Eur J Med Res (2000) 5: 347-355

Local and Systemic Concentrations of Pro- and Anti-Inflammatory Cytokines in Human Wounds*

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Abstract:
Objective: There is a lack of knowledge on the concentrations of cytokines and growth factors in wound healing. The objective was to characterize the pattern of local-tissue and systemic peri-and postoperative dynamics of cytokines and growth factors in a clinical model of a controlled and comparable operative plastic surgery trauma.
Design: Prospective clinical study.
Setting: A University Department of Surgery.
Patients: 28 patients undergoing an elective reduction mammoplasty.
Main outcome measures: IL-6, IL-8, sTNFR-1 and TGF-β levels in plasma and wound fluid.
Results: Levels of cytokines increased only moderately in plasma. Cytokine levels in wound fluid were several fold higher. IL-6 in the wound fluid peaked at 7 hours after the operation (271 ± 135.8 pg/ml); IL-8 after 4 hours (11 ± 9.4 ng/ml); sTNFR-1 at the second postoperative day (11.1 ± 3.4 ng/ml). TGF-β decreased at the first (15.2 ± 8.6 ng/ml) and second (11.7 ± 5.0 ng/ml) postoperative day.
Conclusion: Wound cytokine and growth factor levels are markedly higher than the systemic ones indicating a compartmentalization of the immune response. Cytokines peaked at different time points, probably reflecting the influx of inflammatory cells into the wound and the phase of wound healing. Further studies are necessary to clarify the mechanism of cytokine release in normal postoperative wounds before therapeutic use can be developed.

Key words: Wound healing; cytokines; growth factors

Introduction

The role of cytokines in normal wound healing remains poorly defined. Reduction in inflammatory cytokines in wounds corresponds with increased wound tensile strength which may be achieved by accelerated accumulation of collagen and a reduction in collagenolytic activity [1]. A close relationship of cytokines to wound remodeling and inflammation has been demonstrated in two investigations [1, 2]. Localized production of cytokines may have a beneficial effect on wound healing by promoting an influx of monocytes and T-lymphocytes [3]. Levels of cytokines in chronic wounds are lower than in acute wounds [4] and the balance of cytokines in the wound fluid may ensure the proper regulation of the cytokine network which is necessary for wound healing [5, 6]. While many studies have been performed in animal models, in cell cultures and in normal skin, little is known about the local and systemic kinetics of cytokines in patients undergoing elective operations [7]. This is confirmed by Vogt et al. (1998) who investigated the presence and profile of endogenous growth factors in human split-thickness skin wound enclosed in cutaneous vinyl chambers. The naturally occurring levels at the wound site of cytokines are unknown. The role of cytokines during wound healing has been investigated in animal models and wound healing defects were associated with impaired cytokine expression [8]. Investigations in humans were not yet performed. Nevertheless, growth factors and cytokines have been frequently applied to chronic wounds [9].

We have compared recently the pattern of local and systemic cytokine-response in peritonitis, finding it to be compartmentalized within the peritoneal cavity [10, 11]. Is the same true concerning the surgical wound? We conducted this study with the objective to portray the dynamics of cytokines response (IL-6, IL-8, sTNF-R1, TGF-beta) in plasma and wound fluid, peri- and postoperatively, with regard to time and postoperative complications. We have selected IL-6 and sTNFR-1 because both cytokines can be reliably measured in serum and we had experience in determination of these cytokines in different other studies. Furthermore IL-6 and sTNFR-1 may reflect indirectly TNF-α release. IL-8 which may represent neutrophil activation has been indicated as marker for organ failure and complications in several clinical studies [12]. TGF-β may have an impact in the development of wound scars which are considered to be complications in plastic surgery [13].

Materials and Methods

Samples from plasma and wound fluid in 28 female patients who underwent a reduction mammoplasty were obtained at different time points: preoperatively, during the day of operation and postoperatively- until the 7th postoperative day. The patients received antibiotic prophylaxis with a dose of 2 g Cefotetam. The wound fluid was collected in miniflap drainage systems (McGhan

* This study was supported by a NATO Collaborative Research Grant 910960
# Part of this work has been presented at the 16th Annual Meeting of the Surgical Infection Society, Milwaukee, WI, USA, April 1996

Medical Corporation, Santa Barbara Cal., USA) which were connected to vacuum, endotoxin-free tubes (Chromogenix, Germany). Wound fluid was collected four times daily (10 a.m., 2 p.m., 6 p.m., 10 p.m.) as long as the wound produced drainage. Plasma samples were obtained preoperatively and on the first, second, third, fifth and seventh postoperative day. Samples were collected in heparinized, endotoxin-free tubes (Chromogenix, Essen, Germany), then centrifuged, aliquoted and stored at –80° C until further processing.

IL-6, IL-8, sTNFR-1 and TGF-β were measured with commercial ELISA (R & D System, DPC, Bad Nauheim, Germany). The normal values are: IL-6: normal 3.13-12.5 pg/ml; sensitivity: 0.7 pg/ml, IL-8: normal 0-40 pg/ml; sensitivity 3 pg/ml; STNFR-1: normal 749-1966 pg/ml; sensitivity: 1,5 pg/ml. TGF-β: normal 34.7-63.9 ng/ml; sensitivity: 5 pg/ml.

Data are expressed as mean and standard deviation (SD). Values were compared with Mann-Whitney test using the statistical package (Instat, Santa Monica, CA, USA). Data were considered different if a p value < 0.05 or 0.01 was obtained.

Results

Twenty-eight patients (mean age 26.9 years; range 16 – 64 years) were enrolled in this prospective study.

Soluble TNF Receptor 1 (sTNF-R1) in Plasma

Levels moderately increased from 674.5 pg/ml preoperatively, to 969.2 pg/ml ($p < 0.01$) at the second postoperative day. It remained significantly elevated until the 7th postoperative day (Fig. 1a).

Soluble TNF Receptor 1 (sTNF-R1) in Wound Fluid

Levels increased steadily from 3h (3.3 ng/ml) to reach a maximum at the second postoperative day (11.1 ng/ml; $p < 0.01$). sTNF receptor 1 levels remained significantly elevated throughout the observation period (Fig. 1b).

The concentrations of sTNF-R1 in wound fluid were ten times higher than in the plasma.

IL-6 in Plasma

Levels increased at the first (48.8 ± 45.5 pg/ml) and second (27.1 ± 19.3 pg/ml) post-operative day; the absolute levels were less than half measured in the wound fluid (112.5 pg/ml first postoperative day; 62.6 pg/ml second postoperative day). IL-6 plasma levels remain elevated ($p < 0.0001$) for 7 days; however, the cytokine levels are small when compared to wound fluid cytokine levels (Fig. 2a).

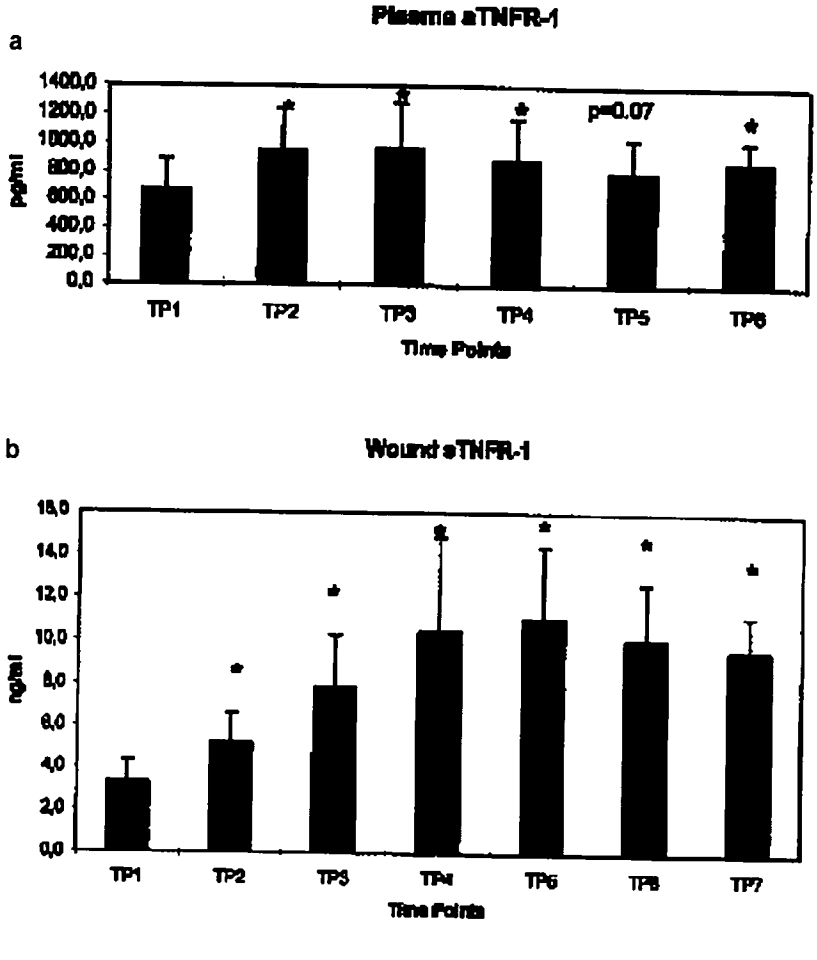

Fig 1.
a sTNFR-1 plasma levels at time point 1 (preoperative), time point 2 (first postoperative day), time point 3 (second postoperative day), time point 4 (third postoperative day), time point 5 (fifth postoperative day) and time point 6 (seventh postoperative day).

sTNFR-1 levels are significantly increased postoperatively ($p < 0.01$) reaching a maximum at the second postoperative day.

b sTNFR-1 levels in wound fluid at time point 1 (3h postop), time point 2 (7h postop), time point 3 (26h post op), time point 4 (34h postop), time point 5 (50h postop), time point 6 (54h postop), time point 7 (58h post op).

sTNFR-1 increases significantly 7 hours after operation and peaks at 50 hours postoperatively

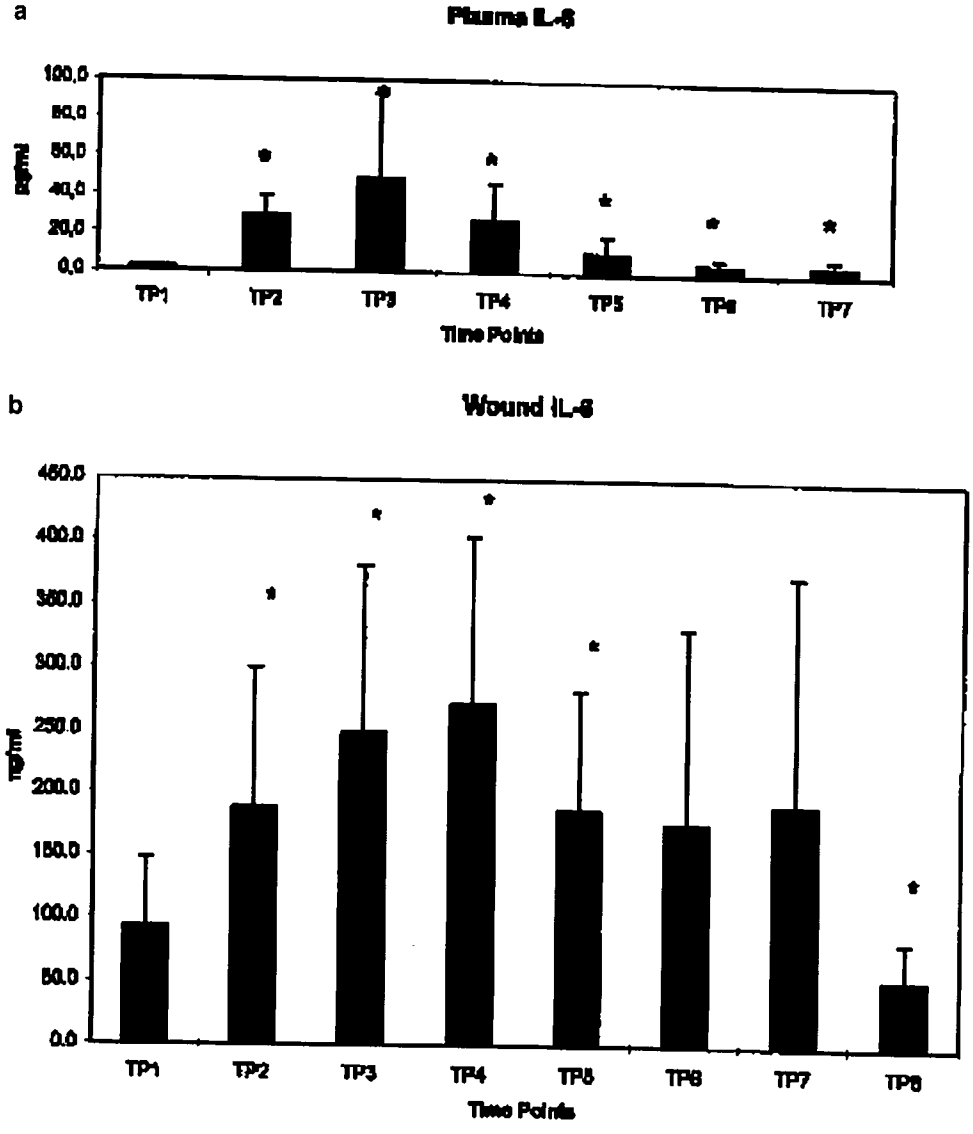

Fig 2. a IL-6 plasma levels at time point 1 (preoperative), time point 2 (day of operation), time point 3 (first postoperative day), time point 4 (second postoperative day), time point 5 (third postoperative day), time point 6 (fifth postoperative day) and time point 7 (seventh postoperative day). IL-6 plasma levels are significantly increased at the day of operation, peak at the first postoperative day and remain elevated until the seventh postoperative day.
b IL-6 levels in wound fluid at time point 1 (3h postop), time point 2 (4h postop), time point 3 (6h postop), time point 4 (7h postop), time point 5 (9h postop), time point 6 (26h postop), time point 7 (34h postop), time point 8 (54h postop). IL-6 peaks at 7 hours postoperatively and declines significantly at 54 hours postoperatively. IL-6 increases significantly at the day of operation and peaks at the first postoperative day.

IL-6 in Wound Fluid

Levels increased from 93.7 ng/ml at 3 h to 271.7 ng/ml at 7h postoperatively (an increase of 300%). They remained significantly elevated 9 hours after the operation when compared to levels at 3 hours after operation. There was a significant decrease in IL-6 levels 54 hours after operation (53.75 ng/ml; $p = 0.03$) (Fig. 2b).

IL-8 in Plasma

IL-8 plasma levels remained within the normal range . The IL-8 determination at the first postoperative day (40.3 pg/ml) was only marginally above the normal range. All other determinations remained at the level of the preoperative determination (Fig. 3a).

IL-8 in Wound Fluid

IL-8 levels in wound fluid were increased showing the highest concentration 4 hours after operation (11 ng/ml) which was almost significantly elevated ($p = 0.055$) when compared to the baseline level at 3 hours. All other determinations of IL-8 did not reveal a difference to the baseline levels (Fig. 3b).

The concentrations of IL-8 in the wound fluid were 10- fold higher compared to the plasma levels.

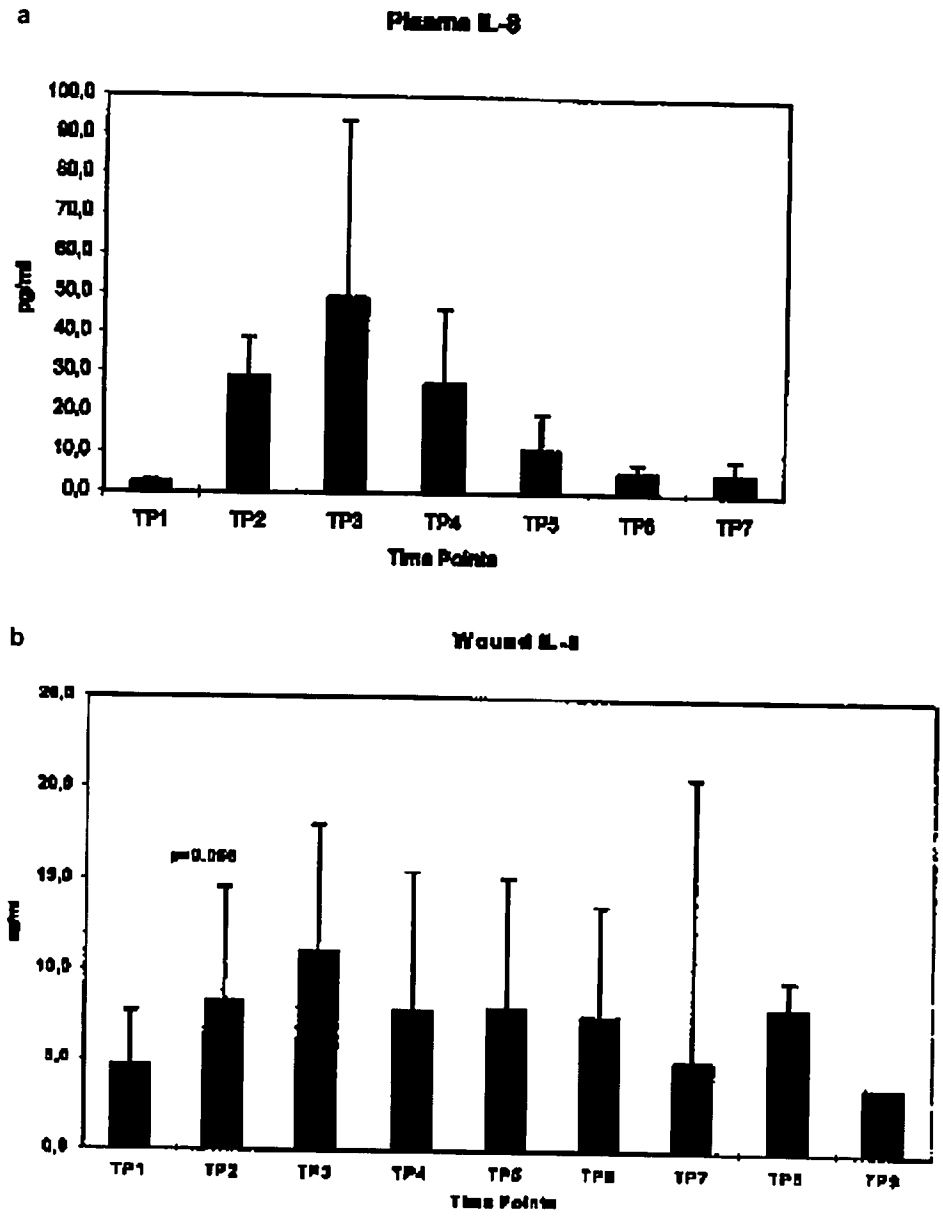

Fig 3. a IL-8 plasma levels at time point 1 (preoperative), time point 2 (day of operation), time point 3 (first postoperative day), time point 4 (second postoperative day), time point 5 (third postoperative day), time point 6 (fifth postoperative day) and time point 7 (seventh postoperative day). IL-8 increases after operation and peaks at the first postoperative day. b IL-8 levels in wound fluid at time point 1 (2 h postop), time point 2 (3h postop), time point 3 (4h postop), time point 4 (4 h postop), time point 5 (6h postop), time point 6 (7h postop), time point 7 (9h postop), time point 8 (26h postop), time point 9 (34h postop), time point 10 (54h postop). There is no significant change in IL-8 wound levels.

Transforming Growth Factor-β (TGF-β) in Plasma

Concentration decreased from the preoperative levels (12.1 ± 3.6 ng/ml) to the first (8.5 ± 3.4 ng/ml; $p < 0.0001$) and second (8.5 ± 2.8 ng/ml; $p < 0.0001$) postoperative day. On the fifth and seventh postoperative day levels were again in normal range (12.4 ± 4.5 ng/ml; 11.2 ± 3.4 ng/ml). (Fig. 4a)

Transforming Growth Factor-β (TGF-β) in Wound Fluid

TGF-β increased almost significantly in the wound fluid 3h (37.9 ± 12.2 ng/ml; $p = 0.07$) and 4h (35.7 ± 10.6 ng/ml; $p = 0.08$) when compared to baseline level at 2 hours (27.8 ± 6.5 ng/ml). There was a steady decrease of TGF-β in the wound fluid 26 hours (15.2 ± 8.5 ng/ml; $p = 0.002$) and 34 hours (11.6 ± 4.9 ng/ml; $p < 0.001$) postoperatively. Lowest levels were observed at 54 hours after operation (9.5 ± 3.5 ng/ml; $p < 0.001$)

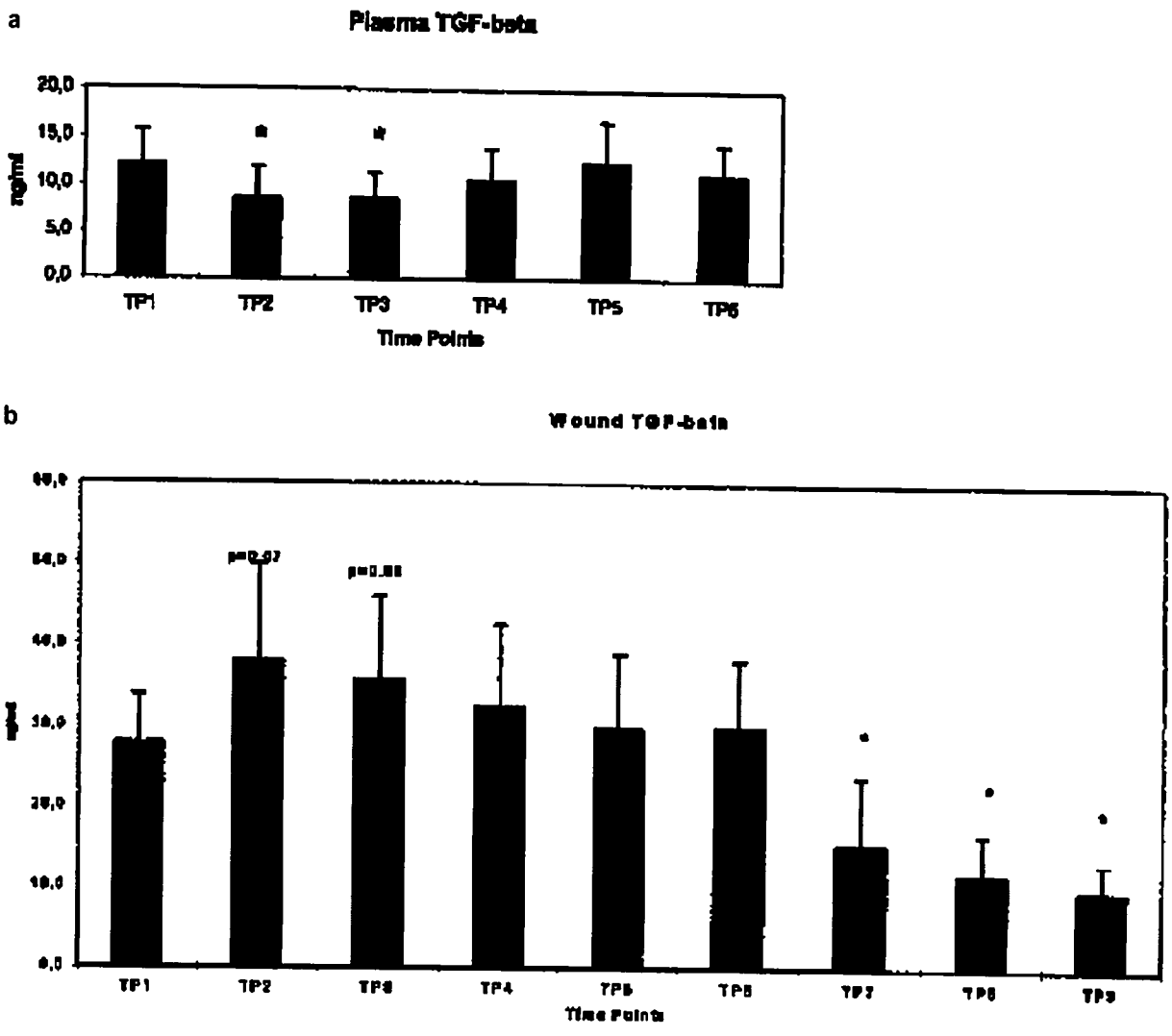

Fig 4. a TGF-β plasma levels at time point 1 (preoperative), time point 2 (first postoperative day), time point 3 (second postoperative day), time point 4 (third postoperative day), time point 5 (fifth postoperative day), and time point 6 (seventh postoperative day). TGF-β decreases significantly at the first and second postoperative day ($p < 0.05$). b TGF-β wound fluid levels at time point 1 (3 h postop), time point 2 (4h postop), time point 3 (6h postop), time point 4 (9h postop), time point 5 (26 h postop), time point 6 (34 h postop), time point 7 (54 h postop). TGF levels are maximum 3 hours postoperatively and decline significantly after 26 hours.

(Fig. 4b).

TGF-β were three times higher in the wound fluid than in plasma. The concentration of TGF-β is in the normal range in the plasma and at the beginning of determination in the wound. There is a decrease of Transforming Growth Factor at later time points which demonstrates that the levels of this factor changed after Interleukin-6 levels changed.

Discussion

Wound healing requires an interaction of epithelial, inflammatory, endothelial cells, platelets and fibroblasts, which interact via transmitter substances and mediators, during the phases of coagulation, inflammation, fibroblasia, matrix deposition, angiogenesis, epithelization and contraction. Early post-traumatic inflammation is an important step for cell replication and the foundation of healing [14]. Activated platelets release into the wound growth factors such as TGF-β and attract fibroblasts and macrophages. The latter produce IL-1, TNF-α, TGF-β and other growth factors [15]. Macrophage secretory products may have different impact on growth of cells: TGF-β can suppress mitosis; TNF-α may increase wound strength in low concentrations but decrease collagen deposition in high concentrations. The control of tissue repair requires temporal and spatial confinement of cytokines [16].

In this study we evaluated the local and systemic concentration of sTNFR-1, IL-6, IL-8 and TGF-β in patients undergoing elective surgery. To our knowledge, wounds levels of TGF-β, TNFR-1, IL-8 and IL-6 were not previously serially measured in a clinical model of controlled operative trauma.

Soluble TNF Receptor 1

TNF-α may have different effects in wound healing – inhibitory and activating [17, 18, 19, 20]. However, it is well known that TNF-α may be difficult to measure in serum. TNF may interact with two receptors, a 55-60 kilodalton (CD 120a) and a 75-80 kilodalton (CD 120b) receptor, which mediate the different effects of Tumor Necrosis Factor alpha. The early inflammatory effects are mediated by the slow p55-60 TNF receptor that is expressed in the vascular system and most organs. The fast p75-80 TNF receptor is the predominant receptor type in activated lymphocytes and monocytes and is involved in the immuno-enhancing effect of TNF. Both receptors release their extracellular part as soluble TNF receptor (sTNFR) into the circulation [21]. sTNFR may function as an inhibitor or carrier protein for TNF to confine any local, e.g., wound, source of TNF. The relationship of TNF and sTNFR has been investigated in several studies [22, 23, 24] and sTNFR levels were associated with systemic complications in a variety of diseases [25, 26] . To our knowledge this is the first study where sTNFR-I was serially determined in the local, postoperative wound and the systemic circulation. Plasma levels are only moderately increased. However, in local wound fluid sTNFR-1 remained elevated for two days postoperatively which may reflect increased pro-inflammatory TNF-α release [27, 28, 29] . The modest operative trauma of mamma reduction plasty is represented by the moderate sTNFR-1 increase in plasma and the visible increase in local wound fluid. The source of sTNFR-1 remains to be elucidated. Peripheral cells, such as lymphocytes and PMN, possess mostly 75 kDaTNFR [30] but in patients with severe trauma and septic shock the serum levels of 55kDasTNFR exceed those of 75kDasTNFR [23]. In this study we have not determined the cells in the wound fluid nor have we determined sTNFR-2 levels. To clarify the function of soluble Tumor Necrosis Factor Receptors in wound healing we need such investigations.

IL-6

The moderate operative trauma in this study is further reflected by small and temporary elevated plasma IL-6 levels. The local trauma leads to increased IL-6 levels in wound fluid (271.7 pg/ml). IL-6 regulates several components of the acute phase response and is involved in the B-cell differentiation and the co-stimulation of T-cells which are involved in wound healing [31] . There is also a strong relationship to antecedent TNF-α release [32] resulting in high levels of IL-6 in early experimental wounds [33] and in burn blister fluids [34]. Increased IL-6 levels correlated with impaired T-cell response and an increased susceptibility to infection in burned patients [35]. IL-6 was also implicated in suppressing fibroblast formation [36]. Fibroblasts again may produce TGF-β. The observed reduction in TGF-β may be caused by the IL-6 induced suppression of fibroblasts. In contrast, high concentrations of IL-6 induced de novo synthesis of active TGF-β by adherent cells or activated macrophages to synthesize or release enzymes capable of activating latent TGF-β [37]. However, monocytes/macrophages appear later in wound healing and it seems unlikely that these cells are responsible for the early local production of TGF-β.

IL-8

The normal levels of plasma IL-8 indicate that no systemic neutrophil activation has occurred. Neutrophil recruitment into inflammatory lesions depends on chemotactic agents such as IL-8 [38]. Elevated IL-8 levels causing neutrophil accumulation were reported in a variety of disease [39, 40, 41, 42]. The IL-8 concentration in wound fluid was increased in this study which may indicate keratinocyte proliferation [43]. The significance of IL-8 in wound healing remains unclear. Only one study deals with effect of IL-8 in wound healing [12]. In hypertrophic scars treated by dressings there was an increase of IL-8 mRNA and a decrease of TGF-β. It was concluded that hydrogel dressing may augment collagenolysis via promotion of inflammation mirrored by increased IL-8 and decreased TGF-β levels. High IL-8 levels as those we observed in the wound are seen in patients with sepsis [44] or primates challenged with E. coli [45, 46]. Our results are in agreement with reports by Ono et al. [5, 6] who measured IL-8 in nanogram range in burn wound blister fluid. The inflammatory response by locally produced IL-8 elicits edema formation due to neutrophil-mediated damage and subsequent plasma leakage [47, 48]. The effect of IL-8, however, is still in dispute. IL-8 may also have anti-inflammatory effects and inhibit the adherence of neutrophils to endothelium [49], intravenous bolus IL-8 caused transient neutropenia and leukocyte sequestration [50]. In this study the increased local IL-8 release may be more a sign of local inflammation than anti-inflammatory action. It is clear to us that the isolation of cells from the wound fluid would further help to clarify the role IL-8 in wound healing.

TGF-β

The concentration of TGF-β in plasma was in the normal range throughout the study period. There was, however, a significant decrease of TGF-β at the first and second postoperative day in the wound fluid. TGF-β may stimulate the proliferation of certain cell types (e.g., connective tissue) while inhibiting proliferation of lymphocytes and epithelial cells [51]. It can be produced by several cell types, e.g., platelets and macrophages, and may promote wound healing [52, 53]. This effect may be achieved by the synthesis and rapid maturation of collagen in early wound [54, 55]. This observation indicates that TGF-β may not be an early player in wound healing. Our results seem to confirm this observation. The decrease of TGF-β at the first and second postoperative day may also be caused by consumption of this factor known to block biologic effects of pro-inflammatory cytokines on matrix remodeling and destruction [56]. It is yet unclear which cell type is primarily responsible for the production of TGF-β; macrophages which follow neutrophils into the wound after two days may also release this factor [57]. Furthermore some of the effects of TGF-β, e.g., chemotaxis for inflammatory cells and

fibroblasts, depend on optimal concentration which we don't know yet [58, 59]. Although growth factors have been applied frequently to chronic wounds in clinical studies, there remains this obvious lack of knowledge of optimal growth factor concentration in wounds.

Cytokines and growth factors play an important role in the inflammatory response during the early phase of wound healing. The absolute levels and the balance between the mediators may decide on the outcome of wound healing. As none of the patients in this study had wound complications the observed pattern of cytokine and TGF-β production can be considered as "normal". The knowledge of the appropriate timing and quantity of growth factor and cytokine application is necessary before widespread application of topical cytokine therapy. The combination of several factors may be more therapeutic than single cytokine application [60].

We conclude that wound cytokine and growth factor levels are markedly higher than the systemic ones. Cytokines peaked at different time points, probably reflecting the influx of inflammatory cells into the wound and the phase of wound healing. Further studies are necessary to clarify the mechanism of cytokine release in normal postoperative wounds before a therapeutic strategy can be developed.

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Received: March 14, 2000 / Accepted: May 18, 2000

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