Chapter 3 Chemokine profiles differ between intracutaneous and subcutaneous locations in early acute wound healing after breast reduction

Kim Kroeze
Sanne de Boer
Frank Niessen
Rik Scheper
Susan Gibbs

ABSTRACT

This study describes chemokines and chemokine gradients which are regulated during the very early hours of normal wound healing by comparing chemokine concentrations in intracutaneous (superficial) and subcutaneous (deep) drainage fluid during the first six hours after surgery. Intracutaneous and subcutaneous drainage fluids were collected at hourly intervals from patients undergoing elective breast reduction surgery and analyzed by ELISA. Lymphocyte-specific chemokines CCL18 (> 17 ng/ml) and CCL20 (0.1 ng/ml) were detected already 2 and 1 hour respectively post wounding indicating that lymphocytes are indeed attracted in the first hours after wounding and might play an important role as early immunological effectors. A very high level of monocyte/ macrophage/ mesenchymal stem cell attracting chemokine CCL5 was also detected at 1 hour post surgery (71 ng/ml). Chemokine CCL2 was expressed at 1 hour after surgery and continued to rise during the 6 hour study period. Neutrophils chemoattractants CXCL1, CXCL8 and IL-6 peaked at 4-6 hours post surgery. Notably, chemokine gradients were detected between the two drain locations. Even though CXCL8 and IL-6 were detected in both intra- and subcutaneous drainage fluids, the relative increase in production of both mediators was much more pronounced intracutaneously than subcutaneously. Also significantly higher CCL27 levels were detected in drainage fluid collected from the intracutaneous compartment compared to the subcutaneous compartment. IL-1α, TNF-α, and CXCL12 were not detected in this study. Since all donors developed normotrophic scars six months after wound closure this study describes the expression of early inflammatory mediators during normal wound healing.

INTRODUCTION

During wound healing a sequence of events takes place, which determines the rate of healing and the quality of the final scar. In normal wound healing, tissue injury triggers an acute local inflammatory response which is followed by cell proliferation, wound closure and remodeling of the tissue (1). In order to understand how adverse wound healing occurs, such as in chronic wounds which fail to close and in deep burns which heal with a hypertrophic scar (2,3), it is important to gain more insight into normal wound healing and in particular into which early inflammatory events occur. Therefore, in this study we investigated wound healing in surgical incision wounds created during breast reduction. In general, surgical wounds heal relatively quickly and with good scar formation and are therefore representative for a normal wound healing process (4,5,6).

During injury, vessels are disrupted releasing platelets into the wound bed. Platelets in turn release a number of cytokines and growth factors such as IL-1α, TNF-α, PDGF, VEGF (4,5,6). Whereas PDGF and VEGF function directly as potent angiogenic and tissue repair factors, IL-1α and TNF-α function indirectly by stimulating a chemokine cascade which initiates the infiltration of leukocyte subsets (neutrophils, macrophages/ monocytes, lymphocytes) from the blood into the wound bed. This inflammatory response targets invading environmental pathogens and also initiates wound repair. Parallel to the release of platelets, damaged skin residential cells (keratinocytes, fibroblasts and endothelial cells) secrete a large number of growth factors, cytokines and chemokines further amplifying the inflammatory response and activating neighbouring skin residential cells to proliferate, migrate and differentiate in order to close the wound (4,5,6). Table 1 summarizes the functions of cytokines and chemokines investigated in this study, which are noted for i) their ability to attract and activate leukocytes and ii) being mediators for various processes involved in tissue repair.

Table 1: Cytokines and chemokines involved in inducing inflammation and tissue repair

Mediator	Function	Target leukocyte	Reference
IL-1α	Pro-inflam; Mit; Hematopoiesis	All leukocytes	12, 13, 14
TNF- α	Pro-inflam; Mit	All leukocytes	12, 13, 14
CXCL1/ Gro- α	Inflam; Ang; Epith; TR	Neutrophils	7, 17
CXCL8/ IL-8	Inflam; Ang; Epith	Neutrophils	7, 21
IL-6	Inflam; Gran; Ang; Mitogen	Neutrophils	15
CCL2/ MCP-1	Inflam; Ang; Epith; TR	Monocytes/ macrophages	3, 10
CCL5/ RANTES	Inflam; Mit; Chemotactic for stem cells	Monocytes/ macrophages	14, 19
CCL18/ PARC	Inflam; Collagen production	Lymphocytes	1, 20
CCL20/ MIP-3 α	Inflam; antibacterial activity	Lymphocytes	22
CCL27/ CTACK	Inflam; Chemotactic for BMDSC	Lymphocytes	22, 24
CXCL12/ SDF-1	Inflam; Ang; Immune responses	Lymphocytes	27

Ang, angiogenesis; Epith, epithelialisation; Gran, granulation tissue stimulating; Inflam, inflammatory mediator, TR, tissue remodelling, BMDSC, bone marrow derived stem cells.

It is thought that events occurring immediately after wounding influence the final quality of the scar (7,8). For example, Ferguson et al. suggested that alterations in the early mediators (e.g.: TGFβ3 or neutralizing antibodies to TGF-β1 and TGF-β2), can have a major long-term effect due to alteration and reduction of downstream regulatory cascades (7). Additionally, Van der Veer et al. showed that activated keratinocytes (showing increased keratin 16 expression) present only in the inflammation phase are associated with hypertrophic scarring, before hypertrophic scar formation is clinically present (9). Still, little is known about the early inflammation events which involve cytokine and chemokine release during the first hours after injury. Most studies focus on the late inflammatory phase by collecting drainage fluid at daily intervals from incision wounds starting from day 1 post surgery (10,11,1,12). In order to gain insight into the very early inflammatory events within the first six hours post surgery, we determined the secretion profile for an extensive panel of cytokines and chemokines (IL-1α, TNF-α CXCL1, CXCL8, IL-6, CCL2, CCL5, CCL18, CCL20, CCL27 and CXCL12) which are reported to regulate inflammation and wound closure. Since most studies described until now collect and analyze deep cutaneous drainage fluid, we also questioned whether this fluid is representative of the dermal wound environment. Therefore, we compared inflammation mediators released deep in the subcutaneous tissue with those released into the intracutaneous tissue of the wound in order to determine which chemokine gradients are present in the wound environment .

METHODS AND MATERIALS

Patient inclusion and drainage fluid collection

Twelve healthy female patients undergoing elective surgery for breast reduction were recruited at the VU University Medical Center and at the Sint Lucas Andreas Hospital, Amsterdam. Their median age was 47.4 years, with a range of 13.2 years. The research protocol was approved by the Health Research Ethics Board of the VU university medical center and in agreement with the ethical guidelines of the 1975 Declaration of Helsinki. Informed written consent was obtained from all participants.

Each patient underwent surgery on both breasts. Incisions were made and drainage fluid was collected from both incisions. Postoperative drainage fluid was collected every hour for up to 6 hours after wound closure. From each incision, drainage fluid was collected subcutaneously and intracutaneously. Subcutaneous drainage fluid samples were collected via a ø 3.3mm end-perforated PUR wound drain (Medinorm, Spiesen, Germany) located between the major pectoral muscle and the subcutaneous fat layer. The drain was placed with a tap in order to obtain drainage fluid directly from the wound each hour. Intracutaneous samples were collected via a ø 2.0mm redon-drain (PFM AG, Köln, Germany) located just beneath the

dermis of the skin. The drain pot was renewed hourly and drainage fluid collected. A protease inhibitor cocktail (final concentration 1:100) (Sigma Aldrich, P8340) was added directly after harvesting drainage fluid. Samples were then centrifuged at 4640 RCF for 6 minutes, aliquoted and stored at -80°C until further analysis.

Enzyme-linked immunosorbent assay for chemokine production

For cytokine and chemokine quantification in wound bed samples, enzyme-linked immunosorbent assays (ELISA) were performed in accordance to the manufacturer's specification. IL-1α, TNF-α CXCL1, CXCL8, IL-6, CCL2, CCL5, CCL18, CCL20, CCL27 and CXCL12 were quantified using commercially available paired ELISA antibodies and recombinant proteins obtained from R&D System Inc. (Minneapolis, Minesota). For CXCL8/ IL-8 and TNF-α a PeliKine compact Kit (CLB, Amsterdam, The Netherlands) was used.

Statistics

Results shown are from 12 patients with wounds in the left and right breast, which were considered as duplicate measurements within one individual. Differences in chemokine and cytokine concentrations were compared using the one-way ANOVA test followed by a Dunnett's test. Differences were considered significant when *P<0.05, **P<0.01, ***P<0.001.

RESULTS

Changes in chemokine concentration during 6 hrs post-surgery

Levels of different chemokines were studied in wound fluid collected from intra- and subcutaneous drains inserted into breast reduction wounds. We first describe chemokine profiles independent of the location of the drain.

During the first six hours after initiation of the inflammatory response CXCL1, CXCL8, IL-6, CCL2, CCL5, CCL18, CCL20, and CCL27 were detectable in all drainage fluid samples, whereas IL-1α, TNF-α and CXCL12 were not detectable (detection limit of ELISAs = 25 pg/ml). When we compared chemokine concentrations at t=1 hrs post surgery (Table 2) the following was observed: i) CCL20, CXCL1 and CXCL8 were detected at <1 ng/ml in both drainage fluids, ii) CCL2, CCL27 and IL-6 at 1-10 ng/ml and iii) CCL5 and CCL18 at > 20 ng/ml in drainage fluids at 1 hr post-surgery. These results demonstrate a large variation in the concentration of the different mediators already at this early time interval. Since it was not possible to obtain drainage fluid at time 0 hr post-surgery absolute basal levels are unknown.

Next, we determined whether chemokine concentrations in drainage fluids increased or decreased within 6 hours post surgery. In all drainage fluid samples CXCL1, CXCL8, IL-6, CCL2 and CCL20 concentrations increased with time (Figure 1), whereas CCL5, CCL18 and CCL27 concentrations showed a trend to decrease (Figure 2). Since a large donor variation was observed (Table 2), levels are depicted relative to the level 1 hr post-surgery (Figure 1 and 2). In spite of the donor variation, duplicate measurements of absolute values (left and right breast) for all chemokines were comparable and each patient showed the same ability to increase or decrease the secretion of a particular mediator during the period studied. This finding is represented in figure 3 for CXCL1 and CCL5.

Table 2: Secretion of wound healing mediators (ng/ ml) 1hr and 6 hrs post surgery

		Absolute concentration (ng/ ml)	
Wound-healing mediator		**intracutaneous**	**Subcutaneous**
IL-1α		not detected	not detected
TNF- α		not detected	not detected
CXCL1/ Gro- α	t=1hr	0,37 +/- 0,23	0,51 +/- 1,61
	t=6hrs	1,63 +/- 1,06	2,25 +/- 1,13
CXCL8/ IL-8	t=1hr	*0,43* +/- 0,43	*2,47* +/- 2,79
	t=6hrs	29,52 +/- 44,32	35,23 +/- 44,80
IL-6	t=1hr	*1,58* +/- 2,24	*9,63* +/- 6,07
	t=6hrs	118,9 +/- 51,83	111,2 +/- 71,90
CCL2/ MCP-1	t=1hr	8,48 +/- 19,22	5,99 +/- 8,65
	t=6hrs	26,55 +/- 23,42	33,09 +/- 21,20
CCL5/ RANTES	t=1hr	71,7 +/-48,71	64,96 +/- 38,71
	t=6hrs	39,17 +/-17,44	35,78 +/- 31,23
CCL18/ PARC	t=1hr	17,15 +/-11,81	22,57 +/- 20,82
	t=6hrs	7,38 +/- 3,78	17,18 +/- 15,91
CCL20/ MIP-3 α	t=1hr	0,1 +/- 0,13	0,07 +/- 0,07
	t=6hrs	2,83 +/- 1,35	5,15 +/- 3,36
CCL27/ CTACK	t=1hr	*10,49* +/- 5,45	*4,92* +/- 2,73
	t=6hrs	5,17 +/- 2,53	3,06 +/- 1,91
CXCL12/ SDF-1		not detected	not detected

The concentration of wound healing mediators was determined by ELISA in intra- and subcutaneous drainage fluid from breast reduction surgery in 12 patients (left and right breast fluid values were averaged). The cytokine and chemokine concentration in drainage fluid at one hour and 6 hour post surgery is given as mean ± standard deviation. Statistical difference between intracutaneous and subcutaneous concentrations was calculated using one-way Anova post hoc Dunnet's and are shown by data being expressed in underlined italics, **P<0.01.

Comparison of intracutaneous and subcutaneous drainage fluid

In addition to the similar chemokine profiles observed in Figures 1 and 2, a number of notable differences were observed when comparing chemokine concentrations collected from intra- and sub-cutaneous drains indicative of chemokine gradients existing between the two drain locations (Table 2). For IL-6 and CXCL8 a difference was already apparent at 1 hour post surgery. The absolute concentrations of IL6 and CXCL8 were much lower (both approximately

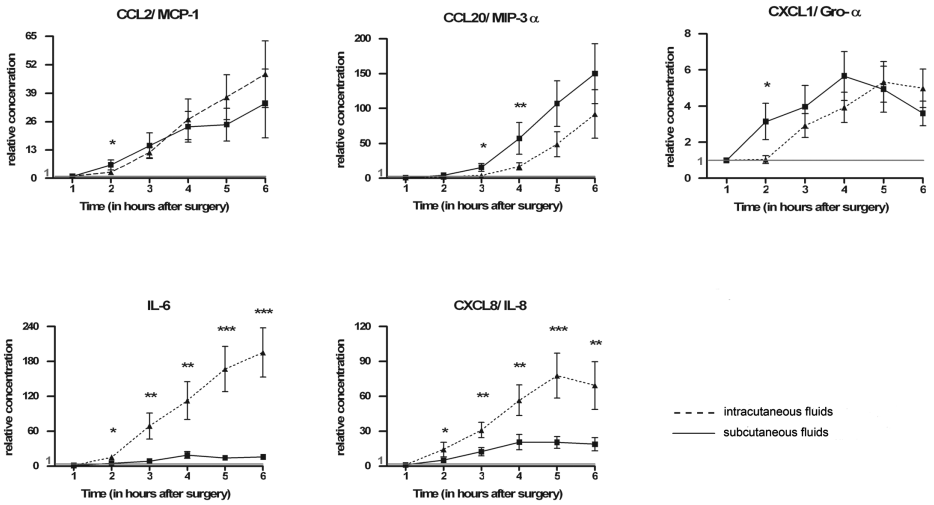

Figure 1: Relative increase in chemokine concentrations in intra- and subcutaneous drainage fluid during six hours post surgery.

The concentration of wound healing mediators was determined by ELISA in intra- and subcutaneous drainage fluid from breast reduction surgery in 12 patients (left and right breast fluid values were averaged) within the first six hours post surgery. Concentrations are given relative to the concentration at one hour after surgery, which is set to 1 as indicated by the grey line. The black lines represents subcutaneous drainage fluid and the dotted lines represent intracutaneous drainage fluid. Differences between the sub- and intracutaneous levels of chemokines in drainage fluid samples on different time points was determined using the ANOVA test followed by a Dunnett's test. *P<0.05, ** P<0.01, *** P<0.001.

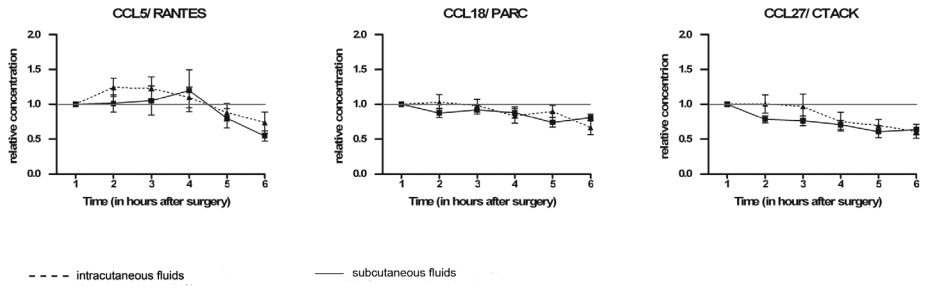

Figure 2: Unaltered relative chemokine concentrations in intra- and subcutaneous drainage fluid during six hours post surgery.

The concentration of wound healing mediators was determined by ELISA in intra- and subcutaneous drainage fluid from breast reduction surgery in 12 patients (left and right breast fluid values were averaged) within the first six hours post surgery. Concentrations are given relative to the concentration at one hour after surgery, which is set to 1 as indicated by the grey line. The black lines represents subcutaneous drainage fluid and the dotted lines represent intracutaneous drainage fluid. Differences between the levels of chemokines in drainage fluid samples on different time points was determined using the ANOVA test followed by a Dunnett's test.

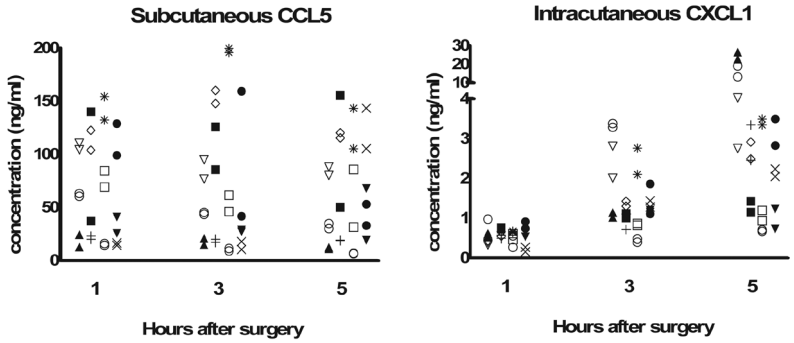

Figure 3: Drainage fluids isolated from duplicate breast surgical incisions show low intra donor variation in chemokine concentrations.

Duplicate measurements for drainage fluid isolated from left and right breast incisions for all 12 patients are depicted using different paired symbols. The concentration of intracutaneous CXCL1 and subcutaneous CCL5 at 1, 3 and 5 hrs post surgery was determined by ELISA.

6 fold) in intracutaneous fluid compared to subcutaneous fluid (IL-6: intra = 1.58 ng/ml and sub = 9.63 ng/ml; CXCL8: intra = 0.43 ng/ml and sub = 2.47 ng/ml). However, at t=6 absolute concentrations of IL-6 and CXCL8 are similar between both tissue compartments (IL-6: intra = 118.9 +/- 51.83 ng/ml and sub = 111.2 +/- 71.9 ng/ml; CXCL8: intra = 29.51 +/- 44.32 ng/ml and sub = 35.23 +/- 35.23 ng/ml). This indicates that the intracutaneous production of IL-6 and CXCL8 during the first 6 hours after surgery was much greater than the subcutaneous production. This is clearly illustrated in Figure 1.

No significant differences were observed between intra-and subcutaneous drainage fluid for the increase in CCL2, CCL5, CCL18, CCL20 and CXCL1 at 1 hr or 6 hr postsurgery, (Table 2). However, a significant difference was observed at early time periods for CCL2 (2 hours), CCL20 (3 and 4 hours) and CXCL1 (2 hours) due to a lag in the induction occurring in intracutaneous fluid compared to subcutaneous fluid. To be noted is that the concentrations of CCL2 and CCL20 were still increasing at 6 hrs post surgery in both intra- and subcutaneous drainage fluid whereas CXCL1 reached maximum concentration after t=5 hrs in intracutaneous drainage fluid and after t=4hrs in subcutaneous drainage fluid (Figure 1, Table 2).

Chemokine CCL27 was the only chemokine that showed a higher intracutaneous concentration compared to subcutaneous drainage fluid at 1 hr post surgery (Table 2). However, since intracutaneous concentrations decreased over the 6 hr study period whilst subcutaneous concentrations remained constant, by the end of the 6 hr study period no difference was observed between intra- and subcutaneous drainage fluids (Table 2).

DISCUSSION

This study compares chemokine concentrations in drainage fluids during the first six hours following elective breast surgery in order to define the early inflammation phase directly after surgery and to identify chemokine gradients existing within the skin immediately after initiating wound healing. Surgical drains were placed intra- and subcutaneously in order to compare inflammatory mediator levels between these different locations.

With regards to lymphocyte-specific chemokines, we found CCL18 and CCL20 to be present already 2 and 1 hour respectively post wounding. These chemokines were reported by others to be quiescent or low 1-4 days post injury (5,13). Our observation might have been missed in these reports since drainage fluid collection began 1 day post surgery. Our results indicate that lymphocytes are indeed attracted in the first hours after wounding and might play an important role as early immunological effectors. Indeed we found the concentration of CCL18 to be remarkably high immediately after injury. CCL18 plays a role in the homing of lymphocytes and dendritic cells, but has also been reported to induce collagen production by fibroblasts (14,15).

Monocytes are recruited to the site of inflammation approximately 8-12 hours after injury where they differentiate into macrophages and dendritic cells in order to elicit an immune response. Here, we demonstrate a very high expression of monocyte/ macrophage attracting chemokine CCL5 already at 1 hour post surgery. Recently we have shown that CCL5/ RANTES, in addition to its known role in attracting monocytes and macrophages, is a potent chemoattractant molecule for mesenchymal stem cells derived from the adipose tissue (16,17). The homing of stem cells into the wound is considered necessary for tissue regeneration and wound healing. Until now, the only monocyte/ macrophage attracting chemokine to be reported in human skin wounds is CCL2/ MCP-1 and this chemokine was detected at 1 day post surgery (5,13,1). Our study shows that CCL2/ MCP-1 is already expressed at 1 hour after surgery (6-8 ng/ml) and that within the first 6 hours after surgery peak levels are still not reached.

Neutrophils are one of the first inflammatory cells to migrate into the wound bed upon tissue damage. Our study shows that the peak levels of CXCL1, CXCL8 and IL-6 already occur at the early time period of 4-6 hours post surgery. This emphasizes a role for these chemokines in the immediate response, most likely in recruiting polymorphonuclear cells within minutes after injury. Until now reports have described CXCL1, CXCL8 and IL-6 expression only at 1 day post surgery coinciding with neutrophil accumulation in the wound bed (5, 13,18,19,20).

In addition to identifying chemokines involved in early inflammation, our study also identifies specific chemokine gradients between the intra- and sub- cutaneous drain location sites. Notably, even though CXCL8 and IL-6 were detected in both intra- and subcutaneous drainage fluids, in agreement with the presence of various leukocyte subsets within the wound area (18,5,20), the relative increase in production of both mediators was much more pronounced

intracutaneously than subcutaneously. This is in line with previous studies that show that CXCL8 and IL-6 are released by keratinocytes and fibroblasts upon tissue damage (21,22,23). Also, CCL27 is a unique chemokine in that it is secreted only by epidermal keratinocytes and is released upon tissue damage (24,20). Our results demonstrate significantly higher CCL27 levels in drainage fluid collected from the intracutaneous compartment compared to the subcutaneous compartment. Taken together, these observations show that chemokine profiles in drainage fluids are dependent on chemokine production of cell types in the vicinity of the drain and that this in turn creates a chemokine gradient within and around the wounded tissue. Additionally it confirms that our methodology used to collect drainage fluid at different depths in a surgical wound is a viable technique. Since most studies to date have reported on wound healing mediators detected in deep surgical drainage fluids (10,25,11,26,12), it can now be questioned in how far deep subcutaneous drainage fluid is representative of an acute dermal wound, particularly when different studies describe different surgical locations. In our study the subcutaneous drainage fluid most probably contains wound healing mediators derived not just from subcutaneous tissue but also from the underlying healing mammary glands.

In our study, TNF-α and IL-1α levels were found to be below the detection limit of our ELISA. Since these potent pro-inflammatory cytokines are secreted by keratinocytes and macrophages upon injury, they would have been expected to be present in wound fluid from surgical wounds (26,1,23,20). However TNFα and IL1α also bind to cell surface receptors and are taken up by target cells in order to initiate the chemokine cascade. The net effect of this would result in these cytokines being continuously removed from the wound fluid.

All donors developed normotrophic scars six months after wound closure (data not shown) indicating that our study describes normal wound healing. However, enormous inter-donor variation was observed with chemokine concentrations even though intra-donor variation was notably very low (between left and right breast incision wounds). This suggests that chemokine levels may be genetically determined or that the results reflect different degrees of trauma during surgery (large vs. small breast reduction). It is also possible that highly variable levels may influence scar formation or the risk of post-operative infections (9).

Our study describes chemokines and chemokine gradients which are regulated during the very early hours of normal wound healing. It indicates that the inflammatory response is initiated very rapidly after wounding (within hours rather than days) in order to attract neutrophils, monocytes, macrophages and lymphocytes into the wound bed. Further examination of chemokines such as these during adverse wound healing, e.g.: hypertrophic and keloid scar formation, may lead to increased understanding of the mechanisms involved in scar formation and improved therapeutic strategies.

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