Effect of Diosgenin on STAT-3 signaling cascade in hepatocellular carcinoma

Fernandez P\textsuperscript{1} and Sethi G\textsuperscript{2}
Department of Pharmacology, Faculty of Science, National University of Singapore, 10 Kent Ridge Road, Singapore 117546

Abstract
The activation of signal transducers and activators of transcription 3 (STAT-3) has been linked with the proliferation of a variety of human cancer cells, including hepatocellular carcinoma cells (HCC). Agents that can suppress STAT-3 activation have potential for prevention and treatment of HCC. In the present study, we tested an agent, diosgenin, found in fenugreek and wild yam, for its ability to suppress STAT-3 activation. We found that diosgenin, a steroidal saponin, inhibited both constitutive and interleukin-6 (IL-6) induced STAT-3 activation in HCC cells. The suppression was mediated through the inhibition of activation of upstream kinases Akt, c-Src, and Janus-Activated Kinase 2 (JAK-2). Diosgenin was also a more potent inhibitor of HCC cell proliferation than AG490 (a well-characterized JAK-2 inhibitor). Overall, these results suggest that diosgenin is a novel blocker of STAT-3 activation that may have a potential in prevention and treatment of HCC and other cancers.

Introduction
HCC, the most common type of liver cancer, is the third leading cause of cancer death worldwide, with 75% of cases occurring in Southeast Asia. The etiology of HCC is likely to involve interactions between multiple risk factors. Signal transducer and activator of transcription (STAT) proteins have been shown to play an important role in tumor cell survival and proliferation (Pandey et al, 2009). For instance STAT-3 is constitutively active in 50% of HCC and it has been reported that the HBx protein of the Hepatitis B virus and the core protein of the Hepatitis C virus, may activate STAT-3. Thus this has greatly increased the interest in this signal transduction pathway for treatment of HCC (Sanchez et al, 2003).

Thus, agents that suppress STAT-3 activation have a potential in prevention and therapy of cancer. Diosgenin, a constituent of fenugreek (Trigonella foenum graecum) and wild yam (Dioscorea villosa), has been linked with suppression of tumorigenesis through a mechanism that is not well understood (Shishodia and Aggarwal, 2006). Since STAT-3 activation has been closely linked with tumorigenesis, we investigated the effect of this saponin on the STAT-3 pathway in HCC cells.

Material & Methods

Reagents
Diosgenin, (chemical structure is shown in Fig. 1A) was purchased from Sigma. A 10 mM solution of diosgenin was dissolved in ethanol and stored in small aliquots at -20°C and then diluted as needed in cell culture medium. MTT, AG490, glycine, SDS, bovine serum albumin (BSA) and β-actin antibody were purchased from Sigma Aldrich. Antibodies to phospho-STAT-3 (Tyr\textsuperscript{705}), phospho-specific Src (Tyr\textsuperscript{416}), phospho-specific JAK-2 (Tyr\textsuperscript{1007/1008}), phosphorylated Akt (Ser\textsuperscript{473}), Src, JAK-2, and Akt were purchased from Cell Signaling Technology. Goat anti-rabbit IgG peroxidase conjugate, rabbit anti-mouse IgG peroxidase conjugate, minimal essential medium (MEM), DMEM and fetal bovine serum (FBS) were purchased from Invitrogen. Bacteria-derived recombinant human IL-6 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

1 Student
2 Assistant Professor
Cell Lines
Human HCC cell lines C3A was obtained from American Type Culture Collection (Manassass, VA). HUH-7 cells were a kind gift from Prof. Hui Kam Man at National Cancer Center, Singapore.

Western Blotting
To detect various proteins, diosgenin treated cells were lysed in lysis buffer, and thereafter, electrophoresis and western blotting were done as described previously (Pathak et al, 2007).

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay
The anti-proliferative effect of diosgenin and AG490 against HCC cell line C3A was determined by MTT dye uptake method as described previously (Pathak et al, 2007).

RESULTS
The present study was undertaken to determine the effect of diosgenin on the STAT3 signaling cascade in HCC. The effect of diosgenin on constitutive STAT3 activation in HCC cell line C3A was investigated.

Diosgenin suppresses STAT3 phosphorylation in a dose and time dependent manner
The ability of diosgenin (structure shown in Fig.1A) to modulate constitutive STAT3 activation in C3A cell line was investigated. C3A cells were incubated with different concentrations of diosgenin for 6h. The whole cell extracts were prepared and examined for phosphorylated STAT3 by Western blot analysis. As shown in Fig. 1B, diosgenin inhibited the constitutive activation of STAT3 in C3A cells in a dose dependent manner, with maximum inhibition occurring between 50 and 100 µmol/L. Diosgenin had no effect on the expression of STAT3 protein (Fig. 1B, lower panel). The incubation time required for diosgenin to suppress STAT3 activation in C3A cells was also determined. Inhibition was time dependent and maximum inhibition occurred at 6-8 h (Fig.1C), again with no effect on the expression of STAT3 protein (Fig.1C, lower panel).

Diosgenin inhibits IL-6 induced STAT-3 and Akt phosphorylation in HUH-7 cells
Since IL-6 is a growth factor for HCC and induces STAT3 phosphorylation, it was determined if diosgenin could inhibit IL-6 induced STAT3 phosphorylation. HUH-7 cells were pretreated with diosgenin for different time intervals (4-8h) followed by treatment with IL-6 for 0.5h. It was observed that the diosgenin pretreatment suppressed IL-6 induced STAT3 activation between 6-8h in HUH-7 cells (Fig.2A). Activation of Akt has also been linked with STAT3 activation. Hence, it was further investigated whether diosgenin could modulate IL-6 induced Akt activation. HUH-7 cells treated with IL-6 showed an increase in expression of phosphorylated Akt, and diosgenin pretreatment suppressed this activation (Fig.2B).
Diosgenin suppresses constitutive activation of Src and JAK-2

Several studies have shown that STAT-3 is activated by soluble tyrosine kinases of the Src kinase family (Pathak et al, 2007). Consequently, the effect of diosgenin on constitutive activation of Src kinase was determined. It was found that diosgenin inhibited the constitutive phosphorylation of c-Src kinase (Fig. 2A) in a time dependent manner, with maximum inhibition occurring between 4-8 h. Several studies have shown that STAT-3 is activated by soluble tyrosine kinases (Pathak et al, 2007). Consequently, it was determined if diosgenin affects constitutive activation of JAK-2 in C3A cells. Fig.2B shows that diosgenin suppressed the constitutive phosphorylation of JAK-2 in a time dependent manner.

Diosgenin is a more potent inhibitor of cell proliferation as compared to AG490

Since diosgenin suppressed the activation of STAT-3 and its upstream soluble tyrosine kinases, its effect on proliferation of C3A cells was also examined. Results of the MTT assay (Fig. 3) show that disogenin suppressed the proliferation in a time and dose dependent manner. Additionally, when the effect of diosgenin and AG490 (commercial JAK-2 specific inhibitor) was compared, it was observed that disogenin was more rapid in inhibiting the proliferation of C3A cells. Diosgenin showed significant inhibition at both 12h and 24h, while AG490 showed a similar extent of inhibition only at 48h.
CONCLUSION

The goal of this study was to determine whether disogenin exerts anti-tumorigenic effects through the modulation of the STAT-3 signaling pathway in HCC cell line. We observed that this saponin was able to suppress constitutive and inducible STAT-3 activation. The effects of disogenin on STAT-3 phosphorylation correlated with the suppression of upstream protein kinases Akt, Src and JAK-2. The results from the MTT assay showed that disogenin inhibited the proliferation of C3A cells and was also more rapid in exerting its anti-proliferative effect as compared to AG490. It has been reported that many types of tumors including HCC, head and neck cancers, breast cancer, multiple myeloma, lymphomas and leukemia exhibit constitutive STAT-3 (Pandey et al, 2009). As such, the suppression of constitutively active STAT-3 in HCC suggests that disogenin might prove effective in inhibiting constitutively activated STAT-3 in other types of cancers as well. Overall, these results suggest that disogenin is a novel blocker of the STAT-3 activation pathway, with a potential role in the prevention and treatment of HCC and other cancers.

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REFERENCES


