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Mechanism of angiogenic effects of saponin from Ginseng *Radix rubra* in human umbilical vein endothelial cells

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1 The effects of saponin from Ginseng *Radix rubra* on angiogenesis (tube formation) and its key steps (protease secretion, proliferation and migration) in human umbilical vein endothelial cells (HUVEC) were examined to elucidate the mechanism of the tissue repairing effects of Ginseng *Radix rubra*. The effect on a wound healing model was also studied.

2 Tube formation was measured by an *in vitro* system. The activity and immunoreactivity of tissue-type plasminogen activator (tPA) as a protease for angiogenesis and the immunoreactivity of its inhibitor, plasminogen activator inhibitor-1 (PAI-1), were measured in conditioned medium of HUVEC stimulated for 24 h with saponin. Cell proliferation was measured by counting the cell numbers at 2–7 days after seeding. Migration was measured by Boyden's chamber method. The effect on wound healing was studied in the skin of diabetic rats.

3 Saponin at 10–100 μg ml^{-1} significantly stimulated tube formation by HUVEC in a dose-dependent manner. Saponin in a similar concentration-range increased the secretion of tPA from HUVEC as estimated by immunoreactivity and enzyme activity. On the other hand, PAI-1 immunoreactivity was slightly increased at 10 μg ml^{-1} of saponin, but then was significantly decreased at 50 and 100 μg ml^{-1}. Cell proliferation was only slightly enhanced by 1–100 μg ml^{-1} of saponin, but migration was significantly enhanced by 10–100 μg ml^{-1} in a dose-dependent manner. Moreover, saponin stimulated wound healing with enhanced angiogenesis *in vivo*.

4 These results indicate that saponin stimulates tube formation mainly by modifying the balance of protease/protease inhibitor secretion from HUVEC and enhancing the migration of HUVEC, and that it is effective *in vivo*.

Keywords: Ginseng; saponin; angiogenesis; tissue-type plasminogen activator; plasminogen activator inhibitor-1; proliferation; migration; endothelial cells; wound healing

Introduction

Ginseng *Radix rubra* is used clinically in Japan for various diseases including atherosclerosis, liver dysfunction, cerebrovascular diseases, hypertension and postmenopausal disorders (Yamamoto, 1988). The major active component of Gingseng *Radix rubra* is saponin, and it includes more than twenty identified ginsenosides (Yamamoto, 1988). In a clinical study we have demonstrated that orally-administered Ginseng *Radix rubra* markedly improves the repair of intractable skin ulcers of patients with diabetes mellitus and Werner's syndrome. Angiogenesis plays an important role in the repair of ulcers. Therefore, the aim of this study was to clarify whether saponin of Ginseng *Radix rubra* stimulates angiogenesis and if so, which steps of angiogenesis are particularly influenced by saponin.

Angiogenesis is considered to consist of four steps (Klagsbrun & Folkman, 1990): (1) Destruction of the basement membrane by proteases secreted by endothelial cells (EC). (2) Proliferation of EC. (3) Migration of EC. (4) Resulting actual tube formation by EC. Recently, tube formation systems that include all or most of the above steps have been developed (Montesano & Orci, 1985; Tsuji & Karaseki, 1985). In this study, we describe the effects of saponin on tube formation, protease secretion, migration and proliferation, using *in vitro* cultured EC and also on an *in vivo* wound healing model.

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Methods

Cell culture

EC from human umbilical vein (HUVEC) were cultured (Jaffe *et al.*, 1972) in MCDB131 medium supplemented with 10% foetal bovine serum (FBS), 10 ng ml^{-1} basic fibroblast growth factor (bFGF) and 90 μg ml^{-1} heparin (growth medium). Cells were subcultured at a split ratio of 1:2, and used at the 2nd–5th passages.

Assay of tube formation

Tube formation of HUVEC was assayed by a slight modification (Tezuka *et al.*, 1993) of a reported procedure (Montessano & Orci, 1985; Tsuji & Karasaki, 1985). Confluent HUVEC on 24-well plates coated with 1% gelatin were overlayed with 1 ml of collagen gel, a mixture of 8 vol. of Vitrogen, 1 vol. of 10 fold concentrated Minimum Essential Medium, and 1 vol. of a solution of 0.05 N NaOH, 200 mM HEPES and 260 mM $NaHCO_3$, in the presence or absence of saponin or bFGF. During incubation in a 5% CO_2 incubator for 2 days, a capillary-like network formed. Light micrographs of three microscopic fields (x100) of the well (the centre and two peripheral fields) were used for the assay of angiogenic activities by measuring the total length of tubes in the area photographed. The mean of the three-field data was calculated. Tube length was measured by computer scanning with a 9801VX computer (NEC Co., Tokyo, Japan), an Image Scanner GT100V (Epson, Tokyo, Japan), and an MKK scan programme (kindly provided by Dr Kosuge). Only the length of tube structures with diameters between 0.5–2.5 mm in the

photographs (x100) was measured. The actual diameters of these tubes were 10–50 μm. Complete capillary-like networks were not found microscopically in control cultures, but some tube-like structures were measured based on this criterion (Tezuka *et al.*, 1993).

Assay of protease and protease inhibitor secretion

We measured the immunoreactivity and enzyme activity of tissue-type plasminogen activator (tPA), an essential protease for the initial step of tube formation by the HUVEC used in this study (Mawatari *et al.*, 1991). The immunoreactivity of plasminogen activator inhibitor-1 (PAI-1) was also measured. HUVEC at confluence in T-25 flasks coated with 1% gelatin were incubated with 4 ml MCDB131 medium in the presence or absence of saponin for 24 h as previously reported (van Hinsbergh *et al.*, 1987). After incubation, the medium was collected and the immunoreactivity and enzyme activity of tPA and the immunoreactivity of PAI-1 were measured. The immunoreactivities of tPA and PAI-1 were measured by ELISA using the kits Imulyse tPA and Imulyse PAI-1, respectively. The activity of tPA was also measured with a kit, Spectrolyse (fibrin)tPA/PAI(v1-1).

Assay of HUVEC proliferation

HUVEC were seeded onto 24-well plates coated with 1% gelatin at $2x10^4$ cells/well in 1 ml growth medium containing various concentrations of saponin and incubated for 2–7 days. Cell numbers were counted in a Coulter counter as reported (Morisaki *et al.*, 1988; Koyama *et al.*, 1994).

Assay of HUVEC migration

The migration of HUVEC was measured by a modification of Boyden's chamber method as described previously (Koyama *et al.*, 1991; 1992a; 1993). Briefly, polycarbonate filters with 5.0 μm pores were coated with 1% gelatin solution. Cultured HUVEC were trypsinized and suspended at a concentration of $5x10^5$ cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Then 50 μl of DMEM containing various concentrations of saponin was placed in the lower chamber. The chambers were incubated at 37°C for 6 h in a CO_2 incubator. The numbers of cells that had migrated to the lower side of the filter were then counted.

Wound healing model

Insulin-dependent diabetes mellitus was induced by the injection of 60 mg kg^{-1} streptozotocin (Kawano *et al.*, 1993) into male Wistar rats weighing 200g, and they were used two months after induction as a model of intractable wound healing. The plasma glucose level was about 500 mg dl^{-1} for the diabetic and 100 mg dl^{-1} for the normal rats. The usual plasma glucose level of normal and diabetic rats is less than 150 mg dl^{-1} and above 300 mg dl^{-1}, respectively (Kanzaki *et al.*, 1987). A full-thickness, dorsal incision wound healing model was used as reported by Mustoe *et al.* (1987). In brief, paired incisions were joined together with three surgical clips and injected once with a total of 25 mg of saponin suspended in 1 ml of 0.3% collagen or with collagen vehicle alone at the time of wounding. The wounds were excised 7 days later and strips perpendicular to the paired experimental and control (collagen alone) wounds were obtained for tensiometry and histological analysis. From each rat, two middle quadrants and two end quadrants were obtained for tensiometry and histological analysis, respectively. Wound breaking strength was estimated by measuring the maximum load tolerated by wounds with a tensiometer on 8 mm skin strips from each of two 6 cm wounds (one experimental, one control) per rat (Mustoe *et al.*, 1987). For histological analysis, strips were taken from the end quadrants of each wound and were immediately placed in phosphate-buffered formalin (10%). Tissues were stained with haematoxylin-eosin.

Statistics

Statistical analysis was carried out with Student's *t* test.

Materials

MCDB131 was a product of Kurorera Corp (Tokyo, Japan). FBS (Lot No. 37k0325) was purchased from GIBCO Laboratories (Grand Island, NY, U.S.A.). Collagen solution for the wound healing model was from Böttger Corp. (Berlin, Germany). Other materials for HUVEC culture and tube formation were as reported (Morisaki *et al.*, 1985; Tezuka *et al.*, 1993). Imulyse tPA, Imulyse PAI-1 and Spectrolyse were purchased from Biopool AB Corp. (Umeå, Sweden). Saponin was separated and purified as described by Kitagawa *et al.* (1989) and supplied by The Conference on Drug Ginseng. In brief, the air-dried root of Ginseng *Radix rubra* was extracted with 80% aqueous methanol and the extract subjected to ethylacetate/water separation. The water phase was extracted with butanol and the extract was adsorbed to a SEP-PAK C_{18} column (Waters, Milford, MA, U.S.A.). After washing with 20% methanol, saponin was eluted with 100% methanol. This

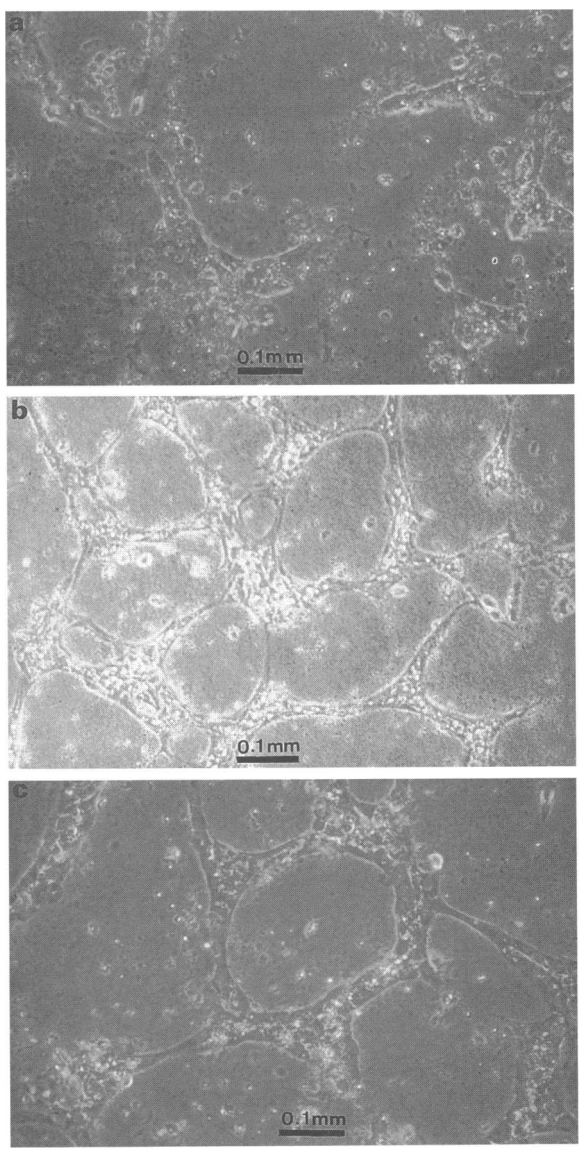

Figure 1 Phase-contrast micrographs of tube formation. Human umbilical vein endothelial cells were incubated in a three-dimensional space for two days in the absence (a) or presence of 10 ng ml^{-1} bFGF (b) or 100 μg ml^{-1} saponin (c). Calibration bar = 0 mm.

saponin contained more than twenty ginsenosides with similar structures (Kitagawa *et al.*, 1983; Yamamoto, 1988).

Results

Effects of saponin on tube formation

Two days after seeding, HUVEC formed capillary-like networks following stimulation with 10 ng ml^{-1} of the angiogenic factor bFGF (Figure 1b). Very few complete network formations occurred in the absence of angiogenic factors (Figure 1a). Networks similar to those seen with bFGF were formed in the presence of 100 μg ml^{-1} saponin (Figure 1c). Tube formation was quantitatively estimated by measuring the tube length of the capillary-like networks of three microscopic fields. Figure 2 shows that 1–100 μg ml^{-1} of saponin stimulated tube formation in a dose-dependent manner, and this stimulation was statistically significant in the range of 10–100 μg ml^{-1}. Saponin at 100 μg ml^{-1} was as effective as 10 ng ml^{-1} bFGF in stimulating tube formation [tube length with bFGF was 6.79 ± 0.99 ($n=6$, mean ± s.d. mm/field)].

Effects of saponin on tPA and PAI-1 secretion

Plasminogen activators are important as proteases for angiogenesis, and tPA is the major activator in HUVEC (Mawatari *et al.*, 1991). HUVEC secreted tPA into the medium as determined at 4–24 h (data not shown). After 24 h incubation, saponin (10–100 μg ml^{-1}) significantly stimulated the secretion of tPA immunoreactivity (Figure 3). The effective concentrations of saponin for the secretion of tPA immunoreactivity were similar to those that stimulated tube formation.

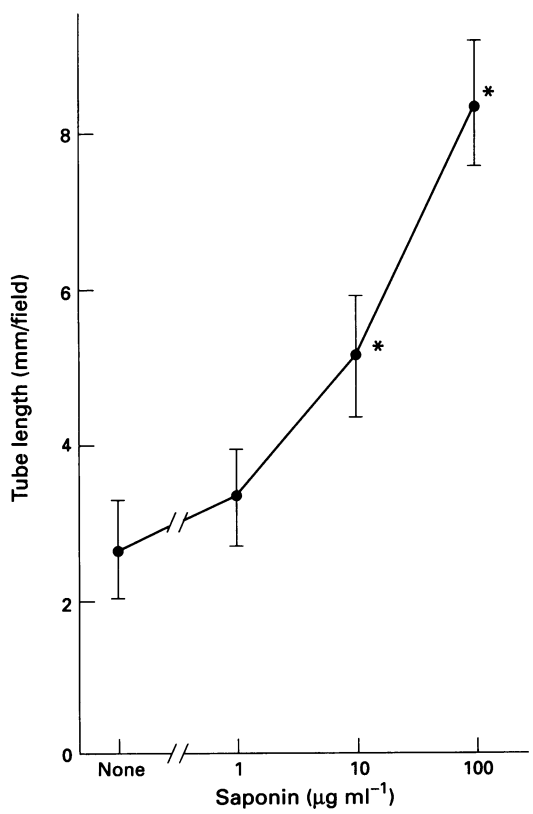

Figure 2 Effects of saponin on the tube formation by human umbilical vein endothelial cells (HUVEC). HUVEC were incubated in a three-dimensional space for two days with various concentrations of saponin. The vertical line shows the tube length formed. Values are mean ± s.d. ($n=9$, 9 cultures from three different strains of HUVEC). *$P<0.01$ (vs. None, i.e. control).

A balance of plasminogen activators (tPA and urokinase-type PA) and inhibitors is important for the actual display of the activities of the plasminogen activators. PAI-1 is important, as such an inhibitor (Eriksson *et al.*, 1988). For that reason, the secretion of PAI-1 immunoreactivity was measured in the same samples as those used for measurement of tPA immunoreactivity. Saponin only slightly increased the secre-

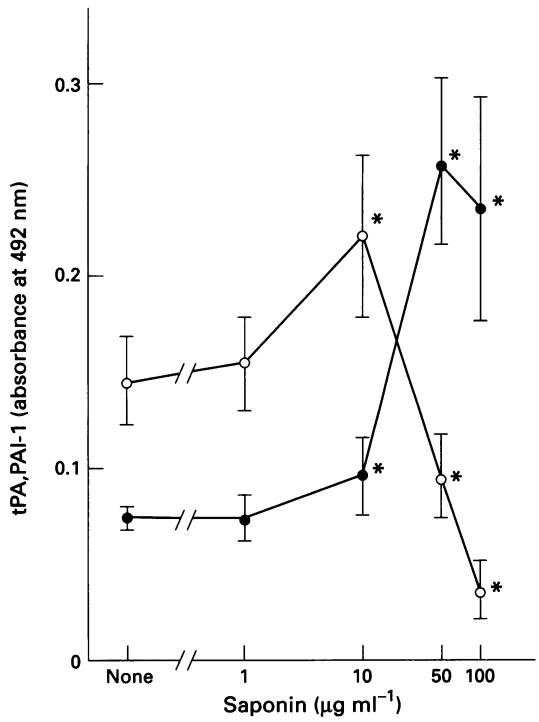

Figure 3 Effects of saponin on the secretion of tPA and PAI-1 immunoreactivities from human umbilical vein endothelial cells (HUVEC). Confluent HUVEC were incubated in MCDB131 medium for 24 h in the absence or presence of saponin. The conditioned media were collected and the immunoreactivities of tPA and PAI-1 were measured by ELISA: (●) tPA; (○) PAI-1. Values are mean ± s.d. ($n=9$, 9 cultures from three different strains of HUVEC). *$P<0.01$ (vs. None, i.e. control).

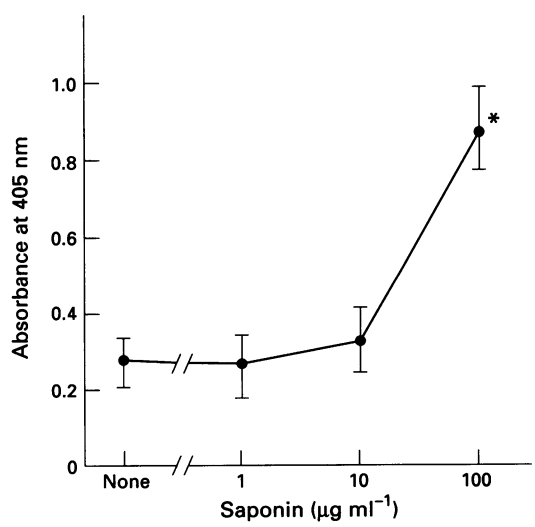

Figure 4 Effects of saponin on the secretion of tPA activity from human umbilical vein endothelial cells. tPA activity in the conditioned media for Figure 3 was measured. Values are mean ± s.d. ($n=9$). *$P<0.01$(vs. None, i.e. control).

tion of PAI-1 immunoreactivity at 10 μg ml^{-1}, but at higher concentrations significantly inhibited it, to the extent of about one-fourth that of control at 100 μg ml^{-1}. These results suggest that saponin changes the balance of activation/inhibition of protease to create a predominance of activation over inhibition especially at the range of 50–100 μg ml^{-1}. Figure 4 shows the tPA activity in the conditioned medium during 24 h incubation. Saponin slightly, but not significantly, increased tPA activity at 10 μg ml^{-1}. However, 100 μg ml^{-1} saponin significantly increased the activity to more than three times that of the control.

Effects of saponin on the proliferation of HUVEC

HUVEC did not grow without bFGF in MCDB131 containing 10% FBS either in the absence or presence of saponin (the cell number of HUVEC incubated with MCDB131 containing 10% FBS and 0–100 μg ml^{-1} of saponin was about 0.2×10^{-4}/well at day 7). HUVEC grew well in the growth medium containing 10 ng ml^{-1} bFGF (Figure 5). The addition of 1–100 μg ml^{-1} saponin to the growth medium increased the growth only slightly by 10–28% at day 2 and by 3–17% at day 7. There was no clear concentration-dependent response in this increase. These data indicate that saponin itself has no mitogenic effect on HUVEC and that it stimulates cell proliferation only slightly in the presence of an EC growth factor, bFGF.

Effects of saponin on the migration of HUVEC

When saponin was added to the lower chambers, it stimulated the migration of HUVEC in a dose-dependent manner (significant at 10–100 μg ml^{-1}) (Figure 6). The effective concentrations of saponin were similar to those that stimulated tube formation. The activity at 100 μg ml^{-1} was about three times that of control, and the effect was similar to that of the optimal concentration (10 ng ml^{-1}) of bFGF [317±48 cells/HPF(n=6), mean±s.d.].

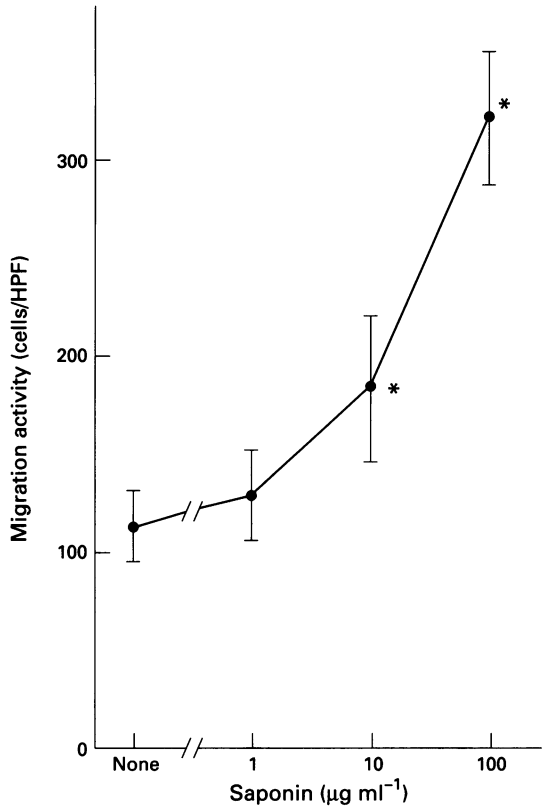

Figure 6 Effects of saponin on the migration of human umbilical vein endothelial cells (HUVEC). HUVEC were incubated in Boyden's chamber for 6 h and cells migrating through the membrane pores were counted. Saponin was added to the lower chamber. Values are mean±s.d. (n=9, 9 cultures from three different strains of HUVEC). *P<0.01 (vs. None, i.e. control).

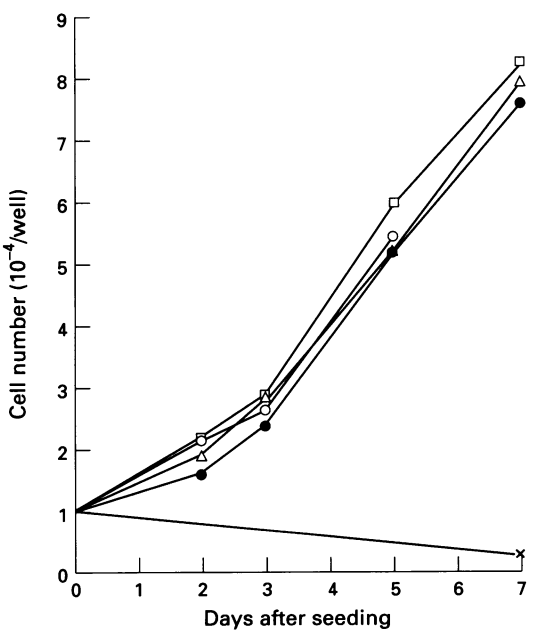

Figure 5 Effects of saponin on the proliferation of human umbilical vein endothelial cells (HUVEC) as a function of incubation time. HUVEC were seeded at 2×10^4/well and cell numbers were counted 6 h after seeding (day 0). Then cells were incubated in MCDB131/10% FBS in the absence (x) or presence (●) of 10 ng ml^{-1} bFGF. HUVEC were also incubated with MCDB131/10% FBS/10 ng ml^{-1} bFGF in the presence of 1 (△), 10 (○) and 100 (□) μg ml^{-1} saponin. Incubation was carried out until day 7. Each point shows the mean of three determinants. Standard deviations were less than 20% of each mean.

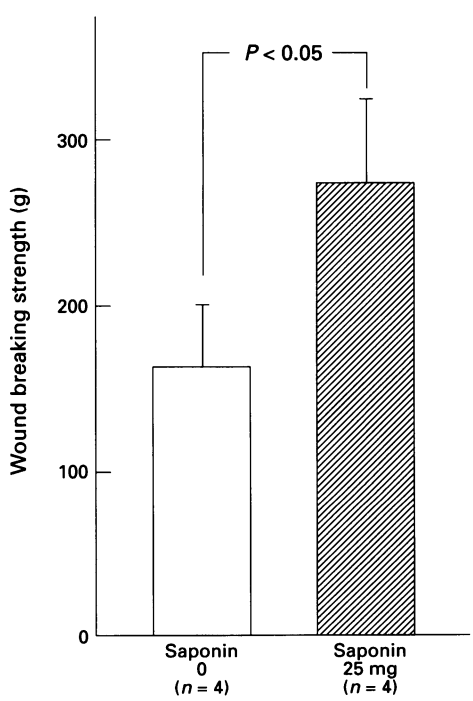

Figure 7 Effects of local administration of saponin on wound healing of diabetic rats. Skins of insulin-dependent diabetic rats were incised and 25 mg saponin in 1 ml of 0.3% collagen (hatched column) or vehicle (open column) was administered to the wound. The breaking strength was measured 7 days later. Columns are mean±s.d. (n=4, four animals with two wounds each).

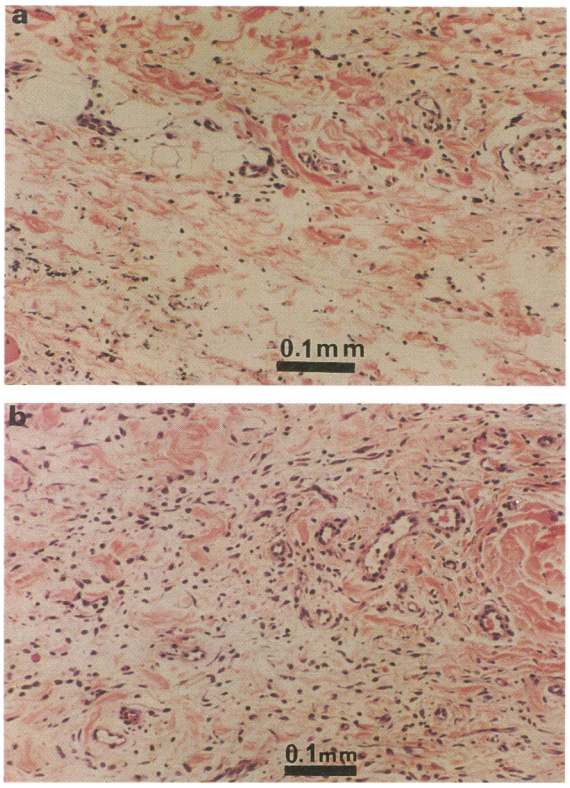

Figure 8 Micrographs of wounds of diabetic rats 7 days after causing wound. See the legend for Figure 7. (a) Control; (b) experimental. Haematoxylin-eosin staining. Calibration bar = 0.1 mm.

Effects of saponin on wound healing and in vivo *angiogenesis*

The breaking strength of skin incisional wounds was measured at 7 days after wounding. The experiment was repeated twice, and a typical experiment is shown in Figure 7. The breaking strengths for experimental incisions (275 ± 65 g/wound, $n = 4$) were significantly ($P < 0.05$) higher than those for control (164 ± 36 g/wound, $n = 4$). Histologically, microvessels were more frequently observed in the experimental incisions than in control (Figure 8). The granulation tissues in association with infiltration of inflammatory cells were well developed in the experimental incisions compared with control.

Discussion

The present study clearly showed that saponin stimulates angiogenesis *in vitro* and *in vivo* , mainly through (1) enhancement of tPA secretion together with supression of PAI-1 secretion from HUVEC, and (2) migration of HUVEC. It was also observed that acceleration of wound healing *in vivo* by saponin is associated with enhanced angiogenesis. Accordingly, saponin may indeed be an angiogenic factor.

The tube formation system used in this study involves 4 steps that are thought to take place during angiogenesis *in vivo*, that is, destruction of the basement membrane by EC, their subsequent proliferation, migration and actual tube formation. Destruction of the basement membrane *in vivo* by EC may correspond to destruction of the collagen gel in our *in vitro* system. The migration of EC must play an important role in the three-dimensional network formation in the collagen gel. Although the tube formation system must also involve the actual process of tube formation, this step is difficult to analyse separately. On the other hand, EC proliferation may not be essential for tube formation, as the cells were already confluent at the beginning of the assay and the incubation time was relatively short, only 48 h. Thus this system is suitable for the screeening not only of the type 1 but also of the type 2 angiogenic factors mentioned below.

The endogenous angiogenic factors so far reported can be classified into two categories: Type 1, factors which stimulate the proliferation of EC as well as protease secretion and migration such as FGF (Esch *et al.*, 1985), vascular endothelial growth factor (VEGF) (Ferrara *et al.*, 1992), and transforming growth factor-α (TGF-α) (Schreiber *et al.*, 1986); Type 2, factors which do not stimulate proliferation but enhance angiogenesis in certain systems such as tumour necrosis factor (TNF) (Frater-Schroder *et al.*, 1987) and transforming growth factor-β (TGF-β) (Roberts *et al.*, 1986). As the effect of saponin on HUVEC proliferation is not prominent, it should be classified among the type 2 angiogenesis factors. If this were the case, saponin may act in cooperation with endogenous type 1 angiogenic factors *in vivo* because EC proliferation is essential for *in vivo* angiogenesis.

The destruction of the extracellular matrix is the initial step of angiogenesis and requires proteases. Among these, PA and matrix metalloproteinase (MMP) (Cawston, 1986) secreted from EC are important for angiogenesis. Besides PA converting plasminogen to plasmin which in turn digests matrices, it also activates the inactive form of MMP. Thus PA plays an especially important role in angiogenesis. Not only proteases but also inhibitors are secreted from EC, such as PAI-1 for tPA and tissue inhibitor of metalloproteinase (TIMP) for MMP (Cawston, 1986). Of importance, then, for the protease activities is the balance of proteases and inhibitors. Saponin stimulates secretion of tPA and inhibits that of PAI-1 at higher concentrations, indicating a tilt of the balance of proteolysis to its inhibition. The tPA activity at 10 μg ml^{-1} of saponin was not significantly increased (Figure 4) in spite of the increased secretion of tPA immunoreactivity at the same concentration of saponin (Figure 3). This may be explained by the fact that PAI-1 secretion was increased at that concentration of saponin (Figure 3).

Whether the effects of saponin on tube formation or its steps (protease and its inhibitor secretion, and migration) in HUVEC are direct or indirect cannot be determined by the present study. The possibility that saponin enhances the expression and secretion of type 1 angiogenic factor(s) such as FGF and indirectly stimulates tube formation is small because: (1) saponin has little effect on HUVEC proliferation whereas FGF stimulates it; (2) FGF lacks a signal sequence for secretion and is primarily cell-associated (Burgess & Macaig, 1989); (3) VEGF and TGF-α are related to transformed cells and are not secreted from normal EC such as the HUVEC used in this study. On the other hand, the possibility that saponin indirectly stimulates tube formation through secretion of type 2 angiogenic factor(s) such as TNF-α and TGF-β cannot be ruled out because these cytokines could be expressed in, and secreted from EC. However, this possibility becomes rather remote as far as these two cytokines are concerned because they inhibit EC proliferation but saponin did not. Finally, this is supported by the relatively short incubation time in the migration assay during which it may be difficult for substantial amounts of autocrine angiogenic factor(s) to be expressed, secreted, and then act on cells. Thus it may indeed be true that saponin directly stimulates tube formation and its steps.

As with other Chinese medicines, the active component of Ginseng *Radix rubra*, saponin, consists of many ginsenosides. It is unclear which of these is (are) responsible for the angiogenic effect. The major ginsenosides of saponin are a group of dammarane saponins which have a similar structure to cholesterol and steroid hormones (Yamamoto, 1988). Sterols affect PA production in EC, as for example, the plant sterols, sitosterol and fucosterol, induce a 3 to 7 fold increase in the production of PA activity, whereas corticosteroids (dexamethasone, cortisone, methylprednisolone, hydrocortisone) decrease PA production (van Hinsbergh, 1988). Therefore, ginsenoside(s) resembling sterols by its structure may be responsible for the angiogenic activity of saponin.

We have shown that saponin inhibits the proliferation and

migration of rabbit cultured aortic smooth muscle cells and that each ginsenoside exerts a different effect on them (Saito *et al.*, 1987; Koyama *et al.*, 1992b). The effects of saponin on HUVEC are different from or even opposite to those on smooth muscle cells as far as proliferation and migration are concerned, suggesting that the effects are specific for EC.

In summary, saponin stimulates tube formation mainly through modification of the balance of the protease/protease inhibitor secretion from HUVEC and the enhancement of the migration of HUVEC. The local administration of saponin helped wound healing in the intractable wound healing model with increased *in vivo* angiogenesis. These results point to the clinical usefulness of saponin in the treatment of intractable ulcers such as diabetic ulcer. The mechanism(s) and active component(s) of saponin will need to be explored further.

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