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Heparin Modulates the Growth and Adherence and Augments the Growth-Inhibitory Action of TNF-α on Cultured Human Keratinocytes

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Abstract Previous works suggest the involvement of mast cells in the epithelialization of chronic wounds. Since heparin is a major mediator stored in the secretory granules of mast cells, the purpose of this work was to elucidate the function of heparin in epithelialization using in vitro culture models. For this, low- and high-calcium media in monolayer and epithelium cultures of keratinocytes were used. Also, an assay based on keratinocyte adherence onto plastic surface was used as well. Heparin (0.02–200 μg/ml) inhibited keratinocyte growth in a non-cytotoxic and dose-dependent manner in low- and high-calcium media, Keratinocyte-SFM® and DMEM, in the absence of growth factors and serum. Also, heparin inhibited the growth of keratinocyte epithelium in the presence of 10% fetal calf serum and DMEM. Instead, in the presence of Keratinocyte-SFM and growth factors, heparin at 2 μg/ml inhibited the growth by 18% but at higher heparin concentrations the inhibition was reversed to baseline. TNF-α is another preformed mediator in mast cell granules and it inhibited keratinocyte growth in monolayer and epithelium cultures. Interestingly, heparin at 2–20 μg/ml augmented or even potentiated this growth-inhibitory effect of TNF-α. The association of TNF-α with heparin was shown by demonstrating that TNF-α bound tightly to heparin-Sepharose chromatographic material. However, heparin could not augment TNF-α-induced cell cycle arrest at G0/G1 phase or intercellular adhesion molecule-1 expression in keratinocytes. In the cell adherence assay, heparin at 2 μg/ml inhibited significantly by 12–13% or 33% the adherence of keratinocytes onto the plastic surface coated with fibronectin or collagen, respectively, but this inhibition was reversed back to baseline at 20 or 200 μg/ml heparin. Also, heparin affected the cell membrane rather than the protein coat on the plastic surface. In conclusion, heparin not only inhibits or modulates keratinocyte growth and adherence but it also binds and potentiates the growth-inhibitory function of TNF-α. J. Cell. Biochem. 92: 372–386, 2004.

Key words: mast cell; mediator; epithelialization; fibronectin; collagen; proteoglycan; cytokine

In the normal skin, mast cells are predominantly located in the upper dermis but only occasionally inside or in contact with the epidermis. However, the number of mast cells is increased in the papillary dermis and in contact with the epidermis in chronic inflammatory skin diseases and in leg ulcers [Green et al., 1977; Bolton and Montagna, 1993; Harvima et al., 1993; Huttunen et al., 2000]. In addition, mast cells have been linked to skin diseases leading to dermal-epidermal separation and blister formation [Briggaman et al., 1984; Kaminska et al., 1999a,b; D'Auria et al., 2000]. Therefore, mast cells and their mediators are assumed to be involved in the epithelialization of chronic wounds and blister bases.

The majority of cutaneous mast cells belong to the MC_{TC} type containing tryptase, chymase carboxypeptidase, and a cathepsin G-like protease in their secretory granules [Irani et al.,

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1989; Harvima et al., 1993; Weidner and Austen, 1993]. In addition, mast cells are a rich source for histamine, heparin proteoglycan, chondroitin sulphate E, and cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-4 [Harvima and Schwartz, 1993]. In fact, mast cells are the predominant cell type in the healthy dermal skin producing, storing and secreting TNF-α [Walsh et al., 1991; Ackermann and Harvima, 1998; Gibbs et al., 2001].

Previously, preformed mast cell mediators, such as histamine, heparin, and TNF-α, have been found to inhibit keratinocyte growth in vitro [Pillai et al., 1989, 1994; Symington, 1989; Piepkorn et al., 1994; Huttunen et al., 2001] suggesting that mast cells could control epidermal growth. TNF-α can inhibit the proliferation of cultured human keratinocytes by inducing differentiation [Pillai et al., 1989] or cytostasis [Symington, 1989]. Heparin can inhibit keratinocyte proliferation without inducing terminal differentiation [Piepkorn et al., 1994; Pillai et al., 1994]. In addition, heparin at 0.1–0.3 mg/ml concentration has been reported to significantly inhibit keratinocyte outgrowth from epidermal biopsies (suction blister roof) placed on contracted fibroblast-collagen lattice [Imaizumi et al., 1996]. Also, heparin efficiently binds mast cell tryptase and chymase which can degrade the basement membrane and induce dermal-epidermal separation [Briggaman et al., 1984; Kaminska et al., 1999a,b]. Soluble tryptase-heparin proteoglycan complexes are likely to reach epidermal keratinocytes after being released from mast cells [Kivinen et al., 2001]. Furthermore, heparin and other proteoglycans can bind cytokines and growth factors, such as basic fibroblast growth factor, amphiregulin, and keratinocyte growth factor, and thereby modify their growth inducing effects on keratinocytes and epithelial cells [Shipley et al., 1989; Ruoslahti and Yamaguchi, 1991; Piepkorn et al., 1994; Putnins et al., 1996; Schönherr and Hausser, 2000]. Keratinocytes adhere avidly to a substratum containing fibronectin [O'Keefe et al., 1985; Kaminska et al., 1999a]. However, fibronectin is susceptible to degradation by mast cell tryptase. On the other hand, fibronectin contains several heparin-binding sites which can affect cell adherence [Wilke et al., 1991; Kaminska et al., 1999a; Mostafavi-Pour et al., 2001]. Thus, heparin not only affects cell growth but it may modulate cell adherence of keratinocytes as well.

The growth of keratinocytes in vitro has mostly been studied in low-calcium culture conditions using proliferating keratinocytes grown as monolayer. The monolayer growth model with unphysiologically low calcium concentration and exogenous growth factors, such as bovine pituitary extract, may not well relate to the growth of the epidermis in vivo, for example, in the epithelialization of wounds and blister bases. Thus, an in vitro growth model in high-calcium conditions in the presence and absence of 10% serum was used in this study and compared to a monolayer model for investigating the effects of heparin on keratinocyte growth. Since heparin can interact with different growth factors and cytokines [Ruoslahti and Yamaguchi, 1991; Schönherr and Hausser, 2000] and different potent mediators are functioning simultaneously after their release from mast cells in skin inflammatory conditions the combined effect of heparin and TNF-α on keratinocyte growth was also studied. Furthermore, a cell adherence assay was employed in studies where the effect of heparin on keratinocyte adherence onto plastic surface was investigated.

MATERIALS AND METHODS

Chemicals

Heparin sodium salt was purchased from Fluka BioChemica (Buchs, Germany), pure plasma-fibronectin and collagen-S 3 mg/ml solution (collagen types I and III from calf skin) from Boehringer Mannheim GmbH (Mannheim, Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and bovine serum albumin (BSA) from Sigma (St. Louis, MO), and recombinant human TNF-α (1.1×10^6 U per 10 μg) from R&D Systems Europe Ltd (Oxon, UK). Heparin-Sepharose® CL-6B was purchased from Pharmacia LKB (Uppsala, Sweden). Hoechst 33258 (bisbenzimide) and propidium iodide are products of Molecular Probes Europe BV (Leiden, The Netherlands). Keratinocyte-SFM medium, Dulbecco's modification of Eagle's medium (DMEM), fetal calf serum (FCS), Dulbecco's phosphate-buffered saline (PBS), and penicillin-streptomycin solution were purchased from Gibco BRL (Life Technologies Ltd, Paisley, UK). Mouse monoclonal anti-involucrin antibody was obtained from Novocastra Laboratories (Newcastle upon Tyne, UK).

Cultivation of Keratinocytes

Proliferating keratinocytes were cultured from human foreskin specimens using Keratinocyte-SFM serum-free medium supplemented with epidermal growth factor (EGF), bovine pituitary extract (BPE), 100 U/ml penicillin, and 100 μg/ml streptomycin as described in detail previously [Harvima et al., 1999; Huttunen et al., 2001]. In every experiment, third to eight passage keratinocytes with a viability of over 90% were used.

Preparation of Epithelium and Monolayer of Keratinocytes for Cell Growth Studies

Culture models for monitoring keratinocyte growth and migration on cell culture plastic were developed. First, four adjacent and thick-walled stainless steel cylinders (inner diameter 6 mm) were placed on the bottom of each well of an uncoated 6-well plate (Falcon, Becton Dickinson, Plymouth, UK) or a fibronectin-coated 6-well plate (Biocoat®, Becton Dickinson). Since the metallic cylinders were sufficiently heavy and thick they were sealed onto the plastic surface without marked escape of keratinocytes. The wells and cylinders were equilibrated in 5 ml of complete (with EGF and BPE) or incomplete (without EGF and BPE) Keratinocyte-SFM medium at 37°C and 5% CO_2. Thereafter, about 30,000 proliferating keratinocytes were added cautiously into each cylinder, and the cells were allowed to adhere onto the uncoated or fibronectin-coated plastic surface overnight. Practically complete confluence of keratinocytes was reached on the following day.

To induce differentiation and epithelium development of monolayer keratinocytes in high-calcium conditions, complete Keratinocyte-SFM medium was changed to 5 ml of 10% FCS, DMEM, 100 U/ml penicillin and 100 μg/ml streptomycin, and the metallic cylinders were removed. DMEM with serum is a well characterized medium for culturing and preparing keratinocyte epithelium—even for clinical purposes with leg ulcer and burn patients [Pittelkow and Scott, 1986; Harvima et al., 1999]. After 1–2 h, modulating agents were added in varying concentrations and combinations to the wells as described in Results. The medium and modulating agents were changed every 1–3 days until the epithelial border was near the wall of the well. The cultures were stopped by removing the medium and subsequently 4% formaldehyde was added to the wells overnight. After fixation, the epithelium was stained with Mayer's hematoxylin overnight.

To study the growth and migration of proliferating keratinocytes as monolayer, the Keratinocyte-SFM medium was replaced with a fresh one after the cells were adhered. Then, the cylinders were removed, and modulating agents were added. The medium and agents were changed as described above.

The growth area of keratinocytes was measured with digital image analysis as described [Huttunen et al., 2001]. The growth area was measured in triplicate for each well and averaged. The final results are given as the percentage of the growth in an unstimulated control well. The initial area of seeded cells was subtracted from the growth area before calculating the outcome of growth.

MTT-Analysis for the Detection of Cytotoxicity and Cell Viability

The modified MTT-analysis [Mosmann, 1983] was used to detect cell viability. Keratinocytes were seeded into the wells of a 96-microwell plate at the density of about 4,000 cells/well using complete Keratinocyte-SFM medium. The optimal cell density was confirmed by using varying numbers of cells. On the following day, the medium was replaced with a fresh one (complete or incomplete Keratinocyte-SFM or DMEM). The adhered keratinocytes were treated with modulating agents for 24 h and then 0.33 mg/ml MTT in incomplete Keratinocyte-SFM was added into the wells at 37°C and 5% CO_2 for 2 h. Thereafter, the MTT-solution was removed and the formed intracellular dye was solubilized by incubating with dimethyl sulfoxide for 15 min. The absorbance of the solution was measured at the wavelength of 550 nm using a micro-ELISA reader. The cultures and analyses were performed using six parallel wells and each experiment was performed at least twice.

Involucrin Expression in Keratinocytes

To study the possibility that heparin or TNF-α induces differentiation of keratinocytes, involucrin immunoreactivity was used as the differentiation marker [Fujimoto et al., 2000]. First, keratinocytes were seeded into the wells of a

4-well chamber slide (Nunc Lab-Tek™, Nunc, Roskilde, Denmark) in the presence of complete Keratinocyte-SFM. On the following day, the medium was replaced with a fresh one and 20 or 200 μg/ml heparin and/or 5,000 U/ml TNF-α were added into the wells. After 2 or 3 days, the culture was stopped by removing the medium followed by washing the cells with PBS. Subsequently, the cells were fixed in cold methanol for 10–15 min and finally stained immunocytochemically with anti-involucrin antibody (1:1,000 dilution) as described [Kivinen et al., 1999].

Keratinocyte Adherence Onto the Surface of Cell Culture Plastic

The wells of 24-well plates were either uncoated or coated with 250 μl of 50 μg/ml plasma-fibronectin in PBS overnight. Alternatively, 30 μl of 3 mg/ml collagen-S solution was spread evenly with a pipette on the bottom of wells, and the solution was allowed to dry up. After coating with fibronectin or collagen-S, non-specific binding was blocked by adding 1% heat-inactivated (30 min at 56°C) bovine serum albumin to the wells for 2 h. Then, the wells were washed twice with PBS and 1 ml of incomplete (or complete) Keratinocyte-SFM with varying concentrations of heparin was added into the wells. After equilibration at 37°C and 5% CO_2 for 30 min, about 65,000 proliferating keratinocytes in complete or incomplete Keratinocyte-SFM were added cautiously into the wells. The cells were allowed to adhere onto the plastic surface for 60 min followed by washing non-adherent cells away with PBS twice. The amount of DNA of adherent keratinocytes was measured as described [Otto, 1994]. Briefly, the DNA was dissolved in a buffer containing 8 M urea, 0.04% sodium dodecyl sulphate, 0.154 M NaCl, and 0.015 M sodium citrate, pH 7.0. After 60 min, the DNA was allowed to react with 0.5 μg/ml Hoechst 33258. Finally, the fluorescence was measured at the excitation wavelength of 355 nm and the emission wavelength of 460 nm. The interfering background of solutions and chemicals was excluded by using wells treated similarly but without cells. The relation between the DNA assay and the number of cells was studied: the fluorescence increased linearly when 10,000–100,000 keratinocytes were added into the wells in complete Keratinocyte-SFM medium. In addition, the DNA of adherent keratinocytes did not produce fluorescence in the absence of Hoechst 33258. The cultures and analyses were performed using quadruplicate wells and each experiment was repeated at least three times. The inter-well variation of fluorescence in quadruplicate wells was $6.4 \pm 3.9\%$ (mean $\pm$ SD, $n = 66$) based on the fluorescence values from 11 individual and randomly chosen experiments. Thus, the inter-well variation can be considered to be low allowing detection of relatively small changes in cell adherence.

CD54 (ICAM-1) Expression of Keratinocytes

CD54 expression was studied using flow cytometry (FACScan, Becton Dickinson, San Diego, CA) equipped with an argon ion laser (488 nm). About 96,000 keratinocytes/well were grown in 6-well plates overnight in complete Keratinocyte-SFM. On the following day, the medium was replaced with a fresh incomplete or complete Keratinocyte-SFM and 1,000 U/ml TNF-α and/or 20 μg/ml heparin were added into the wells. After incubation for 48 h, the cells were trypsinised and washed twice with FACS buffer containing PBS and 2% FCS. After washings, keratinocytes were suspended with 200 μl of FACS buffer and incubated with 10 μl of R-PE-conjugated monoclonal anti-CD54 antibody at +4°C for 20 min. Then, the keratinocytes were washed with 2 ml of FACS buffer, suspended with 600 μl of FACS buffer and analyzed. Fluorecence of R-PE was collected at 575 nm. The living cells were separated into $CD54^+$ and $CD54^-$ populations using unlabeled control cells, without TNF-α and heparin treatments. The effects of different treatments on CD54 expression were analyzed using this gating.

Propidium Iodide Method to Study Changes in Cell Cycle and DNA Content

Keratinocytes were seeded in 6-well plates using complete Keratinocyte-SFM. On the following day, the medium was changed to complete or incomplete Keratinocyte-SFM and 20 μg/ml heparin and/or 1,000 or 5,000 U/ml TNF-α were added to the medium for 1 day. Thereafter, the medium containing possible detached cells was collected to a test tube. The attached keratinocytes were released by incubating in 0.05% trypsin and 0.02% EDTA for 15–20 min. After inactivating trypsin with 10% FCS, the cell suspension was combined with

spontaneously detached cells. The final keratinocyte suspension was centrifuged and washed with D-PBS. For fixation, the cells were suspended in 0.5 ml of cold D-PBS and the cells were added cautiously to 5 ml of ice-cold 70% ethanol using continuous mixing of the solution. After a minimum fixation for 1 day, the cells were centrifuged and suspended in D-PBS, treated with 0.15 mg/ml RNase at 50°C for 1 h, and incubated in 16 μg/ml propidium iodide at 37°C for 2 h. Finally, the cells were analyzed by flow cytometry [Nicoletti et al., 1991; Wingens et al., 1998]. The experiment was performed twice.

Heparin-Sepharose Chromatography to Study the Binding of TNF-α to Heparin

In order to find out if heparin can bind TNF-α heparin-Sepharose CL-6B (Pharmacia) chromatography was used. The heparin-Sepharose column (1.5 cm × 3.5 cm) was equilibrated with a buffer containing 0.1 M NaCl and 10 mM 2(N-morpholino)ethanesulfonic acid (MES), pH 6.1. First, it was confirmed that only trace amounts of BSA (≥99% pure, heat-inactivated as described above) bind to the column from the equilibrating buffer containing 0.1% BSA. Thereafter, 1,000 U/ml TNF-α was added to the buffer containing 0.1% BSA, 0.1 M NaCl, and 10 mM MES, pH 6.1, and this solution was loaded into the column. BSA was included in the TNF-α solution to prevent non-specific binding and disappearance of TNF-α from the solution. Fractions (0.5 ml) were collected throughout the procedure. After loading, the column was washed with the equilibrating buffer and then eluted using the 10 mM MES buffer with increasing concentration of NaCl (up to 1 M NaCl). TNF-α in the collected fractions, containing 0.1% BSA, was analyzed by using a commercial ELISA kit (R&D Systems, Minneapolis, MN). Assays were performed according to the manufacturer's instructions using an ELISA microplate reader at the wavelength of 450 nm. Also, the conductivity of the eluted fractions was measured to determine the concentration of NaCl in fractions.

Statistical Analysis

Student's *t*-test for paired data was used to test statistical significance ($P < 0.05$) when comparing the differences of the means in cell adherence assays.

RESULTS

Heparin Inhibits or Modifies the Growth of Proliferating Keratinocytes in a Monolayer Culture

To study the effect of heparin on the growth of proliferating keratinocytes, the cells were cultured as a monolayer on the fibronectin-coated plastic surface in the presence of complete Keratinocyte-SFM (Fig. 1). The monolayer growth was 91%, 87%, 82%, 83%, or 106% of the growth area in the control well in 0.02, 0.2, 2, 20, or 200 μg/ml heparin, respectively (mean of two independent 3- and 4-day cultures). By using uncoated 6-well plates, the result was essentially similar. No marked changes were noted in the growth area in repeated experiments when the concentration of heparin was increased from 200 to 500 μg/ml.

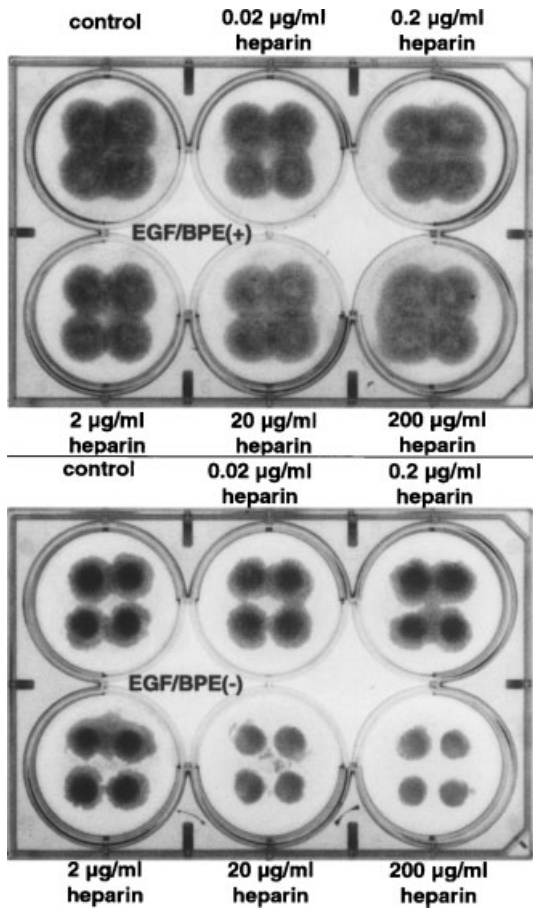

Fig. 1. The effect of heparin on the growth of a monolayer culture of keratinocytes. In this representative 3-day culture, Keratinocyte-SFM® together with epidermal growth factor (EGF) and bovine pituitary extract (BPE) (**upper figure**) or Keratinocyte-SFM alone (**lower figure**) was used as the culture medium as well as fibronectin-coated 6-well plates.

Keratinocytes were also simultaneously cultured as a monolayer on the fibronectin-coated plastic surface in the presence of incomplete Keratinocyte-SFM (Fig. 1). As expected, the monolayer growth in the absence of EGF/BPE was relatively poor and heparin even inhibited the growth in a dose-dependent manner—the monolayer growth was 105%, 86%, 65%, 25%, or 18% of the growth area in the control well in 0.02, 0.2, 2, 20, or 200 μg/ml heparin, respectively (mean of two independent 3- and 4-day cultures). By using uncoated 6-well plates (but keratinocytes suspended in complete Keratinocyte-SFM before addition into the wells in order to allow cell adherence onto the plastic surface), the result was essentially similar.

In order to confirm the result (see Fig. 1), keratinocytes were cultured in incomplete or complete Keratinocyte-SFM medium for 2 days in uncoated 24-well plates in the presence of 20 or 200 μg/ml heparin followed by measuring the DNA amount of cells with Hoechst 33258. In incomplete Keratinocyte-SFM, 20 or 200 μg/ml heparin decreased significantly the amount of DNA to $84 \pm 9\%$ or $84 \pm 5\%$ ($P < 0.05$, paired t-test, four independent experiments), respectively, when compared to cells cultured without heparin. In complete Keratinocyte-SFM, heparin at 20 μg/ml had no significant effect ($99 \pm 7\%$) and at 200 μg/ml, it consistently increased ($P = 0.056$) the amount of DNA to $124 \pm 13\%$. In general, this result agrees with the result mentioned above and demonstrated in Figure 1 showing a change from slight inhibition to stimulation by increasing the concentration of heparin from 20 to 200 μg/ml.

Heparin Inhibits the Growth of Keratinocyte Epithelium

By using uncoated 6-well plates and 10% FCS and DMEM as the medium, heparin was added in increasing concentration to the high-calcium culture of keratinocytes. The epithelial growth in the presence of 0.02, 0.2, 2, 20, or 200 μg/ml heparin was 105%, 110%, 92%, 81%, or 67% of the growth area in the control well, respectively (mean of two independent 5-day cultures, not shown). A higher concentration of heparin (500 μg/ml) did not additionally reduce the growth area.

Likewise, the growth of keratinocyte epithelium cultured on the fibronectin-coated plastic surface in the presence of 10% FCS and DMEM was inhibited by heparin in a dose-dependent manner (Fig. 2). The epithelial growth was $94 \pm 1\%$, $75 \pm 19\%$, $83 \pm 12\%$, $73 \pm 17\%$, or $55 \pm 14\%$ of the growth area in the control well in 0.02, 0.2, 2, 20, or 200 μg/ml heparin, respectively (mean $\pm$ SD, three independent 5-day cultures). In the presence of DMEM but without 10% FCS, the growth of keratinocyte epithelium on the fibronectin-coated plastic surface was poor and destructive changes in the epithelial structure were observed. However, up to 200 μg/ml, heparin still appeared to inhibit the epithelial growth (three independent 5-day cultures) (Fig. 2).

Heparin Augments the Growth-Inhibitory Effect of TNF-α on Keratinocytes in a Monolayer Culture

The addition of TNF-α to the keratinocyte culture grown on the uncoated plastic surface in

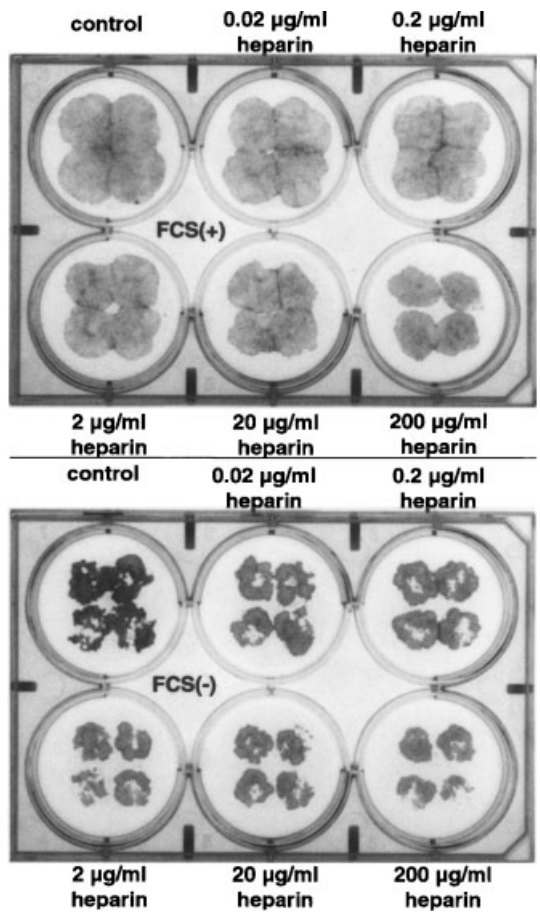

Fig. 2. The effect of heparin on the growth of keratinocyte epithelium. In this representative 5-day culture, DMEM together with 10% fetal calf serum (FCS) (**upper figure**) or DMEM alone (**lower figure**) was used as the culture medium as well as fibronectin-coated 6-well plates.

complete Keratinocyte-SFM resulted in dose-dependent inhibition of keratinocyte growth. The growth area in the presence of 1,000 or 5,000 U/ml TNF-α was $59 \pm 14\%$ or $47 \pm 17\%$ ($n = 6$, 4- or 5-day cultures), respectively, of the growth area in the control well. An increase in the inhibition of keratinocyte growth was apparent by combining 20 μg/ml heparin with 1,000 or 5,000 U/ml TNF-α (Fig. 3) (three independent experiments). However, no marked additional inhibition could be observed by combining 200 μg/ml heparin with 1,000 or 5,000 U/ml TNF-α when compared to the inhibitory effect of these substances alone (Fig. 4).

Heparin Potentiates the Growth-Inhibitory Effect of TNF-α on Keratinocyte Epithelium When the Mediators Act Simultaneously

TNF-α alone exerted growth-inhibitory effects in a dose-dependent manner on keratinocyte epithelium cultured on the uncoated plastic surface in the presence of 10% FCS and DMEM. The growth area at 1,000 or 5,000 U/ml TNF-α was $85 \pm 8\%$ or $68 \pm 12\%$ of the growth area in the control well ($n = 7$, 3- to 5-day cultures). The combination of 2 or 20 μg/ml heparin with 1,000 or 5,000 U/ml TNF-α strongly potentiated the inhibitory effect on epithelial growth when compared to the inhibitory effect of heparin and TNF-α alone (five independent experiments) (Figs. 5 and 6).

In addition to the study on the simultaneous effect of TNF-α and heparin, these mediators were also added to the culture medium (10% FCS and DMEM) in sequential order. First, TNF-α (1,000 or 5,000 U/ml) or diluent control was added to the culture for 1 day. On the following day, the cultures were washed with fresh medium, then 10% FCS and DMEM

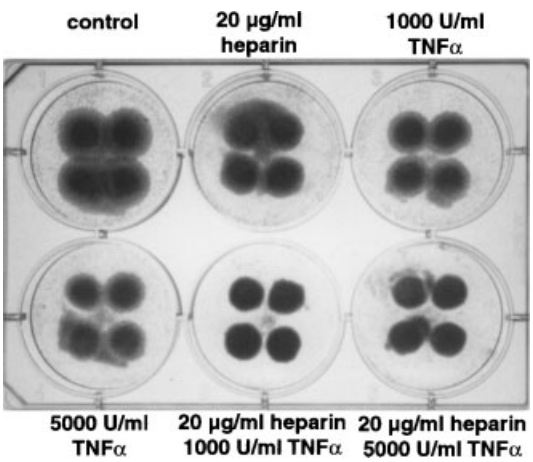

Fig. 3. The effect of 20 μg/ml heparin and TNF-α on the growth of proliferating monolayer keratinocytes. In this representative 4-day culture, Keratinocyte-SFM was used as the culture medium containing epidermal growth factor and bovine pituitary extract. Note that the combination of heparin and TNF-α resulted in increased inhibitory effect.

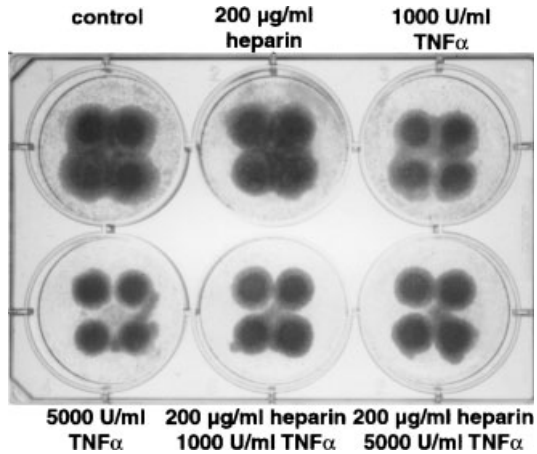

Fig. 4. The effect of 200 μg/ml heparin and TNF-α on the growth of proliferating monolayer keratinocytes. Keratinocyte-SFM medium was supplemented with epidermal growth factor and bovine pituitary extract and the cells were cultured for 4 days. Note that the combination of heparin and TNF-α did not inhibit growth markedly more than TNF-α alone.

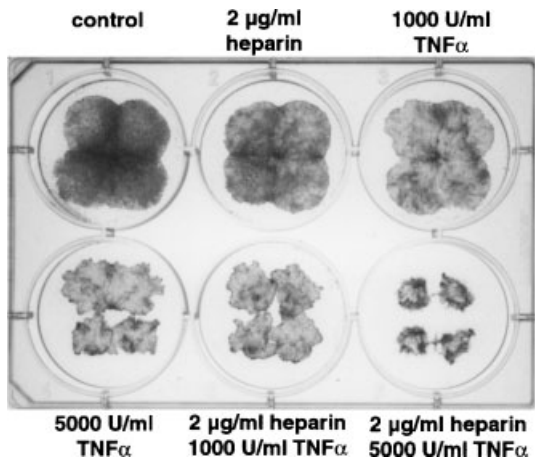

Fig. 5. The growth inhibition of keratinocytes is potentiated when 2 μg/ml heparin and TNF-α act simultaneously in high-calcium medium. In this representative 4-day culture in an uncoated 6-well plate, DMEM was used as the culture medium containing 10% FCS. Well 1 = phosphate-buffered saline as the control (growth area 100%); Well 2 = 2 μg/ml heparin (growth area 91% of the control); Well 3 = 1,000 U/ml TNF-α (growth area 88%); Well 4 = 5,000 U/ml TNF-α (growth area 54%); Well 5 = 2 μg/ml heparin + 1,000 U/ml TNF-α (growth area 50%); Well 6 = 2 μg/ml heparin + 5,000 U/ml TNF-α (growth area 13%).

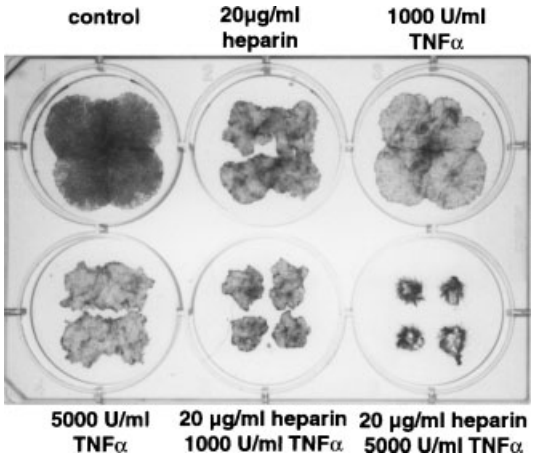

Fig. 6. The growth inhibition of keratinocytes is potentiated when 20 μg/ml heparin and TNF-α act simultaneously in high-calcium medium. In this representative 4-day culture in an uncoated 6-well plate, DMEM supplemented with 10% FCS was used as the culture medium. Well 1 = phosphate-buffered saline as the control (growth area 100%); Well 2 = 20 μg/ml heparin (growth area 63% of the control); Well 3 = 1,000 U/ml TNF-α (growth area 86%); Well 4 = 5,000 U/ml TNF-α (growth area 54%); Well 5 = 20 μg/ml heparin + 1,000 U/ml TNF-α (growth area 27%); Well 6 = 20 μg/ml heparin + 5,000 U/ml TNF-α (growth area 2%).

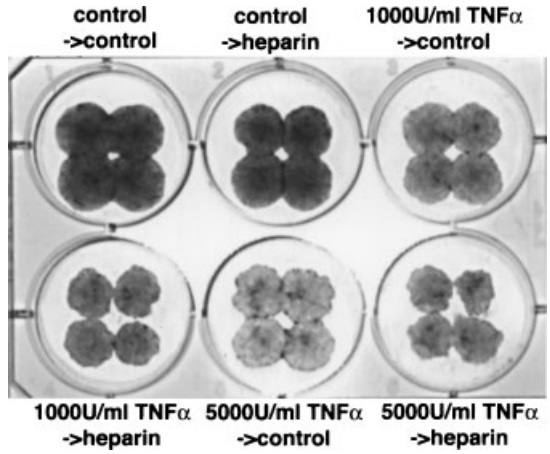

Fig. 7. The sequential effect of TNF-α and heparin on the growth of keratinocytes in 10% FCS and DMEM medium. In this representative culture in an uncoated 6-well plate, TNF-α or diluent control was added to the wells for 1 day. On the following day, the cultures were washed and 0 or 20 μg/ml heparin was included in the culture medium for 2 days. Well 1 = diluent control for 1 day, then diluent control for 2 days; Well 2 = diluent control for 1 day, then 20 μg/ml heparin for 2 days; Well 3 = 1,000 U/ml TNF-α for 1 day, then diluent control for 2 days; Well 4 = 1,000 U/ml TNF-α for 1 day, then 20 μg/ml heparin for 2 days; Well 5 = 5,000 U/ml TNF-α for 1 day, then diluent control for 2 days; Well 6 = 5,000 U/ml TNF-α for 1 day, then diluent control for 2 days. Note that no potentiation in the growth inhibition can be seen when compared to the result illustrated in Figure 6.

containing 0 or 20 μg/ml heparin was added, and the cultivation was continued for 2 more days (three independent cultures). As shown in Figure 7, the pretreatment with TNF-α for 1 day did not appear to render the cells more susceptible to growth inhibition by the subsequent heparin treatment when compared to cells not pretreated with TNF-α. Similarly, the addition of 0, 2, or 20 μg/ml heparin for 1 day followed by addition of 0 or 5,000 U/ml TNF-α for 2 more days did not clearly potentiate growth inhibition (not shown). Thus, the results suggest that heparin and TNF-α should be present simultaneously in the culture to potentiate growth inhibition.

Heparin Modifies Keratinocyte Adherence Onto the Plastic Surface

The effect of heparin on the adherence of keratinocytes onto the plastic surface was variable depending on the assay conditions. As shown in Table I, the fibronectin coat increased significantly ($P = 0.001$) the adherence of keratinocytes, though BSA blocking of unspecific binding sites decreased cell adherence in wells not treated with fibronectin. In experiments using fibronectin coating without BSA blocking, the inclusion of up to 200 μg/ml heparin in the incomplete Keratinocyte-SFM resulted in up to 12% reduction in keratinocyte adherence ($P < 0.02$). In the presence of fibronectin coat and subsequent BSA blocking, the adherence of keratinocytes was first decreased significantly ($P = 0.018$) by 12–13% but then reversed back to baseline adherence at 200 μg/ml heparin. By using collagen-S coating and BSA blocking, 2 μg/ml heparin decreased significantly ($P = 0.024$) the adherence of keratinocytes by about 33%. Instead, heparin at 200 μg/ml rather increased keratinocyte adherence slightly when compared to the control values ($P = 0.066$). In contrast, in the absence of fibronectin or collagen-S coat but in the presence of complete Keratinocyte-SFM heparin increased significantly ($P = 0.023$) the adherence of keratinocytes by about 20%.

In further experiments, it was investigated if heparin can bind to the fibronectin coat and affect cell adherence. Hence, the wells were first coated with fibronectin and then blocked with BSA. Subsequently, increasing (0, 2, 20, or 200 μg/ml in PBS) concentration of heparin was added into the wells for 2 h at 37°C.

TABLE I. Effect of Heparin on the Adherence of Keratinocytes Onto the Plastic Surface Coated or Uncoated With Plasma-Fibronectin or Collagen-S

Fibronectin coat (+/−)	Collagen-S coat (+/−)	BSA blocking (+/−)	EGF/BPE in medium (μg/ml)	Heparin in medium	Fluorescence (%)
−	−	−	−	0	66 ± 26*
+	−	−	−	0	100 (n = 7)
+	−	−	−	2	94 ± 5
+	−	−	−	20	89 ± 9*
+	−	−	−	200	88 ± 10
−	−	+	−	0	5.5 ± 2.3*
+	−	+	−	0	100 (n = 5)
+	−	+	−	2	88 ± 7*
+	−	+	−	20	87 ± 8
+	−	+	−	200	99 ± 8
−	−	+	−	0	14 ± 11*
−	+	+	−	0	100 (n = 4)
−	+	+	−	2	67 ± 40*
−	+	+	−	20	100 ± 10
−	+	+	−	200	109 ± 9
−	−	−	+	0	100 (n = 4)
−	−	−	+	2	120 ± 5*
−	−	−	+	20	116 ± 12
−	−	−	+	200	117 ± 13

About 65,000 keratinocytes were added into the wells of 24-well plates containing Keratinocyte-SFM® medium (+/− EGF and BPE) and indicated concentration of heparin. After 60 min, non-adherent cells were washed away with phosphate-buffered saline. Then, the DNA of adherent cells was dissolved and allowed to react with Hoechst 33258. Finally, the fluorescence was measured. The results are expressed as mean ± SD (four to seven independent experiments).
*Denotes significant ($P < 0.05$) change when compared to control.

Thereafter, heparin solutions were removed and the wells were washed with PBS before the addition of keratinocytes in the presence of incomplete Keratinocyte-SFM. However, the putative third coating layer of heparin did not alter keratinocyte adherence at all (three independent experiments) (not shown). This suggests that heparin does not bind to the fibronectin coat under the present assay conditions.

Growth-Inhibitory Effect of Heparin and/or TNF-α Is Not Cytotoxic

The MTT assay was used to clarify the possibility that the increased growth inhibition by heparin and/or TNF-α could be a consequence of increased cytotoxicity. Heparin (2, 20, or 200 μg/ml) or TNF-α (300, 1,000, or 5,000 U/ml) alone had in repeated experiments no apparent cytotoxic effect on keratinocytes when cultured in complete Keratinocyte-SFM in the presence of these mediators for 1 day. In addition, the combination of 20 μg/ml heparin with 1,000 or 5,000 U/ml TNF-α or the combination of 200 μg/ml heparin with 1,000 U/ml TNF-α revealed no apparent cytotoxicity under these experimental conditions (not shown). The result was similar when keratinocytes were cultured with these agents in incomplete Keratinocyte-SFM for 1 day.

For confirmation, incomplete and complete DMEM was used as the medium and keratinocytes were cultured with heparin (20 or 200 μg/ml) or TNF-α (1,000 or 5,000 U/ml) or with both heparin (20 μg/ml) and TNF-α (1,000 or 5,000 U/ml) for 1 day before the MTT assay. However, no increased cytotoxicity was noted.

No Increase in Involucrin-Positive Keratinocytes After Treatment With Heparin and TNF-α

Heparin (20 or 200 μg/ml) and/or TNF-α (5,000 U/ml) were added to the keratinocyte culture for 2 or 3 days using complete Keratinocyte-SFM. Three individual cultures were performed and involucrin was stained immunocytochemically. However, heparin or TNF-α alone or both in combination had in repeated cultures no marked influence on the number of occasionally observed involucrin-positive keratinocytes (not shown). This suggests that these mediators do not induce marked differentiation of keratinocytes under the experimental conditions used.

TNF-α Induces Cell Cycle Arrest in Keratinocytes at G0/G1 Phase but Heparin Does Not Augment This Change

In order to find out whether TNF-α (1,000 or 5,000 U/ml), heparin (20 μg/ml), or both in combination can modulate cell cycle or induce

apoptotic cell death (subdiploid events in the cell cycle analysis), keratinocytes were cultured with these mediators for 1 day using both incomplete and complete Keratinocyte-SFM. The adhered and detached cells were combined and analyzed by flow cytometry after propidium iodide staining. The results are the mean of two independent experiments with essentially similar results. TNF-α at 1,000 and 5,000 U/ml slightly but consistently increased the cell cycle arrest at G0/G1 phase in incomplete (the proportion of cells increased from 55% to 62% maximally already by 1,000 U/ml TNF-α) and especially in complete (from 58% to 68%) medium, but heparin did slightly so only in one culture in incomplete medium (not shown). However, the simultaneous presence of heparin and TNF-α did not yield any additional increase in the cell cycle arrest at this phase. Neither heparin nor TNF-α or both in combination could induce the apoptotic cell death above the control level (3.9% in incomplete and 0.9% in complete medium).

CD54 (ICAM-1) Expression Induced by TNF-α Is Not Augmented by Heparin

CD54 expression of keratinocytes was analyzed by flow cytometry. The results of repeated experiments are illustrated in Figure 8. There was a remarkable increase (seven- to tenfold) in the CD54 expression of keratinocytes after the treatment with TNF-α (1,000 U/ml) when compared to the control cells. However, heparin (20 μg/ml) did not enhance TNF-α-induced expression of CD54. In fact, the CD54 expression by TNF-α and heparin in combination

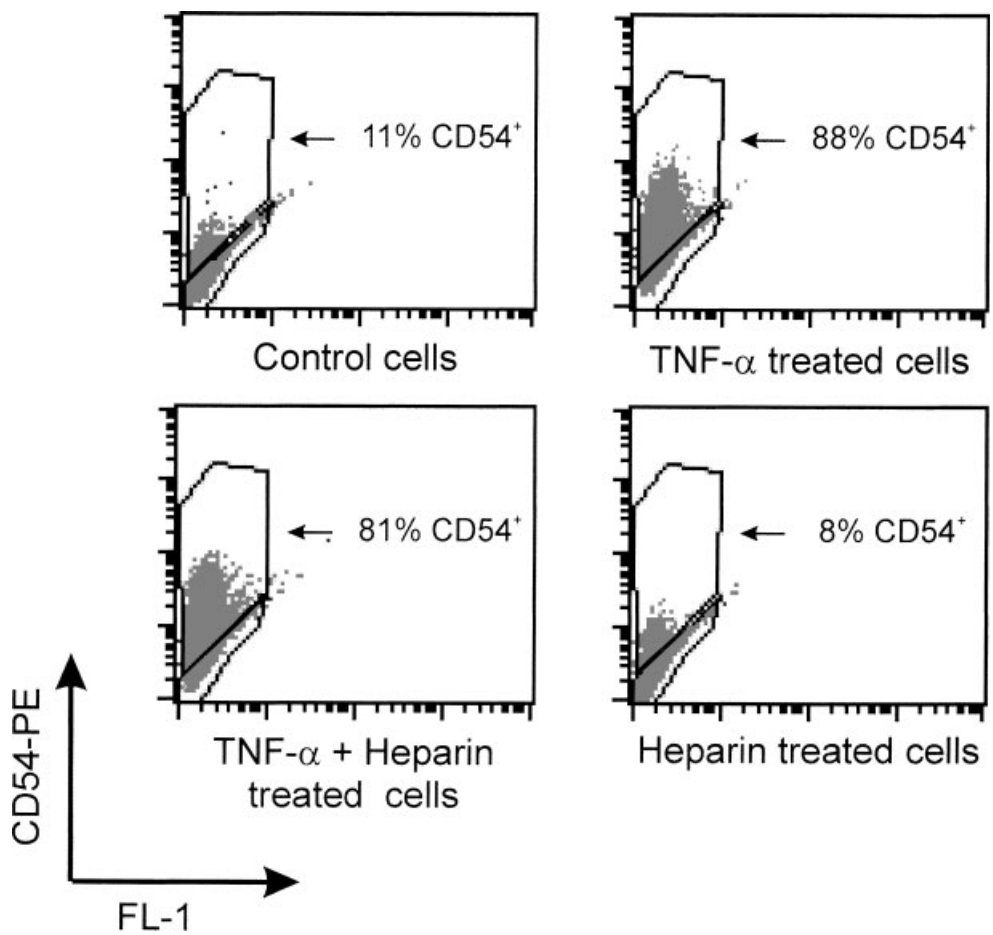

Fig. 8. The expression of ICAM-1 (CD54) on keratinocytes induced by TNF-α and heparin using flow cytometry. The keratinocytes were labeled with R-PE-conjugated monoclonal anti-CD54 antibody after cultivation for 48 h in complete Keratinocyte-SFM medium in the presence of control solution (control cells), 1,000 U/ml TNF-α alone (TNF-α treated cells), 1,000 U/ml TNF-α and 20 μg/ml heparin (TNF-α + Heparin treated cells), or 20 μg/ml heparin alone (Heparin treated cells). For each sample, 10,000 cells were collected and analyzed. The CD54-positive and -negative populations were defined using unlabeled and untreated control cells.

remained at the same level or even slightly lower than the expression induced by TNF-α alone. Similarly, the treatment with heparin per se did not increase CD54 expression over the values of the control cells (Fig. 8). Essentially similar results were obtained in both complete and incomplete Keratinocyte-SFM.

TNF-α Binds Efficiently to Heparin-Sepharose Column

The binding of TNF-α to heparin was studied using the heparin-Sepharose column, and this experiment was performed twice. During loading of the TNF-α solution into the column, no TNF-α immunoreactivity could be detected in the effluent fractions, whereas BSA passed the column, suggesting complete binding of TNF-α to the column material at 0.1 M NaCl. During elution with increasing concentration of NaCl, the peak immunoreactivity of TNF-α was detected in fractions containing about 0.7–0.9 M NaCl. This indicates high affinity binding of TNF-α to heparin-Sepharose material.

DISCUSSION

Previously, we have found that heparin at up to 200 μg/ml inhibits ^{3}H-thymidine incorporation by up to 33% in keratinocyte culture using incomplete Keratinocyte-SFM. However, in the presence of complete Keratinocyte-SFM heparin first inhibited ^{3}H-thymidine incorporation slightly, by up to 12%, but the inhibitory effect was reversed to baseline at a higher (200 μg/ml) heparin concentration [Huttunen et al., 2001]. Similarly, in the present study, heparin reduced keratinocyte growth and DNA amount in a dose-dependent manner in incomplete Keratinocyte-SFM. Instead, in complete Keratinocyte-SFM heparin at 2 μg/ml inhibited the growth by 18% but at 200 μg/ml the inhibitory effect was abrogated and reversed to baseline. The result was essentially similar regardless of the fibronectin coat on the plastic surface. This suggests that heparin exerts direct inhibitory effect on keratinocyte growth in a monolayer in the absence of exogenous growth factors. One probable target of heparin is amphiregulin, that is, an autocrine growth factor secreted by keratinocytes [Piepkorn et al., 1994]. However, the growth factors or other proteins present in the supplements (EGF and BPE) of Keratinocyte-SFM medium apparently interact with heparin and the slight growth inhibition is reversed to growth stimulation if the heparin concentration is increased from 2 to 200 μg/ml. This may be possible since heparin can interact with several growth factors, for example, basic fibroblast growth factor, amphiregulin, and keratinocyte growth factor, and protect some of them from proteolytic degradation [Ruoslahti and Yamaguchi, 1991; Piepkorn et al., 1994; Putnins et al., 1996; Berman et al., 1999]. Nevertheless, the results suggest that the growth-inhibitory effect of heparin was not due to increased cytotoxicity or differentiation. Furthermore, heparin has not been found to be cytotoxic or induce differentiation in keratinocyte culture in a previous study [Pillai et al., 1994]. Heparin can bind to specific cell surface receptors on rat vascular smooth muscle cells, a binding which leads to suppression of an early response gene, *sgk*, [Delmolino and Castellot, 1997] and to induction of a growth arrest gene, COP-1 [Delmolino et al., 2001]. Heparin can also induce matrix metalloproteinase expression in epithelial cells in vitro, an induction which is enhanced by keratinocyte growth factor [Putnins et al., 1996].

In a previous study, 1 mg/ml heparin inhibited by 35% the adherence of cultured synovial cells onto plasma-fibronectin-coated substratum [Hino et al., 1996]. Co-coating of plastic surface with 10 μg/ml plasma-fibronectin and 0.1–5 μg/ml heparin resulted in significant inhibition in the adherence of rat smooth muscle cells, the occurrence of which was maximal at 1 μg/ml heparin [Lundmark et al., 2001]. The results of the present study revealed that heparin inhibited significantly, although by only about 12%, the adherence of keratinocytes onto the fibronectin-coated but BSA-unblocked plastic surface in the presence of incomplete Keratinocyte-SFM. After blocking non-specific binding sites with BSA, the pattern of keratinocyte binding onto the fibronectin-coated plastic surface was different: heparin at 2 μg/ml first significantly inhibited keratinocyte adherence by 12%, but not more so at the concentration of 200 μg/ml. This feature is not specific to the fibronectin coat since heparin modified keratinocyte adherence onto the collagen-S-coated plastic surface in a similar fashion. The result became more complex when the effect of heparin on keratinocyte adherence onto the uncoated plastic surface was studied in the presence of complete Keratinocyte-SFM. A significant

increase in keratinocyte adherence was noted already at 2 μg/ml heparin. It is possible that the uncharacterized protein mixture of BPE contains, yet an unknown, substance that interacts with heparin and keratinocytes resulting in enhanced adherence. The third precoating of wells with heparin had no effect on keratinocyte adherence, a result which is similar to that published on rat smooth muscle cells [Lundmark et al., 2001]. Thus, it is likely that heparin interacts with the cell membrane rather than with the fibronectin coat. This conclusion also agrees with the finding that both fibronectin and collagen-S coats gave similar adherence results. However, these findings can also explain, in part, the varying effect of heparin on the growth and ^{3}H-thymidine incorporation [Huttunen et al., 2001] of keratinocytes in a monolayer culture.

In high-calcium conditions using 10% FCS and DMEM, heparin inhibited in a dose-dependent manner the growth of keratinocyte epithelium by up to 33–45% regardless of the fibronectin coat. This is in contrast to the results obtained with complete Keratinocyte-SFM showing a switch from slight inhibition to stimulation along with increased heparin concentration. An explanation for the growth inhibition of epithelium induced by heparin may be that exogenous heparin enhanced the function of inhibitory substances in FCS or prevented the action of growth-stimulatory factors. By using DMEM alone without FCS, heparin apparently deteriorated the growth of keratinocyte epithelium paralleling the result obtained in the presence of incomplete Keratinocyte-SFM. This suggests a direct influence of heparin on growth and migration, adherence properties, or autocrine growth factors. Thus, it may be possible that heparin can impair the re-epithelialization of blister bases and chronic ulcers in pathologic processes. However, the system is evidently more complex since in skin organ cultures, in the presence of 10% FCS and DMEM, 2 μg/ml heparin first inhibited by 27% the outgrowth of keratinocyte epithelium from skin specimens but then stimulated the outgrowth by about 30% at the concentration of 200 μg/ml [Huttunen et al., 2001]. In contrast to this finding, heparin at 100–300 μg/ml has been reported to inhibit keratinocyte outgrowth from epidermal biopsies placed on contracted fibroblast-collagen lattice [Imaizumi et al., 1996]. Therefore, the outcome depends on the concentration of heparin and the mixture of growth factors, cytokines, and matrix proteins.

In addition to heparin also TNF-α is a powerful mediator of mast cells [Walsh et al., 1991; Ackermann and Harvima, 1998; Gibbs et al., 2001]. Based on the morphologic findings, it is likely that these mediators can reach the epidermis after being released from mast cells and can affect keratinocytes simultaneously. Significant levels of TNF-α have been measured in blister fluids of patients with pemphigoid or toxic epidermal necrolysis [Paquet and Pierard, 1998; Rhodes et al., 1999]. It is noteworthy that TNF-α itself can induce mast cell activation and mediator release [Overveld van et al., 1991], and TNF-α production can be induced in keratinocytes themselves [Luger and Schwarz, 1990]. Therefore, heparin derived from mast cells may interact with TNF-α derived not only from mast cells but also from keratinocytes or other cells. In this study, heparin at 20 μg/ml augmented the growth-inhibitory effect of TNF-α on keratinocytes cultured in complete Keratinocyte-SFM. Instead, 200 μg/ml heparin, combined with TNF-α, could not markedly increase the growth inhibition above that induced by TNF-α per se. Possibly heparin at higher concentrations enhanced the function of growth factors present in EGF/BPE and/or increased the adherence of keratinocytes onto the substratum. Nevertheless, the inhibitory effect of TNF-α was fully retained at 200 μg/ml heparin. Based on the MTT and propidium iodide assays and involucrin staining, the inhibitory effect of heparin-TNF-α combination was not due to increased cytotoxicity, cell cycle arrest, apoptosis, or differentiation. In addition, heparin could not modulate another well-known action of TNF-α, that is, induction of ICAM-1 in keratinocytes [Ackermann and Harvima, 1998]. Previously, TNF-α has been found to induce cell cycle arrest at G0/G1 phase in keratinocytes [Vieira et al., 1996], a result which is similar with that in this study.

The combined effect of heparin and TNF-α was more pronounced when the high-calcium medium, 10% FCS and DMEM, was used. Heparin at 2 and 20 μg/ml strongly potentiated the growth inhibition induced by 9 ng/ml (1,000 U/ml) and 45 ng/ml TNF-α. The simultaneous presence of these mediators in the culture appeared to be a requirement for this potentiation. It is likely that TNF-α binds to heparin

similarly to many other preformed mediators stored in the secretory granules of mast cells. The present result of heparin-Sepharose chromatography supports this conclusion. Consequently, heparin may stabilize and protect TNF-α from proteases during longer cultivation leading to prolonged survival and action time of the cytokine. Heparin can also stabilize other mediators of mast cells such as tryptase and basic fibroblast growth factor [Reed et al., 1995; Harvima et al., 1999; Schönherr and Hausser, 2000]. Alternatively, the binding of putative TNF-α-heparin complex to the TNF-receptor may be enhanced as has been observed in other ligand-receptor systems [Ruoslahti and Yamaguchi, 1991; Schönherr and Hausser, 2000]. In addition, it may be possible that heparan sulphate on the cell membrane can capture and function as a reservoire of TNF-α, but heparin keeps the cytokine available for the receptor. The potentiating effect of heparin in high-calcium culture seems to be specific for this sulphated glycosaminoglycan since we have not found any similar effect when using hyaluronic acid with TNF-α under similar culture conditions (unpublished results). The possible relation of the present finding to the poor re-epithelialization of chronic ulcers or blister bases is of interest since, for example, in blister fluids of pemphigoid elevated levels of tryptase, histamine and TNF-α have been measured [Katayama et al., 1984; Rhodes et al., 1999; D'Auria et al., 2000]. The results suggest that in addition to the cell cycle arrest at G0/G1 phase also the migration and adherence are simultaneously affected resulting in poor keratinocyte growth by the heparin-TNF-α mixture.

In conclusion, heparin can inhibit keratinocyte growth in a non-cytotoxic and dose-dependent manner in low- and high-calcium medium in the absence of growth factors and serum. Heparin per se inhibits dose-dependently the growth of keratinocyte epithelium in the presence of FCS and high-calcium medium. Instead, heparin apparently interacts with the growth factors present in BPE and EGF and therefore modulates the growth of proliferating keratinocytes in complete Keratinocyte-SFM. Heparin showed a variable effect on keratinocyte adherence depending on culture conditions and heparin concentration—both decreased and increased adherence was noted. Novel findings were that TNF-α binds tightly to heparin-Sepharose and heparin potentiates the growth-inhibitory effect of TNF-α especially in high-calcium medium.

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