RESEARCH COMMUNICATION

Quercetin Mediated Reduction of Angiogenic Markers and Chaperones in DLA-Induced Solid Tumours

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Abstract

Diet-derived flavonoids, in particular quercetin, may play advantageous roles by preventing or/and inhibiting oncogenesis. Evidence suggests that quercetin can elicit various properties depending on the cell type. The aim of this study was to evaluate its effects on Dalton's lymphoma ascites (DLA) induced solid tumours and to identify the target(s) of action. We addressed this question by inducing subcutaneous solid tumours in Swiss albino mice and investigated whether the quercetin affects essential biological processes that are responsible for tumour growth, morphology, angiogenesis and apoptosis. We also studied influence on several heat shock proteins (HSPs). Our findings demonstrate that intra-tumour administration of quercetin results in decreased volume/weight. Furthermore, we demonstrate that quercetin promotes apoptosis of cancer cells by down-regulating the levels of Hsp90 and Hsp70. Depletion of these two chaperones by quercetin might result in triggering of caspase-3 in treated tumours. Moreover, it also down-regulated the expression of major key angiogenic or pro-angiogenic factors, like HIF-1α and VEGF In addition, H&E staining together with immunofluorescence of fixed tumour tissue provided evidence in support of increased cell death in quercetin -treated mice.

Keywords: DLA - quercetin - VEGF - HIF-1α - apoptosis - heat shock proteins

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Introduction

Solid tumor is an abnormal mass of tissue that does not contain cyst or liquid and are mostly epithelial in nature. Almost all tumours are hypoxic in nature as compared to surrounding normal tissue and can proliferate even in severe nutrient deprivation in spite of poor blood supply (Dachs et al., 1997). The transcriptional activator, hypoxia induction factor-1(HIF-1) increases due to an imbalance in O_2 homeostasis (Semenza et al., 1998). It induces many genes (Semenza et al., 2003) that encode proteins, involved in the tumour progression as well as in the cell immortalization, pH regulation, growth/survival, metastasis, invasion, resistance to chemotherapy and angiogenesis. Angiogenesis is a process of new micro vessel formation from the pre-existing vasculature. However, in tumour progression, it is usually activated and the newly formed vessel helps to sustain and expand neoplastic growth. Therefore, inhibition of blood vessel formation leads to shrinkage of tumor and consequently, inhibits the tumour development beyond 1-2 mm3 (Folkman et al., 1990). The angiogenic switch is regulated by the different factor either induces (VEGF, MMP, and bFGF) or acts as a repressor (TIMPs; Prothrombin kringle-2) of neovascularization (Carmeliet et al., 2000).

Another major problem associated with cancer is the differential role of highly conserved heat shock proteins (HSPs). Many tumour shows increased level of HSPs (Ciocca et al., 1993; Ralhan et al., 1995; Whitesell and Lindquist, 2005). These proteins promote tumour cell survival, growth and metastasis even in growth factor deprived condition by allowing continued translation and cellular proliferation (Whitesell and Lindquist, 2005). In this context, Hsp90, Hsp70, Hsp60 and Hsp27 play a key role. The molecular chaperone, Hsp90, expressed both constitutively as well as in an inducible manner that critically involved in maintaining the stability, integrity and function of key oncogenic proteins, responsible for an angiogenesis event (Staufer et al., 2010), apoptosis (Giommarelli et al., 2010; Trepel et al., 2010), HIF-1 stability (Isaacs et al., 2002; Katschinski et al., 2002) and significantly involved in adaptation of cancer cells to many environmental stresses. Hsp70 and 27 are abundantly expressed in malignant tumour of various origins, including those which are epithelial in nature (Ciocca et al., 1993; Nanbu et al., 1998; Jaattela et al., 1999), and stimulates tumorigenesis via its pro-survival function (Ralhan and Kaur, 1995; Ciocca and Calderwood, 2005). They also help in inhibiting cell death induced by a wide range of stimuli including several cancer-related stress, apoptosis and anticancer drugs (Jaattela et al., 1992; 1993; Fuqua et al., 1994; Mosser et al., 1997).

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Quercetin, a well-studied flavonol is ubiquitously distributed in various foods. It is widely consumed flavonoid (Lamson et al., 2000) in the human diet and abundant in onion, apple, tea and red wine (Hertog et al., 1992). Being the most potent antioxidant in the entire flavonoid group (Rice-Evans et al., 1996) it has a wide range of pharmacological properties together with anti-tumour activity for a variety of cancer cells (Ranelletti et al., 1992; Avila et al., 1994; Larocca et al., 1995; Prajda et al., 1995, Granado et al., 2006; Lee DH and Yong J, 2008; Xiao et al., 2011). Various studies have highlighted the anti-cancerous property of quercetin by down regulating the expression of mutant p53 protein (Avila et al., 1994), VEGF, HIF-1 (Lee and Lee, 2008), COX-2 (Xiao et al., 2011) HSPs and induced apoptosis (Hosokawa et al., 1990, 1992; Jakubowicz-Gil J et al., 2002; Granado et al., 2006). Despite the promising action of quercetin and its anti tumour activities, the molecular mechanism underlying these effects are still unsettled due to the differential nature of various types of cancer. Based on previous observations, quercetin has been negative (Wilson et al., 2002) as well as positive effects (Jeon et al., 2007) on HIF-1 and VEGF expressions in different type of cell and several uncertainties have raised regarding the absorption and availability of this drugs on tumour. Therefore, it is necessary to understand the mechanism of quercetin on different types of cancer.

In the present study, we evaluated the response of quercetin on DLA induced subcutaneous solid tumour by a different mode of drug administration and examined the possible role of some of the HSPs. Our result showed that quercetin significantly suppresses HIF-1α, VEGF, Hsp70 and Hsp90. However, it does not have any effect on Hsp27 and 60. It also induces Caspase-3 activation. We, moreover, provide evidence in this study that it has an anti-neoplastic effect in tumour morphology. We are also reporting that intra tumour (i.t.) injection of quercetin regulates various proteins responsible for angiogenesis and apoptosis in solid tumour.

Materials and Methods

Chemical and Reagents

High-purity quercetin (Q4951) was purchased from Sigma Aldrich. All other chemicals used in this study were purchased from Merck, Sigma and of highest grade and purity. Anti-caspase-3 (9662) antibody was purchased from Cell Signalling (USA) while anti-Hsp27, anti-Hsp60, anti-Hsp70 anti-Hsp90 anti-Hif-1α and anti-VEGF were purchased from Santa Cruz biotech (USA). HRP conjugated (secondary antibody) anti-rabbit and anti-mouse polyclonal immunoglobulin was purchased from DAKO (DENMARK).

Chicken Chorioallantoic Membrane (CAM) Assay

Angiogenic activity was assayed using the chick embryo chorio-allantoic membrane (CAM) assay. Fertilized eggs were incubated at 37°C in a humidified and sterile atmosphere for 3 days (eggs were rotated 5-6 times a day). On 4th day a thin groove of approximately 1.5x1.5 cm was made on the surface of the egg and sealed using sterile surgical tape. The eggs were then further incubated until 5th day and after that, a window was made to check for the normal development of the embryo. Eggs possessing 80% similarities in their vasculature were selected for the experiment. Quercetin was applied onto the CAM at different concentrations (2.5-10 μg/CAM) using a sterile filter disk. Apart from negative control (solvent only), a positive control (retinoic acid, 1μg/CAM) was also included in this experiment. The CAM was inspected after 24 hours for changes in the density of blood vessels and photo-graphed under dissecting microscope attached with digital camera. For each concentration, 10 eggs were used.

Maintenance of the Cell Line

The Dalton's lymphoma ascites cell was obtained through the courtesy of Amala cancer research centre, Thrissur, Kerala, India and cells were maintained in the peritoneal cavity of female Swiss albino mice through serial passaging in an ascitic form at an interval of 9-10 days in our laboratory. The freshly aspirated cells from the mouse peritoneum were washed with phosphate-buffered saline (PBS) under sterile conditions, and their number was determined using a hemocytometer before model generation.

Experimental Animals

Female Swiss albino mice (6-8 weeks, 22-24 gms) were obtained from the VIT animal house and maintained in sterile polycarbonate cages (n=6 mice per cage). Animals were acclimatized to experimental room having controlled standard environmental conditions (25 ± 2ºC, 60-70 % relative humidity, 12-hour light and 12 hour dark cycle). They were fed with commercially obtained mouse chow and water *ad libitum*. All experiments were carried out as per the guidelines of the institutional animal ethics committee. VIT University animal ethical committee has approved this experimental study (registration number: 1333/C/10/CPCSEA) dated 09.07.2011 Approval number is: VIT/IAEC/IVth/016/2011.

Solid Tumour Model and Treatment

Swiss albino mice were divided into two groups (n=6 animals/group). Solid tumor was induced by injecting DLA cells (1×10^6 cells/animal, suspended in 0.1 ml PBS) subcutaneously to the right hind limb of experimental animals. Tumours were allowed to grow for a period of 8 days to reach an average size of 1±0.33 cm^3, and mice were randomized into two experimental groups. Group I was treated with quercetin (25 mg/kg body weight) while the second group was kept as vehicle control. Mice were then injected with 22-gauge needle intratumour (i.t.) (50 μl) with an excipient (quercetin dissolved in 1N NaOH). For i.t. administration, drug was injected slowly (10 μl/min) in a total volume of 50 μl excipient into left and right sides of tumor (25 μl at each side) at each day of injection (Husain et al., 1999). Control mice were injected with same volume of 1N NaOH.

After completion of the experimental treatment on 28th day, all the mice were deprived of food overnight and euthanized by cervical dislocation under ketamine-

xylazine anaesthesia.

Effects of Quercetin on Solid Tumour Development

The radii of developing tumors were measured using vernier calipers at different time intervals (12th, 20th and 28th day) and tumor volume was calculated using the formula: $V = 4/3\pi r1 2r^2$ Where, r1 and r2 is the radius of the tumours at two different directions (Preethi et al., 2006). Tumours were excised 24 h after the last injection, washed with PBS and weighed using standard weighing balance. Tumour weight of treated and its untreated counterparts were recorded and compared.

Hematoxylin-eosin Staining

The excised tumours from each experimental group after proper fixation with 10% buffered formaldehyde were embedded in paraffin. The prepared paraffin blocks were cut at 3 µm thickness and then stained with hematoxylin-eosin after serial dehydration with graded ethanol solutions.

Western blot analysis: DLA solid tumours were dissected and lysed by homogenization in 0.1% Triton X-100 lysis buffer followed by incubation on ice. Proteins (30 µg) from soluble fraction were denatured in 2X Laemmli sample buffer at 95°C for 20 minutes and resolved on 10% SDS–PAGE followed by blotting onto PVDF membrane (GE healthcare). Membranes were blocked in 5% (w/v) non-fat dry milk and incubated for three hours at room temperature with the indicated primary antibody (1:500–1000 dilution) and goat anti-mouse IgG or goat anti-rabbit IgG HRP-conjugated secondary antibody (1:2000 dilution) for one hour, washed and later the autoradiography signals were visualized using ECL advance western blotting detection kit (RPN2135) on the X-ray film (GE Healthcare).

Immunofluorescence

Immunohistochemical detection of Caspase-3, in 5 µm thick tumor section was performed. In short, mice were sacrificed, and the tumour was fixed in 10% buffered formaldehyde followed by paraffin wax embedding. 5 µm sections were cut using a microtome. Sections were deparaffinized and rehydrated. Following permeabilization with 0.05% Triton X-100, and blocking, immunodetection was carried out. Sections were incubated with rabbit anti-caspase3 (1:300) and primary antibodies were marked using appropriate FITC-labeled secondary antibodies (1:300). Antibodies were dissolved in Tris-buffered saline (pH 7.2) containing 3% bovine serum albumin, and this solution was also used for blocking. Sections were embedded in Fluoromount and imaged using a 100× objective. Negative control sections were processed simultaneously with the omission of the primary antibodies. All sections were dehydrated, mounted with cover slips and viewed under a light microscope.

Densitometric and statistical analysis

For *in vivo* model, six animals were considered for statistical analysis. All data were expressed as means standard errors of the means (Mean±SEM). Student t-test was used to test the significance of difference between tumour volume and weight of treated versus non-treated using Graph Pad prism software (Instat software package). $P < 0.05$ was considered as statistically significant.

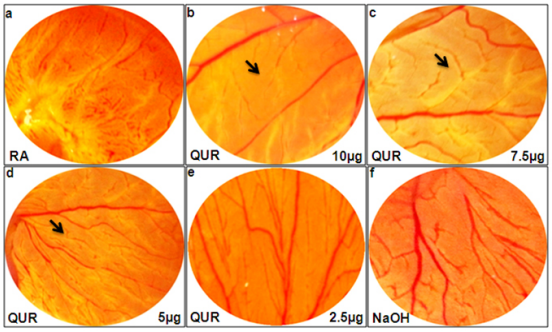

Figure 1. Antiangiogenic Effects of Quercetin at Different Concentrations. (a) Retinoic acid (1µg/CAM) as a positive control (b) 10μg/CAM of quercetin (c) 7.5μg/CAM of (d) 5μg/CAM of quercetin (e) 2.5μg/CAM of quercetin (f) vehicle or negative control

Results

Quercetin elicits Anti-angiogenic Properties

CAM assay is relatively easier to perform and having extensive vascularization. Moreover, it is a widely used model to screen anti-angiogenic properties. We screened various concentrations of quercetin along with a well-known anti-angiogenic drug retinoic acid (RA) as positive control Figure 1 (a). Upon drug administration, a marked reduction in new blood vessel's formation has been observed in Fig. 1 (b-d) in a dose dependent manner, however maximum inhibitory activity has been observed at a concentration of 10μg Figure 1 (b). At the same time, in case of 7.5μg Figure 1 (c) quercetin was less anti-angiogenic then 10μg. In addition, no significant inhibition of blood vessels has been observed in 5 and 2.5μg Figure 1 (d, e) as compared to solvent control Figure 1 (f). Among all the concentration used in CAM assay, 10μg showed the best inhibition as compared to positive control.

Anti-tumour Effect of Quercetin on Tumor Development

Tumor volume of mice treated with drug was found significantly lesser than that of untreated vehicle control. The average volume of vehicle control on 28th day was 4.3±0.93 cm^3 whereas tumor volume of animals treated with quercetin was 1.041±0.36 cm^3 on the same day (Figure 2B), which was higher compared to treated groups. We measured the increase in tumor volume on 20th day as compared to 12th day and found an increase in 51% (vehicle control) and 26% (quercetin treated) animals. On 28th day, a reduction in tumour volume has been seen by 76% as compared to control of same day and 60 % with drug treated (20th day). These observations revealed that quercetin can ability to inhibit tumour progression. To confirm these findings, we also recorded the tumour weight after excising. A significant reduction in average weight of solid tumour was found in drug treated animals than the vehicle control animals. In quercetin treated group, there was a reduction in tumour weight by 64% on 29th day as compared to the control on same day (Figure 2A)

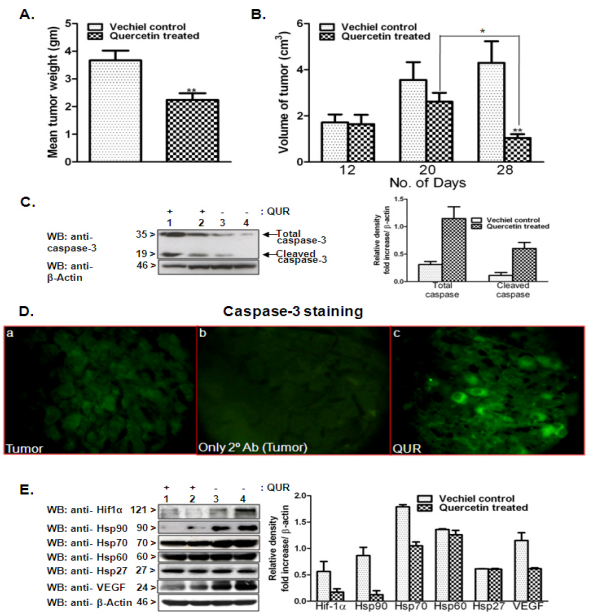

Figure 2. Tumour Suppression Effect of Quercetin on Solid Tumour Developed from DLA Cells. Mice were divided into two groups (n=6) and i.t given with quercetin/ solvent (25mg/kg body weight) as mention under material and methods. Tumour weight and volume were compared with control group. (A) Drug treated mice have lesser mean tumour weight than untreated group (B) Tumour volume were calculated by using V= 4/3πr12r2 on day 12, 20 and 28. (C and E) Quercetin suppresses intracellular level of HIF-1α, VEGF HSPs and activates Caspase-3 expression: Cell lysate from excised tumour containing equal amount of protein (30μg) were separated by SDS-PAGE and immunoblot with anti-HIF-1α, anti-VEGF, anti-Hsp90, anti-Hsp70, anti-Hsp60, anti-Hsp27, and anti-caspase-3. β-actin was used as internal loading control. This is a representative of three individual blots. Densitometric analysis was shown with respect to fold increase/ β-actin (relative density). Each bar indicate Mean±SEM.*p<0.05 significant difference between treated and control group. Immunohistochemistry of (D a-c) Casapse-3 in tumor sections: Mice were treated with QUR (25 mg/kg body weight) started from day 10th of tumor implantation and continued till 28 days. After 28th days mice were sacrificed and solid tumor was excised and processed for immunohistochemical studies. Tumor sections from three different tumors in each group and 10 randomly selected areas from each tumor were analyzed. (a) Vehicle tumor control, (b) Negative control sections with the omission of the primary antibodies and (c) Apoptosis indicated by green fluorescence in quercetin treated tumor

Selective Effects of Quercetin on Caspase Activation in DLA-induced Solid Tumours

To further confirm, proteins (30 μg) from Triton X-100 soluble fractions from both the groups were separated on 10% SDS–PAGE and probed with anti-Caspase-3, a pro-apoptotic protein marker. The immunoblot of Caspase-3 (Figure 2C) expressed an enhanced level of total and cleaved Caspase in quercetin treated tumour (Figure 2C. Lane 1, 2 compared with 3, 4 of Figure 2C). Although cleaved caspase has been observed in vehicle control (Figure 2C, lane 3) which is far lesser than drug treated group suggests a basal apoptosis because of the body's own defence mechanism.

Moreover, to validate this effect, we have done

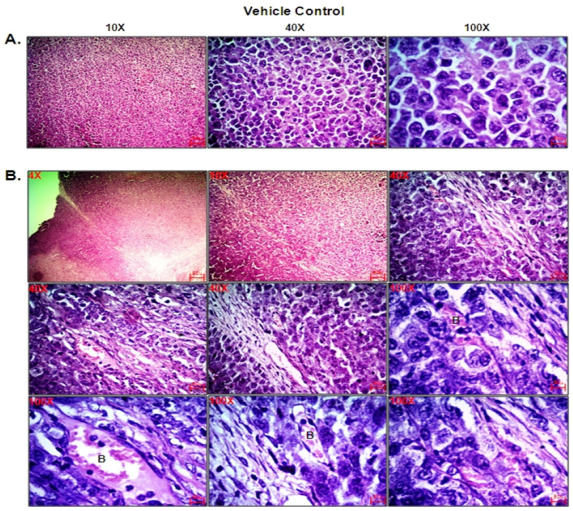

Figure 3. Histochemical Analysis of Control and Treated Solid Tumour (DLA -induced). Section of paraffin embedded tumour from vehicle control was stained with H&E. Neoplasm tissue were photomicrograph at x4, x10, x40 and x100 magnification. (A) Representative H&E staining show well differentiated and large area of confluent tumour cells with solid sheet like pattern morphology Very little or no tumour tissue necrosis was observed. (B) Microphotograph showing invading cords of malignant cells and large tumor cells in untreated animal Blood vessels are evident toward the central part of tumor away from tumor edge.

immunostaining of solid tumor sections for an apoptosis cell marker, Caspase-3. It was observed that treatment with quercetin at 25 mg/kg body weight enhances the expression of Caspase-3 in the cytoplasmic region of tumour cells (Figure 2D-c), whereas control tumors (Figure 2Da) injected with the excipient did not exhibit any apoptotic cells. These data along with the histopathological illustrations demonstrate that direct injection of drugs into a tumor bed causes cell death through necrosis and apoptosis pathways.

Expression of HSPs in Quercetin-treated Solid Tumours

Commonly, high expression of HSPs has been associated with cancer metastasis, poor prognosis and resistance to chemotherapy and radiation therapy (Ciocca DR et al., 1993, 2005; Fuqua et al., 1995; Vargas-Roig et al., 1998). Hence, we were interested to observe the effect of quercetin on various HSPs in solid tumour. Proteins from tumour of both groups were resolved on SDS-PAGE, transferred onto PVDF membrane followed by incubation in primary antibody against Hsp27, Hsp60, Hsp70 and Hsp90. β-actin was used as an internal control for equal loading of proteins in each lane (Figure 2E). Comparative expression analysis of various HSPs revealed that quercetin significantly down regulates Hsp70 (by one and half folds) and Hsp90 (by around three fold) level (lane 1 and 2, Figure 2E) with respect to control sample (lane 3 and 4, Figure 2E) as expected (Hosokawa et al., 1992; Rong et al., 2000) but there are no significant changes in expression level of Hsp27 and Hsp60 as compared to control group (Figure 2E Densitometric analysis).

Quercetin Down-regulates Hif-1α and VEGF Expression in Solid Tumours

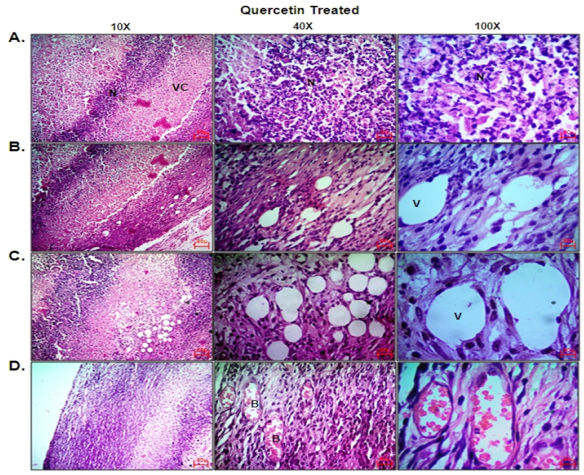

Figure 4. Histological Evidence of Tumour Necrosis and Vacuole Formation after Quercetin Treatment. After final injection of drug tumour were excised, formalin fixed and sections were stain with H&E. treated group shows distinct necrosis like morphology (A), had large vacuole near tumour edge (B) as well as towards central part (C). Blood vessels are observed near the periphery only (D). N- Necrosis like morphology, VC-viable cell, V-Vacuole, B-Blood vessels

Chronic hypoxia, a hallmark of many tumours is associated with angiogenesis, tumour progression, metastasis/invasion, metabolic adaptation and resistance to apoptosis (Semenza GL, 2003). Expression of many HIF-1α target genes such as VEGF is induced by hypoxia in most cancer cell types. To examine the effect of drug on Hif 1α and angiogenic marker expression level in solid tumour immunoblot were performed.

Immunoblot results explained the marked upregulation of Hif 1-alpha and VEGF in the untreated group (Figure 2E, lanes 3 and 4), while upon quercetin administration, these levels were significantly down-regulated (Figure 2E, lanes 1 and 2). From this observation, we may predict one of the possible ways by which quercetin inhibits tumour progression is by down regulating Hif-1α and VEGF.

Assessment of Histopathology Results

Subcutaneous tumours were processed for histological examination. For assessment, tumours were harvested from both treated and non treated animals, fixed in formalin and embedded in paraffin. Sections from the centre of each tumour were stained with hematoxylin and eosin (H&E) (Figure 3 and 4). Tumour tissue from control mice had large areas of confluent tumour cells with little or no evidence of tumour necrosis as (Figure 3A) compared to drug treated (Figure 4A). It is observed as a hilic (solid) with a solid sheet like pattern (Figure 3A).

Control mice tumour revealed the presence of occasional perfuse blood vessels found within the tumour region, mainly seen away from the tumour edge and invading cords of malignant cells (Figure 3B). In contrast treated tumour had large vacuoles near the tumour edge (Figure 4B) as well as away from edge (Figure 4C), an indication of necrosis, which is characterized by large central tumour regions of contagious cells with pyknotic nuclei that stained homogeneously dark with hematoxylin. Necrotic regions were also characterized by significant thinning of the cell cytoplasm, disruption of tissue architecture and loss of cell to cell contact. Distorted blood vessels are seen only at the edge of processed drug treated tumour indicate a possible anti-angiogenic effect of drugs (Figure 4D) which is further proven by down regulation of Hif-1alpha, VEGF and Hsp90 in immunoblot. At the same time, blood vessels are evident towards the central region as well as near the tumour boundary in the control group (Figure 3B).

Discussion

Carcinogenesis in human is a multistage process leads to progressive transformation of normal cell into malignant. Capability of limitless replication potential, evading apoptosis, invasion, metastasis, supporting angiogenesis, self-sufficiency in growth signal and resistant to the growth inhibitors makes cancerous cells different from healthy cells (Hanahan et al., 2000). Depending upon the type of tumour, the mechanisms by which cancer cells acquire this ability vary considerably that leads to alteration of signal transduction pathways associated with most of the physiological changes. Epidemiological and statistical data indicate that about 35% of human cancer mortality is attributable to diet (Doll et al., 1981). It is well accepted that the consumption of food products containing high amounts of flavonoids, a diet rich in fruits and vegetables, is associated with a reduced risk of cancer (Tadjalli-Mehr et al., 2003). Several reports confirm that quercetin inhibits the growth of other tumor cell lines as well (Rong et al., 2002). Previous studies also suggested multi targeting effect of quercetin on cancer cells (Hosokawa et al., 1992; Ranellettiet et al., 1992; Avila et al., 1994; Larocca et al., 1995; Prajda et al., 1995; Jakubowicz-Gil et al., 2002; Granado et al., 2006; Lee and Lee, 2008; Xiao et al., 2011). In an effort to identify the molecular targets of quercetin in solid tumour induced by subcutaneous injection, we used the proteomic approach as well as tissue histology to explore this aspect of our study. For instance, the expression level of VEGF correlates with the level of angiogenesis (Carmeliet et al., 2000) therefore; we investigated the therapeutic effect of quercetin in angiogenesis. In several animal models, higher expression of VEGF in tumor cells induces tumor growth and metastasis by triggering the neo-vascularization process. Based on this, a variety of assay methods have been developed to investigate both the pro and anti-angiogenic effects of potential new drugs. Moreover, expression of VEGF is considered to be essential for tumor cell growth and proliferation (Carmeliet et al., 2000). VEGF binds to VEGF receptor-2 (VEGFR-2) on the endothelial cells and phosphorylate that will in turn activate the signalling pathways of Akt. These cascades are closely associated with the cell growth, proliferation and survival (Kang et al., 2004). A strong stimulus for expression of the VEGF is mediated primarily by the Hif1α, a master regulator of angiogenesis (Mizukami et al., 2007). In this study, we observe that quercetin inhibits hypoxia-induce HIF-1α expression level.

The intracellular level of Hif1α is synchronized by protein translation and proteasome-dependent degradation, whereas hypoxia-induced Hif1α accumulation occurs due

to the impairment of the proteasomal degradation system in the absence of oxygen (Tanimoto et al., 2000; Peng et al., 2006) where HSPs play a decisive role.

In continuation, we also investigated the expression of different heat shock proteins. There are several reports indicating the role of HSPs in cancer progression, inhibition, angiogenesis and apoptosis (Jaattela et al., 1999; Rong et al., 2000; Rane et al., 2003; Giommarelli et al., 2010; Staufer et al., 2010; Trepel et al., 2010). In our study, we have shown that quercetin inhibits the Hsp90 expressions that plays a critical role in stabilization of several client proteins implicated in the control of cell growth and malignant transformation including HIF-1α (Isaacs, et al., 2002; Katschinski et al., 2002; Giommarelli et al., 2010; Staufer et al., 2010; Trepel et al., 2010). It is conceivable that the down-regulation of Hsp90 by quercetin can lead to modulation of its chaperonic activities, thereby accelerating degradation of numerous oncogenic associated proteins that participate in the regulation of cell proliferation, survival, and angiogenesis (Giommarelli et al., 2010; Trepel et al., 2010). Similar observations were described by Zhang et al. where Hsp90 client proteins in breast and colon cancer cells were down-regulated upon Hsp90 inhibition.

In addition to the above observations, we also demonstrated that drug treatment down regulates Hsp70 expressions, a key regulator which helps cancerous cell to escape from apoptosis by inhibiting caspase activation. Although Hsp60 as well as Hsp27 has a role in cancer but in this study, quercetin did not have any effect on their expression, which requires further investigation at RNA level. In contrast, both tumour morphology and caspase-3 activity in quercetin treated group supported our findings that apoptosis was responsible for quercetin-induced cell death.

Cell death in a multi cellular organism can occur by two distinct mechanisms, apoptosis or necrosis. Apoptosis plays an important role in growth homeostasis. Both extensive and intrinsic pathways converge to a same terminal or execution pathways and the activation of a downstream effecter caspase-3 that results in DNA fragmentation, formation of apoptotic bodies and finally uptake by phagocytic cells. (Susan Elmore, 2007). There is accumulating evidence suggesting that the efficacy of antitumor agents in relation to the intrinsic propensity to target the tumor is apoptotic dependent (Gorczyca et al., 1993). Our Western blot, and immunoflurosence data have provided an evidence that caspase activation in quercetin treated group induces the apoptosis. Based on above and other's findings it is clear that quercetin is capable of tumor inhibition.

To conclude, quercetin has the property to prevent and/or to retard the tumor growth that might be a multifactorial phenomenon. In each treatment group, tumor growth was suppressed significantly where quercetin was intra tumour injected, resulting in a significant reduction in the growth of DLA induced tumour cells. Moreover, quercetin attenuated the expression of Hsp90, Hsp70, VEGF and HIF-1α in tumor mode, allow us to assume that the retardation of tumor growth is due to the anti-angiogenic activity.

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