Chapter-V

Soy Isoflavones inhibits TPA-induced proinflammatory cytokines production, expression of COX-2, ki-67 and activation of NF-kB in mouse skin
Introduction

Inflammation, one of the important universal physiological responses associated with the process of carcinogenesis, catalyses the conversion of pre-malignancy to the malignancy. Chronic inflammation acts as mediator of various degenerative disorders including skin cancer. Improper regulation of redox sensitive signal transduction pathways induce by various inflammatory stimuli have been implicated in tumor promotion and carcinogenesis. Exposure to various physical and chemical agents are known to induce different biological events like infiltration of neutrophils which are key producers of reactive oxygen species (ROS) and reactive nitrogen species (RNS), cytokines and prostaglandins (Murakami, 2000). So for evidence from various approaches shows the critical role of TNF-α, IL-6, IL-1β, cycloxygenase-2 and the ubiquitous redox sensitive transcription factor nuclear factor-kappa B (NF-kB) in inflammation driven carcinogenesis (Allavena, 2008).

Development of cancer is one of the highly complex heterogenic and multistage viz., initiation promotion and progression, process. Promotion, a reversible and long term process, appears to be practically best for the intervention strategies of cancer development.

Topical application of phorbol ester induces inflammatory responses, mediated by different cytokines and regulatory factors, which are closely associated with promotion phase of carcinogenesis. In response to proinflammatory stimuli, activated inflammatory/immune cells generate ROS and RNS, over production of ROS leads to oxidative stress, a condition which is critically associated with most of the human diseases, and plays important role in inflammation-driven carcinogenesis (Cerutti and Trump, 1991).

Promotion phase of carcinogenesis/ cancer development is a complex step that involves cell proliferation, inflammation and oxidative stress-mediated signal transduction. Double TPA application, one of the most frequently used ideal system to study the
molecular, biochemical, cellular and histological alterations associated with promotion stage of carcinogenesis and also to understand the role of inflammation and oxidative stress in cancer promotion (Nakadate, 1989; Khan et al., 2012; Ha et al., 2006).

Naturally occurring compounds and their formulations are used for the treatment of different chronic diseases (Anthony et al., 1998; Barnes, 1998), particularly those with inflammatory mechanism in which reactive moieties are formed. Epidemiological findings reveal that use of natural compounds is a well promising approach for the chemoprevention and management of human cancers (Nakachi et al., 1996). Chemoprevention is a strategy of cancer control by administration of one or more naturally occurring and/or synthetic compounds to block initiation or suppress or delay promotion/progression of carcinogenesis (Surh, 2003).

Isoflavones are the one of them and exerts protective effects against a series of cancer modules in vivo and in vitro (Messina et al., 1994). Soybeans, most widely used in Asian countries, are the rich source of biologically active isoflavones like genistein (4, 5, 7-trihydroxyisoflavone) and daidzein (4,7-dihydroxyisoflavone) known to have spectrum of biological activities (Tikkanen,1998; Setchell and Adlercreutz, 1998). Epidemiological observations reveal that use of soy products associated with reduced incidence of breast and prostate cancers, heart disease etc., (Clarkson et al., 1995).

On the basis of above facts we hypothesize that intervention strategies targeting inflammatory pathways may prevent inflammation-induced early tumor promotional changes and provide some insights to understand the underlying mechanism of action of soy isoflavones against inflammation induced ill effects. So we have assessed the protective effects of SIF pretreatment on TPA induced cutaneous oxidative stress, epidermal hyperplasia and inflammation.

**Treatment protocol**

Animals were divided into five groups (I-V) of six animal each (n=6). Dorsal skin of all the animals was shaved with an electric clipper two days prior to the start of the
experiment. All the treatments were carried out topically onto the shaved area of dorsal skin.

**Group I**- Animals of this group were given topical application of vehicle (0.2 ml acetone) only and served as control group.

**Group II**- Mice were treated with the topical application of TPA (10 nmol) in 0.2 ml acetone. **Group III**- Animals of the group III were given pre-treatment of SIF at the dose of 1.0 μg (D 1) in 0.2 ml acetone, 30 min before TPA [(10 nmol) in 0.2 ml acetone] application.

**Group IV**- Animals of the group IV were given pre-treatment of SIF at the dose of 2.0 μg (D 2) in 0.2 ml acetone, 30 min before TPA [(10 nmol) in 0.2 ml acetone] application.

**Group V**- Animals of this group were given topical application of higher dose (D2) of SIF.

The treatments were carried out for 2 days at an interval of 24 h. Animals of the all the group were sacrificed by cervical dislocation 1 h after the last TPA treatment and skin tissue was processed for the evaluation of different parameters.

**Results**

**Effect of SIF pretreatment on TPA-induced cutaneous lipid peroxidation**

We have observed that SIF pre-treatment inhibits lipid peroxidation caused by TPA application in terms of TBARS (MDA) formation, a well known biomarker of oxidative stress. Double application of TPA causes significant elevation in the level of MDA in group II as compared to vehicle treated group I (p<0.001). Pre-treatment with soy isoflavones in group III and IV half an hour before TPA application significantly (p<0.01, p<0.001) suppressed the level of MDA when compared to group II. There was no significant change observed in the level of MDA between group I and V (Figure 1).

**Effect of SIF and TPA on the cutaneous GSH content**

TPA application causes significant depletion in the level of GSH in group II when compared with only vehicle treated group I (p<0.001). Pre-treatment with SIF in group
III and IV have showed significant (p<0.05, p<0.01) increase in the level of GSH as compared to group II. There was no significant difference observed in the GSH content between group I and V (Figure 2a).

**Effect of SIF and TPA on the cutaneous antioxidant enzymes**

The effect of SIF pre-treatment on TPA induced alteration in the activity of antioxidant enzymes (SOD and CAT) was assayed and the results were shown in figure 2 b and 2c respectively. We have observed that there was a significant (p<0.001) depletion in the activity of both the antioxidant enzymes in TPA treated group II as compared to only vehicle treated group I. However, pre-treatment with SIF in the group III and IV before TPA application leads to the significant (p<0.05) restoration in the activity of antioxidant enzymes when compared with the TPA treated group II. There was no significant difference observed between the group I and V as far as the activity of antioxidant enzymes were concerned.

**Effect of SIF pre-treatment against TPA-induced cutaneous TNF-α, IL-6 and IL-1β**

We have assessed the effect of topical application of SIF on TPA induced cutaneous TNF-α, IL-6 and IL-1β production, as shown in figure 3a, 3b and 3c respectively. We found that there was a significant (p<0.001) increased in the level of all the three cytokines in TPA treated group II in comparison to only vehicle treated group I. Pre-treatment with SIF has showed significant inhibition of cytokines production in the group III and IV when compared with the TPA treated group II (p<0.05, p<0.01, p<0.001). There was no significant difference found between group I and V.

**Effect of SIF on the TPA-induced cutaneous Immunohistochemical expression of NF-kB (p65), COX2 and ki-67**

TPA induced cutaneous expression of NF-kB (p65), COX2 and ki-67 in group II have been shown in the figure 4, 5 and 6 respectively. The intensity of the brown color in the
TPA treated animals (group-II) clearly indicates the more number of cells having NF-kB, COX-2 and ki-67 expression. Pre-treatment with SIF in the group III and IV shows less number of cells having the expression of NF-kB, COX-2 and ki-67. However there was no significant difference observed in the expression of these proteins in the group V as compared to group I. For immunohistochemical analyses, brown color indicates specific immunostaining of NF-kB, COX-2 and ki-67 and light blue color indicates haematoxylin staining. Original magnification: x40 and x100.

Effect of SIF on TPA-induced cutaneous histological alterations

Effect of topically applied SIF was further observed on the skin histological alteration caused by TPA application with reference to neutrophil infiltration and epidermal hyperplasia as shown in figure 7. We have found that TPA application leads to considerable increase in the leukocyte infiltration and epidermal thickening in group II (Figure 7 B) as compared to group I (Figure 7 A). Pre-treatment with SIF in the group III (Figure 7 C) and group IV (Figure 7 D) diminishes TPA induced leukocyte infiltration as well as hyperplasia. There was no distinguished change observed between the group I (Figure 7 A) and group V (Figure 7 E) as far as leucocytes infiltration and hyperplasia is concern. Original magnification: x10.

Discussion

Skin, being the largest organ of the body, provides first line of defense against different harmful exogenous exposure. So far, it has been observed that consumption of natural food have alleviating effects on human health including skin diseases. One of the approaches to prevent the malignancies including that of skin is chemoprevention. Chemoprevention is a means of cancer control that is based on the use of specific natural or synthetic chemical substances that can suppress, retard or reverse the process of carcinogenesis. Polyphenols are the most promising group of compounds possessing anti-inflammatory, immunomodulatory and antioxidant properties and have shown considerable protection against skin cancer (Nichols and Katiyar, 2010).
Carcinogenesis consists of three distinct stages initiation, promotion and progression. Promotion stage has gained attention for the intervention of cancer development by cellular and molecular approaches. In this study we have examined the attenuative effects of soy isoflavones with reference to TPA induced cutaneous damage which is closely associated with promotion step of cancer development.

Inflammatory responses, play important roles in skin tumor promotion and infiltration of neutrophils, are the pivotal source of ROS production leading to oxidative stress which is critically associated with carcinogenesis. Our findings also show the antioxidant effects of soy isoflavones. We have found that application of TPA causes oxidative stress that ultimately leads to membrane damage which is evident from enhanced lipid peroxidation. Level and activity of different antioxidants were also found distorted that shows the presence of milieu crucial for the promotion stage of tumor development. Pre-treatment with isoflavones shows protective response as the result implicates. Present study outcomes are in accord with previous research on soy isoflavones (Sharma and Sultana, 2004; Khan and Sultana, 2004). These effects may be directly because of the molecule’s phenolic ring (Dugas et al, 2000), which protects against lipid peroxidation, or to its ability to up-regulate anti-oxidant genes (Siow et al., 2007).

It has been observed from the present study findings that soy isoflavones have strong anti-inflammatory potential. Proinflammatory cytokines are the important mediators of inflammation and have distinguished roles in the promotion stage of cancer development. TNF-α, IL-6 and IL-1β have been implicated in various cellular and molecular mechanisms associated with most of the inflammation associated chronic human diseases including cancer (Kundu and Surh, 2008). Their association with inflammation driven tumorigenesis was confirmed by the previous findings that mouse deficient in TNF-α, IL-6 and IL-1β are resistant to skin tumor formation and chronic inflammation, neoplasia and tumor metastasis respectively (Moore et al., 2009; Tricot,
2000; Vidal-Vanaclocha et al., 2000). Further, studies on human and mouse shows that the proinflammatory cytokines play important role in the development of microenvironment for tumor progression (Smith et al., 1998). The data of the present study evidently supports that soy isoflavones effectively suppress the production of cytokines which implicates their anti-inflammatory and thus anti-tumor promoting potential.

COX, one of the important proinflammatory enzymes catalyzes the formation of prostaglandins from arachidonic acid, play vital role in inflammation (DuBois et al., 1998). Two well known forms of the COX viz., COX-1 and COX-2 have been identified. The expression of COX-1 is ubiquitous in most of the cells and involved in normal homeostasis of prostaglandins. While, expression of COX-2 is inducible and was found to increases in response to various stimuli like growth factors, cytokines and oncogenes (Smith et al., 2000). Substantial role of COX-2 has been recognized as it critically involved with inflammation, tumor promotion and carcinogenesis (Lai et al., 2007). A number of stimuli like pro-oxidant /proinflammatory cytokines, anomalous regulation of signaling regulated by kinases and transcription factors results in abnormal expression of COX-2 (Chun et al., 2004). Different approaches for cancer prevention have demonstrated the critical importance of COX-2 in tumor promotion and so the targeted inhibition of COX-2 emerges as one of the promising approaches to inhibit inflammation and carcinogenesis (Philip et al., 2004). Since the aberrant expression of COX-2 is recognized as a connecting link for the tumor promotion the findings of the current study reflect the anti-inflammatory property of soy isoflavones which attenuates TPA induced cutaneous expression of COX-2 in mouse.

NF-κB, one of the ubiquitous redox sensitive transcription factors, regulates many biological processes including cellular proliferation, differentiation and inflammation. The main inducible form of NF-κB is heterodimeric consisting of the p50/p65 subunits.
In resting cells NF-κB resides along with its cytosolic repressor inhibitory protein IκB. Exposure with different stimuli like oxidative stress/phorbol esters causes the phosphorylation and degradation of IκB by cytoplasmic IκB kinase (IKK) which results in the nuclear translocation of NF-κB. In the nucleus, it regulates the transcription of array of target genes including proinflammatory mediators, such as iNOS, COX-2, various cytokines, chemokines, and adhesion molecules (Pahl et al., 1999; Baeuerle and Baltimore, 1996; Chao et al., 2010). Previous findings implicate that activation of NF-κB alleviates / triggers the transcriptional up-regulation of COX-2 and proinflammatory cytokines like IL-6, IL-1β, and TNF-α (Wu et al., 2008). The present study findings show that cutaneous application of TPA leads to the activation of NF-κB in mouse which was strongly suppressed by soy isoflavones pretreatment suggesting the strong anti-inflammatory and therefore anti-tumor promoting potential of soya isoflavones.

Immunohistological staining of ki-67 also support the anti-proliferative effects of soy isoflavones on TPA induced tumor promotion. The above mentioned findings corroborated with the histological data which exhibited the protective effects of soy isoflavones against TPA induced cellular anomalies like disorganization of epithelium, presence of necrotic cells and focal proliferative areas with epidermal thickness, neutrophils infiltration and intercellular edema.

In conclusion the biochemical, molecular and histological findings of present study reveals the antioxidant and anti-inflammatory and anti-proliferative potential of soy isoflavones against TPA induced alterations in mouse skin. Inhibition of COX-2 expression, production of proinflammatory cytokines and activation of NF-κB provides the molecular basis of the anti-inflammatory potential of soy isoflavones.
Figure 1. Effect of SIF pre-treatment on TPA-induced cutaneous lipid peroxidation

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in Group II [TPA (10 nmol)] as compared to Group I (only vehicle). ##p<0.01, ###p<0.001 shows the significant difference in the Group III [SIF (1μg) + TPA (10 nmol)] and Group IV [SIF (2μg) + TPA (10 nmol)] as compared to Group II.

Fig.2. Effect of SIF pre-treatment on endogenous anti-oxidants: (a) GSH level (b) Superoxide dismutase (SOD) activity (c) Catalase (CAT) activity.

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in Group II [TPA (10 nmol)] as compared to Group I (only vehicle). #p<0.05, ##p<0.01 shows the significant difference in the Group III [SIF (1μg) + TPA (10 nmol)] and Group IV [SIF (2μg) + TPA (10 nmol)] as compared to Group II.
Fig. 3. Effect of SIF pre-treatment on TPA induced cutaneous proinflammatory cytokines. (a) TNF-α (b) IL-6 (c) IL-1β

Values are expressed as mean ± SEM. (n = 6). ***p<0.001 shows significant difference in TPA treated Group II [TPA (10 nmol)] as compared to Group I (only vehicle). #p<0.05, ##p<0.01, ###p<0.001 shows the significant difference in the Group III [SIF (1μg) + TPA (10 nmol)] and Group IV [SIF (2μg) + TPA (10 nmol)] when compared with Group II.
Fig. 4. Effect of SIF pre-treatment against TPA-induced cutaneous NF-kB expression

Representative photomicrographs (magnification ×40), (A) only vehicle (Group I), (B) TPA (Group II), (C) SIF (D1) + TPA (Group III), (D) SIF (D2) + TPA (Group IV) and (E) SIF (D2) only (Group V). Brown color indicates NF-kB specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more NF-kB immunopositive staining (arrows) as compared with vehicle treated group (Group I). SIF pretreatment (Group III and IV) reduces NF-kB expression as compared to Group II. However there was no significant difference in the NF-kB immunostaining in Group V as compared to Group I.
Fig.5. Effect of SIF pre-treatment against TPA-induced cutaneous COX-2 expression

Representative photomicrographs (magnification ×100), (A) only vehicle (Group I), (B) TPA (Group II), (C) SIF (D1) + TPA (Group III), (D) SIF (D2) + TPA (Group IV) and (E) SIF (D2) only (Group V). Brown color indicates COX-2 specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more COX-2 immunopositive staining (arrows) as compared with vehicle treated group (Group I). SIF pretreatment (Group III and IV) reduces COX-2 expression as compared to Group II. However there was no significant difference in the COX-2 immunostaining in Group V as compared to Group I.
Fig. 6. Effect of SIF pre-treatment against TPA-induced cutaneous ki-67 expression

Representative photomicrographs (magnification ×40), (A) only vehicle (Group I), (B) TPA (Group II), (C) SIF (D1) + TPA (Group III), (D) SIF (D2) + TPA (Group IV) and (E) SIF (D2) only (Group V). Brown color indicates ki-67 specific staining and blue color indicates haematoxylin staining. TPA treated group (Group II) shows more ki-67 immunopositive staining (arrows) as compared with vehicle treated group (Group I). SIF pre-treatment (Group III and IV) reduces ki-67 expression as compared to Group II. However there was no significant difference in the ki-67 immunostaining in Group IV as compared to Group I.
Fig. 7. Effect of SIF pretreatment on TPA-induced cutaneous histological alterations

Representative photomicrographs (magnification ×10), (A) only vehicle (Group I), (B) TPA (Group II), (C) SIF (D1) + TPA (Group III), (D) SIF (D2) + TPA (Group IV) and (E) SIF (D2) only (Group V). There was marked infiltration of neutrophils and epidermal thickness in TPA treated animals [(Group II, 7(B)] as compared with only vehicle treated animals [(Group I, 7(A)]. SIF pretreatment in [(Group III, 7(C)] and [(Group IV, 7(D)] attenuates TPA induced alterations significantly as compared to TPA treated animals (Group II). However there was no significant difference in the cutaneous histology in the animals only treated with SIF [(Group V, 7 (E)] as compared to only vehicle treated animals (Group I).
SIF- Soy Isoflavones
TPA- 12-β-tetradecanoylphorbol-13-acetate

Graphical representation of the mechanism of action of SIF against TPA-induced cutaneous oxidative stress and inflammation.