8.1. Effect of [6]-shogaol on NF-κB/AP-1 signaling pathway

Introduction

Cancer is a chronic disease that is caused by defective genome-surveillance and signal-transduction mechanisms (Hanahan and Weinberg, 2000). Early stages of inflammation enhance tumour development, through signal-transduction mechanisms that influence factors involved in either malignant conversion or cancer surveillance. The transient of signalling pathways rely on specific protein phosphorylation, ultimately lead to the activation of transcription factors that enhance the expression of appropriate target genes such as inflammatory, cell cycle regulatory genes and deregulation of apoptosis genes implicated in carcinogenesis (Kundu and Surh, 2004).

Nuclear transcription factor kappa-B (NF-κB), a redox-sensitive transcription factor tight association with NF-κB inhibitory-protein IκB sequestered in the cytoplasm under neutral state. These transmit signals can be activated by a wide variety of stimuli such as cytokine binding to its receptor endotoxin, carcinogens (such as cigarette smoke), UV radiation and tumor necrosis factor (TNF-α) and oxidative stress (Karin and Greten, 2005). These factors subsequently activation of IκB kinase (IKK) complex which leads to rapid phosphorylation and proteolytic degradation of the IκB-α, and form free NF-κB dimerization. Further, this free form NF-κB translocation into the nucleus and binds to its target DNA site, which regulate the expression of NF-κB target genes that play a crucial role in neoplastic transformation (Kavitha et al., 2013). Another transcription factor Activator protein-1 (AP-1) are primarily dictated by the dimer composition of the AP-1 family proteins, these dimers can bind on AP-1 DNA specific site. It has been regulated of several oncogenes involved in inflammation, apoptosis and proliferation may promote by activating the down stream molecules (Kim et al., 2009).
Prostaglandins are originated from arachidionate and some other highly unsaturated fatty acids which are oxygenated-lipid signaling molecules. The major role of prostaglandins is assigned to regulate a wide variety of physiological processes including, wound healing, blood clotting, bone metabolism, immune responses, nerve growth and development and inflammation (Aggarwal and Shishodia, 2006). COX-2 is the catabolic agent to synthesis prostaglandins. Up-regulation of prostaglandins synthesis (COX-2) causes enhancement of inflammation, cellular proliferation, suppression of apoptosis and increase invasiveness and angiogenesis (Dannenberg et al., 2003). iNOS and COX-2 enzymes is a key molecules for inflammatory response. Hence, the transcriptional activation NF-κB/AP-1 signaling pathway in order to activation of inflammatory, cell cycle regulatory anti-apoptotic and deregulation of pro-apoptosis genes (Herencia et al., 1999; Monteghirfo et al., 2008).

The multistage process of cancer is initiated by inflammation and uncontrolled proliferation of cells. In general cell proliferative markers such as PCNA, Cyclin D1 and Ki-67 which are involved in cell cycle regulation. This protein is frequently amplified and over expressed in patients with oral squamous cell carcinoma (Cheng et al., 2007; Staibano et al., 1998). Ki-67 is admirable diagnostic marker in cancer and it detected throughout the all phase of cell cycle with the exception of early G1 phase (Gerdes et al, 1984). Deregulation of these proteins suggesting uncontrolled proliferation of the would-be malignant cell. In this study, we examined the effect of [6]-shogaol suppression of inflammatory and cell proliferation through inhibition of NF-κB/AP-1 signaling on DMBA induced HBP carcinogenesis.
8.2. Results

8.2.1. Effect of [6]-shogaol on inflammatory protein analysed by Western blot

The immunoblotting expression pattern of TNF-α, IkB-α, NF-κB, AP-1, COX-2 and iNOS in control and experimental hamsters were depicted in Fig. 8.1. In DMBA painted animal shows higher expression of TNF-α, NF-κB, AP-1, COX-2, iNOS and significantly decreased expression of IkB-α as compared to control. However, oral administration of [6]-shogaol significantly decreased expression of TNF-α, NF-κB, AP-1, COX-2, iNOS and restrained degradation or phosphorylation of IkB-α in DMBA treated hamsters. Similar expression pattern of above noticed inflammatory markers were observed in control and [6]-shogaol alone treated hamsters.

Fig. 8.1. Effect of [6]-shogaol on DMBA induced inflammatory markers such as TNF-α, IkB-α, NF-κB, AP-1, COX-2 and iNOS were analysed by Western blot. Above mentioned protein were normalized to β-actin. (b) The representative graph shows the relative protein expression of band intensities and fold changes were quantified by Image-studio software (LI COR, USA.). Values are expressed as mean ± SD for three independent experiments. Values that do not share a common superscript letter (* and **) between groups differ significantly at p < 0.05 (One way ANOVA followed by DMRT).
8.2.2. Effect of [6]-shogaol on inflammatory and EGFR gene analysed by RT-PCR

The mRNA expression pattern of IL-6, IκB-α, NF-κB, AP-1 and EGFR in control and experimental hamsters groups were depicted in Fig. 8.2. In DMBA painted animal shows over expression of IL-6, EGFR, IκB-α, NF-κB, AP-1 as compared to control. However, oral administration of [6]-shogaol to DMBA treated hamsters shows significantly decreased expression of IL-6, EGFR, IκB-α, NF-κB, AP-1 as compared to DMBA alone. Similar expression pattern of above noticed inflammatory markers were observed in control and [6]-shogaol alone treated hamsters.

Fig. 8.2. (a) Effect of [6]-shogaol on DMBA induced mRNA expressions of IL-6, EGFR, IκB-α, NF-κB and AP-1 were analyzed by RT-PCR. (b) The densitometric analysis was done by Kodak GL 100 imaging system. The densitometry data represent means ± SD from three independent experiments and are shown as relative density of mRNA normalized to respective GAPDH mRNA. Values not sharing a common marking (* and **) differ significantly at \( P < 0.05 \) (One way ANOVA followed by DMRT).
8.2.3. Effect of [6]-shogaol on cell proliferative markers

The immunoexpression pattern of cell proliferative markers (PCNA, Cyclin D1 and Ki-67) in control and experimental hamsters in each group are depicted in Fig. 8.3. Over expression of PCNA, Cyclin D1 and Ki-67 were noticed in DMBA treated hamsters (Group 2). However, Oral administration of [6]-shogaol (20 mg/kg b.wt) to DMBA treated hamsters shows significantly decrease the expression of cell proliferative markers such as PCNA, Cyclin D1, and Ki-67 as compared to DMBA alone. Hamsters treated with [6]-shogaol alone revealed expression similar to that of control hamsters. Consequently, the protein expression of PCNA, Cyclin D1 and Ki-67 were analysed by Western blotting.

![Image](image_url)

Fig. 8.3. Effect of [6]-shogaol on cell proliferative marker expression. (a) The Immunoexpression expression pattern of Cyclin D1, PCNA and Ki-67.  (b) Western blotting analysis of Cyclin D1, PCNA and Ki-67, normalized to β-actin loading control.  (c) Band intensities and fold changes were quantified by Image-studio software (LI COR, USA). Values are expressed as mean ± SD for three independent experiments. Values that do not share a common superscript letter (* and **) between groups differ significantly at $p < 0.05$ (One way ANOVA followed by DMRT).
8.3. Discussion

Development of chemotherapeutic drug can be achieved by the use of natural phytonutrients with negligible toxicity that reverse, suppress and/or prevent the multistage carcinoma. Naturally occurring dietary ingredients are considerable attention to improve health and reduce risk of cancer (Jones et al., 2009; Siu, 2011). The multistep process of carcinogenesis can be activated by various ideological factors such as smoking, chemicals and radiation. These carcinogenic factors are well known to alter the transcription factors. The activation of NF-κB/AP-1 signaling pathway leads to involved in oncogenic process of inflammation, cell proliferation and apoptosis (Baldwin Jr, 1996). Accumulated scientific evidence shows that the phytochemicals have been shown to inhibit the activation of NF-κB and AP-1 signaling pathways, which is known to sustain a homeostatic balance between cell proliferation and apoptosis (Han et al., 2001; Aggarwal and Shishodia, 2006). Present study, we aimed to investigate the mechanism of [6]-shogaol could inhibit NF-κB/AP-1 signals and its target molecules in DMBA induced HBP carcinogenesis.

Chronic inflammation is initial stage of neoplastic transformation regulated by various transcriptional factors such as NF-κB and AP-1. NF-κB plays a central role in pathological alteration of inflammation as well as tumorigenesis (Surh et al., 2001). Several signal mediators such pro inflammatory cytokine and growth factors transmit signals to cell surface of its receptor to activate target molecules. The pro inflammatory cytokine IL-6, TNF-α induce rapid phosphorylation or proteolytic degradation of IκBα to form free NF-κB. Thus NF-κB subsequently translocate into the nucleus where they coordinate the transcriptional activation of several hundred target genes resulting in tumor development or progression (Karin et al., 2005).

Aberrant activation of NF-κB and its target molecules has been implicated in neoplastic transformation (Postler and Ghosh, 2015). In our results, oral administration of [6]-shogaol could inhibit NF-κB activation by the inhibition of upstream molecules IL-1, IL-6, EGFR and TNF-α expression in
DMBA induced hamsters. Similarly, Tan et al. (2013) reported that [6]-shogaol suppressed NF-κB activity through inhibit IκB-α degradation and activation of PPARγ in breast and colon cancer cells.

AP-1 affords various mechanistic links between inflammation and cell proliferation in cancer, thus pre-neoplastic transformation inhibited by the dietary agents (Aggarwal and Shishodia, 2006). The cytosolic located AP-1 is directly activated by TNF-α and other cytokine, thus activated AP-1 translocate into nuclease to binding AP-1 DNA recognize site. Furthermore, activation of AP-1 down stream events to develop cancer. Many experimental models evidenced that over expression AP-1 and its down stream molecules leads to pathological out come of inflammation, cell proliferation and apoptosis resulting in cancer (Shaulian and Karin, 2001).

In our study, we observed over expression of AP-1 in DMBA induced hamsters. Consequently, pre-treatment of [6]-shogaol significantly decreased expression of AP-1 in DMBA treated hamsters. Notably, Ling et al. (2010) reported that [6]-shogaol mightily suppressed activation of AP-1 transcription through restrained phosphorylation of MAPK family members. Similarly, structural related compounds of [6]-gingerol and [6]-paradol exert inhibit epidermal growth factor (EGF) induced AP-1 activation of neoplastic transformation cells (Bode et al., 2001). Several investigations suggested that structural specificity of [6]-shogaol has α, β-unsaturated carbonyls are excellent vulnerable to nuceophilic addition reaction with thiols group such as gluthione which may excellent interaction with biological molecules could inhibit NF-κB/AP-1 activation (Ishiguro et al., 2008; Lawrence et al., 2006).

Inflammation is one of the early pathological alterations occurring in cancer, is mediated by various pro-inflammatory genes, such as iNOS, COX-2 and cytokines through the activation of signaling molecules (Takahashi et al., 2000; Murakami and Ohigashi, 2007). These pro-inflammatory genes of COX-2 and iNOS have been shown to be regulated by NF-κB and AP-1 (Nanjji et al., 2003). This inflammatory responsible genes has been prevented by bioactive
phyto-components through suppression of signalling pathway (Park et al., 2007; Kaefer and Milner, 2008; Macha et al., 2015). The aberrant expression of COX-2 and iNOS in oral squamous tissue revealed that the enhanced expression in oral premalignant and malignant lesions (Kim et al., 2004). According to this evidence, we observed increased COX-2 and iNOS expression in DMBA induced hamsters. Conversely, oral administration of [6]-shogaol inhibit the expression of COX-2 and iNOS via inhibition of NF-κB/AP-1 transcriptional activation in DMBA induced HBP carcinogenesis. Similarly, [6]-gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-κB transcription factors in phorbol ester-stimulated mouse skin (Kim et al., 2004; Kim et al., 2005). In addition, same class of phenolic nutrient of curcumin has been diminished COX-2, iNOS activity by suppressing a transcription factor of NF-κB (Surh et al., 2001). Taken together, these data suggest that [6]-shogaol has been exert their anti-inflammatory effects by suppressing NF-κB and AP-1 dependent pro-inflammatory mediator gene expression.

Cell cycle regulation is one of the major mechanisms for understanding of cancer progression. Cell cycle regulator proteins Cycline D1, PCNA, and Ki-67 play important role in cell proliferation and it’s over expression in DMBA induced HBP of experimental model (Garg et al., 2008). Thus, evidence of elevated expression of cycling D1 and PCNA is strongly correlated with hyperproliferation and failure DNA repair. Over expression of PCNA implies two aspect of clinical significance such as genotoxicity to cell cycle regulate protein and DNA-breaking progression. Our findings, [6]-shogaol significantly inhibit cell proliferation (Cycline D1, PCNA and Ki-67) through suppression of NF-κB/AP-1 expression in DMBA induced hamsters. Despite many previous studies have demonstrated the efficacy of phytochemicals can modulate cell cycle regulatory proteins like Cyclin D1 and PCNA in carcinogenic experimental model (Weng et al., 2012).