and treated with or without lupeol (10 ng/ml). On day 12, the eggs were opened and the tumors formed were excised, tumor size were determined and processed for H&E.

2.8. Reverse transcription (RT)-PCR

DL cells treated with or without lupeol *in-vivo* were harvested and total RNA was isolated using TRI reagent. RT was performed using oligo(dT) primers and superscript reverse transcript following the manufacturer's recommendation and amplified using MMP-2 forward primer (fwd): 5′-ACAGTGACACCACGTGACAA-3′ and reverse primer (rev): 5′-GGGATGGCATTCCAGGAGTC-3′, MMP-9 fwd: 5′-CGCTCATGTACCCGCTGTAT-3′ and rev: 5′-TGTCTGCCGGACT CAAAGAC-3′, HIF-1α fwd: 5′-TTCACTGCACGGGCCATATT-3′ and rev: 5′-TCCACCTCTTTTGGCAAGCA-3′, Flt-1 fwd: 5′-TGGGCAGTCAAGT CCGAATC-3′ and rev: 5′-GTGCAAACTCCCACTTGCTG-3′, VEGFa fwd: 5′-GGGGTGTCCCATAGGGGTAT-3′ and rev: 5′-CGCCTTGGCTTGTCA CATTTT-3′. Normalization of angiogenic gene expression was achieved by comparing the expression of GAPDH fwd: 5′-CGCTC ATGTACCCGCTGTAT-3′ and rev: 5′-TGTCTGCCGGACTCAAAGAC-3′ for the matching sample. PCR product were resolved by using 1.5% agarose gel and identified by ethidium bromide staining.

2.9. VEGF – ELISA

The serum (100 μl) from solid DL bearing animals treated with or without lupeol was collected and VEGF levels were quantified by using anti VEGF antibody by ELISA [19].

2.10. Gelatin zymography

Expression of MMP-2 & 9 in whole cell lysate from DL tumor tissue treated with or without lupeol was assessed by gelatin gel zymography as previously described [20]. In brief, the equal concentration of total protein from with or without lupeol treated tumor tissue was resolved in 11% SDS-PAGE gels containing 0.1% (w/v) gelatin type A porcine skin. The gels washed with zymography renaturing buffer (2% Triton X-100), then incubated for 18 h at 37 °C in reaction buffer (50 mM Tris–HCl, 200 mM NaCl, and 5 mM $CaCl_2$). The gels were then stained with Coomossie brilliant blue R 250. Gelatinase activity in the gel slab was quantified by Bio-rad Gel Documentation™ XR+ Imaging System.

3. Results

3.1. Lupeol inhibits the neovascularization in non tumor model systems

To investigate the antiangiogenic activity *in-vivo*, lupeol was tested in different angiogenesis models induced by $rVEGF_{165}$. In CAM model a clear avascular zone around the implanted disc with lupeol was clear evident for the regression of neovessels in the developing embryos in both *in-vivo* and *ex-vivo* CAM (Fig. 1A and

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