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# Downregulation of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 by grape seed proanthocyanidin extract

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ABSTRACT

Urokinase plasminogen activator (uPA) system, comprising of uPA, its receptor uPAR and inhibitor, type 1 plasminogen activator inhibitor (PAI-1), plays a vital role in various biological processes involving extracellular proteolysis, fibrinolysis, cell migration and proliferation. The timely occurence of these processes are essential for normal wound healing. This study examines the regulation of uPA and PAI-1 by a natural polyphenol-rich compound, grape seed extract (GSE). GSE is reported to have beneficial effects in promoting wound healing. Fibroblast cells exposed to different doses of GSE for 18 hours were processed for further studies such as ELISA, RT-PCR, western blotting, fibrinolytic assay, cell surface plasmin activity assay and *in vitro* wound healing assay. GSE treatment caused a significant downregulation of uPA and PAI-1 expression, both at the RNA and protein levels. ELISA also revealed a dose-dependent decrease in uPA and PAI-1 activities. Functional significance of the downregulation was evident in decreased fibrinolytic activity, concomittant with decreased cell-surface plasmin activity. *In vitro* wound healing studies showed that GSE also retarded the migration of cells towards the wounded region.



## Introduction

The serine protease, urokinase-type plasminogen activator (uPA) plays an important role in tissue remodeling, cell migration and wound healing (Toriseva and Kahari, 2008; Watanabe et al., 2006). It catalyzes the conversion of inactive zymogen plasminogen to the enzymatically active plasmin. Plasmin participates in fibrinolysis by degrading fibrin and in tissue remodeling by degrading extracellular matrix (ECM) and activating other matrix degrading proteases. The main physiological inhibitor of uPA is plasminogen activator inhibitor-1 (PAI-1). Besides inhibiting plasminogen activation, PAI-1 has multi-faceted roles with relation to cell adhesion/ migration and thus is involved in wound healing (Lijnen, 2005). uPA-PAI-1 system is implicated in the early phase of wound healing that requires dissolution of fibrin clot and cellular migration into the wound region.

Proanthocyanidins are a group of biologically active polyphenolic bioflavonoids synthesized by many plants, and are known to facilitate wound healing (Hupkens et al., 1995). Grape seed extract (GSE) is a rich source of proanthocyanidins. Recent studies suggest that GSE facilitates wound healing by regulating oxidant-induced changes in keratinocytes and improving wound closure (Khanna et al., 2001, 2002). To date, the potential influence of GSE on uPA system has not been evaluated. The aim of the present study was to examine the effects of GSE on uPA and PAI-1 in cultured human fibroblast cells. We report that GSE downregulates uPA and PAI-1, with functional consequences of decrease in fibrinolytic activity as well as cellular migration towards wounded region.

## Materials and Methods

### *Cell culture and reagents*

Human fibroblast cells TIG 3-20 (HSRBB Cell bank, Osaka, Japan) were cultured in Modified Eagles Medium. Semi-confluent cells between passages 3 and 5 were used for the studies. GSE (Vinofelon$^{TM}$) was a kind gift from Tokiwa Phytochemical Co., Ltd, Chiba, Japan. Dried ground seeds (500 kg) of Grape (*Vitis spp.*) were refluxed for 1 hr in aqueous ethanol (80% v/v, 5000 l) twice and the combined alcoholic extract was evaporated and filtered. The filtrate was adsorbed onto porous synthetic resin (DIAION HP-20, 500 l) and eluted with water (1000 l). Proanthocyanidin fraction was then collected by elution with aqueous ethanol (70% v/v, 1000 l). The fraction was evaporated and dried by spray dryer. The obtained solid was crushed, mixed and passed through a sieve to obtain powder of proanthocyanidin-rich extract (15 kg).

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Although the chemical composition of GSE has not been specified completely, the product analysis sheet provided by Tokiwa Phytochemical Co., Ltd. shows proanthocyanidin content as not less than 90% of total flavanol content on dry matter basis. Following is the procedure provided by Tokiwa Phytochemical for standardization of purified proanthocyanidins. Total flavanols in the GSE were determined by the standard vanillin-HCl method (Broadhurst and Jones, 1978) using (+)-catechin (Kurita Kogyo Co., Ltd, Japan) as standard. Briefly, 20 mg of GSE was weighed and dissolved in water to get a sample solution of 100 ml. To 1 ml of the sample solution in a brown tube, 9 ml of 2% vanillin/HCl-methanol reagent (2 g vanillin dissolved in 12 N HCl-methanol (1:2) solution to get final volume of 100 ml) was added, immediately capped, mixed for 10 seconds and incubated at 18–22°C for 20 min. Absorbance of this solution was measured by spectrophotometer at 500 nm (reference: water) (ABS-S). To correct influences of anthocyanidins in sample, HCl-methanol (1:2) was added instead of 2% vanillin/HCl-methanol reagent to each sample solution (ABS-C). Proanthocyanidins (as total flavanol) contents was calculated from the value of (*ABS-S*) – (*ABS-C*) – (*ABS-BLANK*) by using working curve. Working curve was obtained as follows: 1, 2, 3 mg of (+)-catechin was dissolved in water to a final volume of 10 ml (the standard solution). One ml of each standard solution was taken in a brown tube and 9.0 ml of 2% vanillin/HCl-methanol reagent was added, immediately capped, mixed for 10 seconds and incubated at 18–22 °C for 20 min. Absorbance of this solution was measured at 500 nm by spectrophotometer (reference: water) (*ABS-CAL*). In case of blank, water was used instead of standard solution (*ABS-BLANK*). Working curve was obtained with correcting values; (*ABS-CAL*)-(*ABS-BLANK*).

GSE was dissolved in double distilled water and filtered prior to experiments. Cells were incubated with different doses of GSE in serum free medium for 18 hours. Conditioned medium was collected, centrifuged at 8000 g for 10 min to remove cell debris and stored at −70 °C until further assays.

### *Cell viability assay*

Effect of GSE on cell viability was analyzed by CellTiter Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) following manufacturer's protocol.

### *Reactive Oxygen species (ROS) assay*

Effect of GSE on generation of intracellular reactive oxygen species was estimated using dichlorofluorescein diacetate (DCFH-DA) (Balasubramanyam et al., 2003). Following incubation of fibroblasts with different doses of GSE for 18 hours, 10 μM DCFH-DA was added to the cells. Non-fluorescent DCFH-DA is converted to fluorescent DCF, in proportion to the amount of ROS generated in the cells. The fluorescent signal was measured using FP-6200 spectrofluorometer (Excitation 485 nm; Emission 530 nm).

### *Quantification of uPA and PAI-1 antigen levels by ELISA*

The levels of uPA and PAI-1 antigens in the conditioned medium from cells incubated with GSE were measured by ELISA using scuPA ELISA reagent kit (Technoclone, Mullnergasse, Vienna, Austria) for uPA and Assay Max Human PAI-1 ELISA kit (AssayPro, St. Charles, MO, USA) as per manufacturers' protocols. The results of antigen assays are expressed as mean ± SD.

### *Western blotting*

Total protein extracts from cells were obtained by lysing cells in mammalian protein extraction reagent (M-PER, Pierce Biotechnology Inc, IL, USA). Twenty microgram proteins were resolved over 10% SDS–PAGE gels and electroblotted onto nitrocellulose membrane using Trans-blot SD, semi dry transfer cell (Bio-Rad Laboratories, CA, USA). The membrane was subjected to western blotting using anti-uPA, anti-PAI-1 and anti-β-actin antibodies, as the case may be. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Expression of proteins was detected by chemiluminescence using ECL Plus Western Blotting Detection System (Amersham Life Science, Inc., Buckinghamshire,

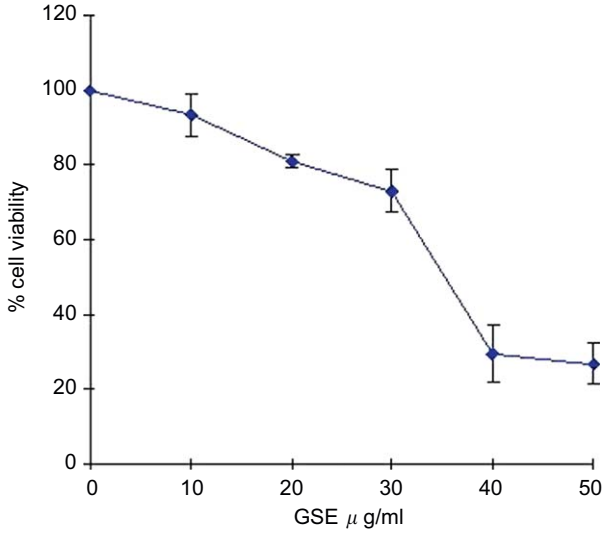


**Fig. 1.** Cytotoxic profile of GSE on TIG 3–20 fibroblasts: Semi-confluent cells were treated with various doses of GSE for 18 hours. Cell viability was analyzed using Cell titer Glo Luminescent Assay. Values are mean ± SD of six independent experiments.

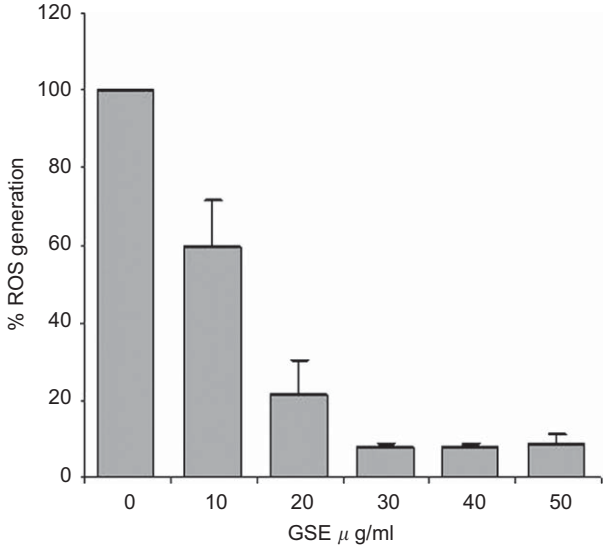


**Fig. 2.** Anti-oxidant property of GSE: TIG 3-20 cells were treated with different doses of GSE for 18 hours. Cells were treated with 10 μM DCFH-DA for 45 minutes. Intracellular ROS generation was measured using spectrophotometer (Exc 485 nm; Emi 530 nm). Data is represented as percentage ROS generation. Values are mean ± SD of six independent experiments.