Effects of enoxaparin and onion extract on cytokine production in skin fibroblasts

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Abstract

Hypertrophic scars and keloids are fibroproliferative disorders of the skin which present serious problems in esthetic dermatology and dermatosurgery. It is believed that an increased secretion of cytokines and growth factors including IL-6, VEGF and TNF-α plays a pivotal role in the pathogenesis of hypertrophic scars and keloids. Prevention and treatment of problematic scars includes topical preparations with heparin and onion extract. Nevertheless, the effectiveness of these agents is still controversial. Here we show that enoxaparin and onion extract reduced the level of both IL-6 and VEGF in the culture of fibroblasts. This effect was the strongest in 20 μg/ml of enoxaparin and 50 μg/ml of onion extract. Our results may add to the explanation of beneficial effects of enoxaparin and onion extract on keloid scars in vivo.

Key words: *LMW heparin, enoxaparin, onion, keloids, cytokines.*

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Introduction

Hypertrophic scars and keloids are fibroproliferative disorders of the skin, resulting from abnormal process of healing response after skin injury. Keloids present serious problem in esthetic dermatology and dermatosurgery [1]. It is believed that an increased secretion of cytokines and growth factors play a pivotal role in the abnormal growth of keloid fibroblasts [2, 3]. As compared to nonkeloid fibroblasts, central keloid fibroblasts have been shown to secrete increased levels of IL-6, VEGF (Vascular Endothelial Growth Factor) and TNF-α (Tumor Necrosis Factor). It is believed that these cytokines may contribute to pathogenesis of keloids and hypertrophic scars [2, 4, 5]. In the prevention and treatment of problematic scars very often topical preparations are used. These drugs are ease to use, comfortable, noninvasive and cheap which makes them very popular among patients [6]. Topical preparations contain different active agents. For example ointments containing onion extract alone (Mederma, Merz Pharmaceuticals, USA) or with heparin and allantoin (Cepan, Unia, Poland; Contractubex, Merz, Germany), are very popular in Europe. Nevertheless, the effectiveness of these agents in the treating of keloids and scars is still controversial [1, 7, 8]. The aim of this study was to examine the effect of dry onion extract and low molecular weight (LMW) heparin (enoxaparin) on the cytokine production. LMW heparin has been chosen as a better candidate for cutaneous penetration than unfractionated heparin.

Materials and Methods

Chemicals

The peeled fresh onions bulbs (*Allii cepae bulbus* var. Armstrong) were extracted by maceration with 95% (v/v) ethanol (1:1) for 5 days. The liquid extract was filtered and concentrated with a rotary vacuum evaporator to reduce ethanol content to approximately 20% and was finally spray-dried (B-290 Büchi spray-dryer, Flawil, Switzerland). The extract contained flavonoids (mainly spiraeoside) and saponins as the main active compounds (data not shown).

Low-molecular-weight heparin (enoxaparin) was a kind gift from Welding (Hamburg, Germany). All solutions used

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in experiments were prepared with phosphate buffered saline (PBS) solution (pH 7.4) in sterile conditions.

Culture conditions

Human fibroblasts cell line (46 BR.1N) were obtained from European Collection of Cell Cultures (ECACC). Cell line was originally derived from skin individual with hypogammaglobulinaemia and was transformed with the plasmid pSV3neo, and now is immortal. Fibroblast were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), with 4500 mg/L glucose, 584 mg/L L-glutamine, sodium pyruvate, and sodium bicarbonate. Medium contained 15% FCS and was supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich). Cells were cultured in humidified atmosphere with 5% CO_2 at 37°C. Cells for experiments were seeded at a density $1 \times 10^4/cm^2$ in 60 mm diameter (growth surface area 21 cm^2) culture dishes (Corning) and grown for 24 h in the medium with FCS (15%). Thereafter cells were washed in PBS, medium was changed to DMEM without serum and chemicals were added obtaining appropriate final concentration. Cells were cultured in the stimulation conditions for 48 h. After this time the supernatant of each plate was removed and stored frozen (-70°C) until analyzed for IL-6, VEGF, TNF-α.

Measurement of cytokines

Concentrations of interleukin IL-6, VEGF and tumour necrosis factor α (TNF-α) in the supernatants were determined using cytometric bead array (CBA) flex set according to the manufacturer's protocol (BD, Franklin Lakes, NJ, USA). In brief, 50 µl of all samples and serial dilutions of cytokine standards were incubated in multiplexed antibody-conjugated beads for 1 h. Thereafter, the PE detection reagent was added, and samples were incubated for additional 2 h, washed, and detected within the range 10–2500 pg/ml in a LSRII flow cytometer (BD). The data were analyzed with FCAP Array Software (BD). The theoretical detection limits for VEGF, IL-6 and TNF-α were 4.5 pg/ml, 1.6 pg/ml, 0.7 pg/ml respectively.

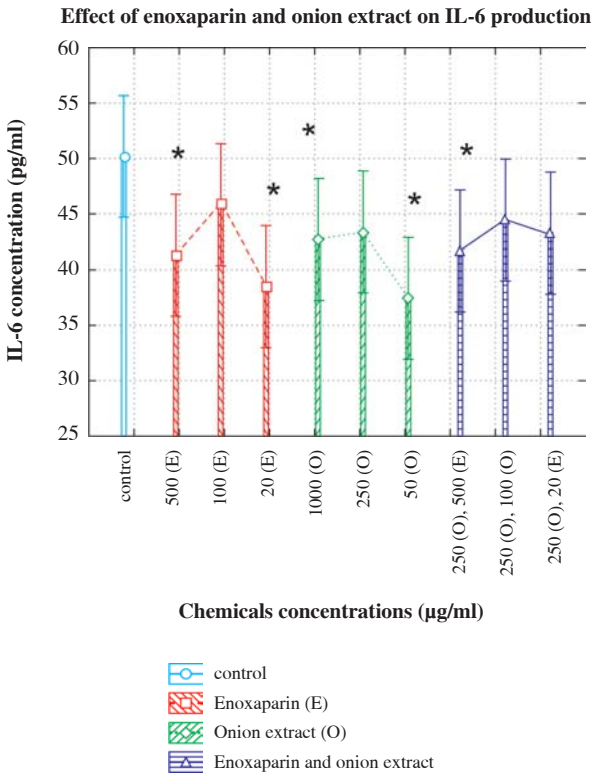
Statistics

Data were computed using the software Statistica 8.0 (Statsoft, Poland). The analysis of data obtained from the cytokine concentrations was based on the ANOVA test as indicated by data distribution. When more than two groups were evaluated, analysis of variance (ANOVA) followed by lowest significant differences post hoc tests were performed. P<0.05 was recognized as significant.

Results

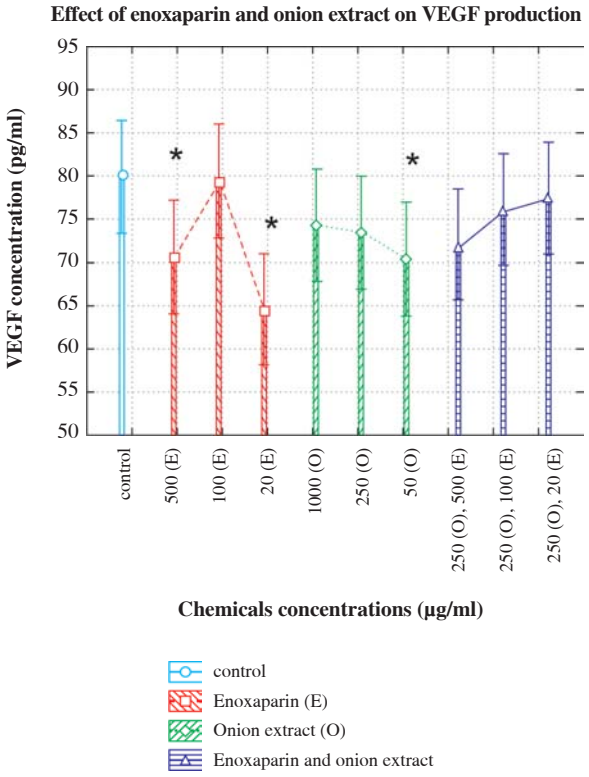
Analysis of the supernatant cytokine profile showed that fibroblasts used in the experiments and cultured for 48 h produced basal level of IL-6 and VEGF (50.2 ± 5.6 and 79.9 ± 7.2 pg/ml respectively). The basal concentration of TNF-α in media was below the theoretical detection limit of assay (0.7 pg/ml). Treatment of the cells with enoxaparin reduced the levels of both IL-6 and VEGF in the supernatants. Analysis revealed reduction in the level of IL-6 which was significant for 20 µg/ml ($p<0.01$) and 500 µg/ml of enoxaparine ($p<0.01$) (Figs. 1 and 2). There was no significant difference in the concentration of IL-6 in cells treated with enoxaparin in 100 µg/ml compared to non-treated cells. The levels of VEGF declined in the presence of enoxaparine in 20 µg/ml and 500 µg/ml ($p<0.03$ and $p<0.05$ respectively). Additionally, there was significant difference between concentration of VEGF in cells treated with enoxaparine in 500 µg/ml and 100 µg/ml ($p<0.01$).

Onion extract in the concentrations 50 µg/ml ($p<0.03$) and 1000 µg/ml inhibited significantly the production of IL-6 ($p<0.03$). The level of VEGF dropped down only in the cultures treated with onion extract in the concentration 50 µg/ml ($p<0.05$). This effect was significant also in comparison to 100 µg/ml of enoxaparin ($p<0.05$).



*Different from non-treated cells (control), p < 0.05

Fig. 1. IL-6 levels in the supernatants of cultures of fibroblast treated with enoxaparin and onion extract. Experiments were performed in triplicate, expressed as a mean concentration of IL-6 in fibroblast supernatants ± SD (pg/ml)



*Different from non-treated cells (control), p<0.05

Fig. 2. VEGF levels in the supernatants of cultures of fibroblast treated with enoxaparin and onion extract. Experiments were performed in triplicate, expressed as a mean concentration of VEGF in fibroblast supernatants ± SD (pg/ml)

Among different concentrations of onion extract and enoxaparine added together, only combination of 250 µg/ml of onion extract and 500 µg/ml of enoxaparine showed significant reduction of IL-6 production ($p<0.03$). Other combinations of these two compounds did not reveal any effect on cytokines production.

Neither enoxaparin nor onion extract or combinations of these two affected production of TNF-α in fibroblasts (data not shown).

Discussion

A growing body of evidence suggests the involvement of cytokines and growth factors in the development and maintenance of keloids and hypertrophic scars. Here we studied the effect on enoxaparin and onion extract on IL-6, VEGF and TNF-α production. Heparins are highly-sulfated, negatively charged glycosaminoglycans which are administered mainly as anticoagulants. In addition to their well-known anticoagulant effects, heparins also bind to growth factors (such as VEGF) and extracellular matrix proteins and affect proliferation and migration of cancer cells. Moreover, heparins can affect angiogenesis, cancer cell oncogene expression and keloids fibroblast proliferation [9, 10]. Mechanism of action of onion extract is still unknown. Onions are known to contain a lot of chemical compounds, such as flavonoids (quercetin and its glucosides), saponins, or sulphur-containing compounds (alkyl cysteine sulphoxides and associated breakdown products, formed during tissue damage) and are believed to have anticarcinogenic, antiproliferative and antibiotic properties [11].

Here we showed that enoxaparin (LMWH, Low Molecular Weight Heparin) and onion extract inhibit IL-6 and VEGF production and do not induce TNF-α production. IL-6 is a proinflamatory cytokine that play an important role in controlling the immune system. IL-6 has been shown to induce the proliferation of human fibroblasts and collagen synthesis [12, 13]. The elevated level of IL-6 in keloids suggests that the cytokine contributes to the formation of this abnormal structure. Due to this fact reduction in the level of IL-6 may be more directed to keloid tissue than normal tissue. Additionally, the inhibition of IL-6 activity may affect fibroblast proliferation and moderate skin inflammation [14].

One of the factors important in the pathogenesis of keloids is VEGF. This factor is considered to be instrumental for endothelial cells to the formation of new sprout and affects temporary fibrin matrix formation, endothelial cell migration and proliferation [15]. Density of the caplillary network in hypertrophic scar tissue is higher than in normal skin. Moroever, the vessels are often dilated which may suggest ongoing neovascularization [16]. Therefore, reduction in the level of VEGF in the skin may affect development and expansion of keloids.

Surprisingly, the treatment of fibroblasts with enoxaparin and onion extract together resulted in low response of cells. Undoubtedly, this phenomenon requires more precise further studies.

Possible mechanism of the action of onion compounds on reduction of cytokines level may be connected with modulation of intracellular signaling pathway. It has been proven than some onion substances may inhibit NF-kappaB activity [17]. Enoxaparin and onion extract may also inhibit proliferation of cells [18] and possess the ability to bind different growth factors, such as VEGF [9, 10].

Since there is a possibility of different response of immortalized fibroblast cell line and fibroblasts isolated from keloids, further studies should take into consideration experimental model for drugs testing. Taken together, these data may offer the link between the beneficial effect of enoxaparin and onion extract and keloid scars formation *in vivo*. However, it is very likely that variety of other cytokines and factors contribute to the pathogenesis of keloids. Thus, further studies are required to establish whether the substances are beneficial in the treatment of keloids.

Acknowledgments

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