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Differential Expression of Chemokines in a Mouse Model of Wound Healing

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Abstract. Macrophages have a multifaceted role in wound healing. While their initial activity may be in the degradation and elimination of damaged tissue, macrophages also produce and secrete a variety of mediators that can participate in the repair process as well. To perform these functions, macrophages must be recruited to a wound site. Our purpose was to examine the temporal and spatial expression of macrophage chemoattracting cytokines (chemokines) at a surgical wound site. A surgical wound was prepared on the dorsal aspect of B6AF1/J mice. Biopsies were obtained from the wound and a comparable nonwounded area between 6 and 72 hr after wounding. The presence or absence of various chemokine mRNAs was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR). Immunohistochemical staining and in situ RT-PCR determined localization of cells producing chemokines. In wounded tissue, both macrophage chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) were detected; however, the time of expression differed for each molecule. MCP-1 mRNA was detected at 6 hr after wounding, with decreased expression at subsequent time periods. In contrast, MIP-1 messages were not observed until 24 hr after wounding, and steadily increased thereafter. MCP-1 and MIP-1 mRNA and protein were localized predominantly in keratinocytes. The rapid and strong expression of MCP-1 and MIP-1 messages within the wound site suggests a pivotal role for these chemokines in the repair process. The differences in appearance and level of expression over time, however, suggest distinctive functions for each chemokine and indicate that the local milieu, rather than a single cytokine, influences macrophage recruitment and/or activation.

Keywords: Chemokines, wound healing, chemoattractant, MIP-1, MCP-1, TGF-β

Introduction

Healing of wounds requires a network of repair mechanisms that influence the recruitment and infiltration of leukocytes at the wound site. During the inflammatory phase of the repair process, macrophages are found in increasing numbers and have been shown to be necessary for wound repair [1,2]. Yet certain growth factors and cytokines that are produced and secreted by activated macrophages have also been implicated in the scarring that occurs during wound healing [3]. Understanding the early mechanisms by which macrophages are recruited to a wound site could assist in managing the potential adverse effects of macrophage activation, without inhibiting the positive functions that macrophages perform in wound repair.

The ability to attract monocytes from blood into tissue, where monocytes then differentiate into activated macrophages, is proposed to be one function of the chemoattracting cytokines, called chemokines. The various chemokines appear to be both pleotrophic and redundant with respect to their target cells and biological activities, at least when studied in vitro [4]. Local expression of cytokines is required for the migration of monocytes to extravascular sites [5-7]. While macrophage chemoattractant protein-1 (MCP-1)

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[5,6] and macrophage inflammatory protein-1 (MIP-1) [6-9] have both been observed within wounds, correlation of their appearance has not been explored. To investigate the roles of these monocyte-attracting chemokines in wound healing, we have used a murine model of wounding to establish their temporal and site-specific expression early in the repair process.

Methods and Materials

Animals. Female B6AF1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in a conventional animal facility, and given food and water ad libitum. Animals were used at 9-16 weeks of age. All experiments were approved by the Institutional Animal Care and Use Committee of Marshall University School of Medicine.

Experimental design. Mice were anesthetized, the hair on the dorsal aspect was shaved, and the skin was prepared for surgery by swabbing with iodine solution. A single 25-30 mm full-thickness incision was made and the wound was closed with stainless steel staples. On some animals, a second site on the dorsal aspect was designated as nonwounded; here the hair was shaved and iodine was applied but no wound was made. This site was at least 8 mm from the wounded site. At 6, 24, 48, and 72 hr after wounding, the wound and nonwounded sites were excised. Control untreated skin, that is, skin from animals not shaved nor swabbed with iodine solution, was also used. Each excised site was divided into three pieces. One piece was place in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A second piece was cut into small segments and snap-frozen in liquid nitrogen for RNA extraction. The third piece was frozen in liquid nitrogen for in situ RT-PCR and immunohistochemistry.

RNA extraction and RT-PCR. Tissues were homogenized with a Tekmar SDT Tissumizer (Tekmar, Cincinnati, OH) and total cellular RNA was extracted using TRIzol Reagent (Life Technologies, Grand Island, NY), according to the manufacturer's instructions. The extracted RNA was stored at -70°C. RNA in RNase-free water was first incubated for 3 min at 70°C and chilled on ice. cDNA was synthesized from 1 μg total RNA in a 30 μl reaction volume using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) and oligo $d(T)_{16}$. Four μl aliquots of the cDNA reaction products were amplified by PCR in 50 μl reactions using Taq polymerase (PE Applied Biosystems, Foster City, CA) and 0.4 μM each of the respective primers. The reaction consisted of 23 (MCP-1) or 35 (all other reactions) cycles in a Perkin-Elmer DNA Thermal Cycler Model 480, with denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. After the final cycle, the temperature was maintained for an additional 7 min at 72°C. PCR products were analyzed by electrophoresis on 3% NuSieve/1% SeaKem agarose gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. Starting quantities of 1-6 x 10^{-10} μg of chemokine/cytokine specific cDNAs produced detectable PCR products. Photographs of the gels were taken with Polaroid 667 film. The prints were scanned using Adobe Photoshop software and inverted images were printed onto Hewlett Packard transparency film (Palo Alto, CA). Band intensities were quantified by densitometry using a Personal Densitometer (Molecular Dynamics Inc, Sunnyvale, CA) with an OD_{260} limit of sensitivity of 0.01 units. After subtracting background OD_{260}, the relative level for each chemokine or cytokine was calculated as OD_{260} for the chemokine or cytokine band, divided by the OD_{260} for the β-actin band. β-actin (540 base pair product), MIP-1 (279 base pair product), and TGF-β (525 base pair product) primers were purchased from Clontech (Palo Alto, CA). The MCP-1 and oligo $d(T)_{16}$ primers were synthesized at the Marshall University DNA Core Facility. The MCP-1 primer sequences were as follows:
5'primer 5'AGCACCAGCCAACTCTCACT3' and 3'primer 5'TCTGGACCCATTCCTTCTTG3' (301 base pair product).

In situ (IS) RT-PCR. IS RT-PCR was performed using IS PCR glass slides and the GeneAmp in situ PCR system 1000 (PE Applied Biosystems). Ten-μm frozen sections were placed on slides and fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) for 15 min on ice. In some experiments,

sections were permeabilized with 0.1% saponin in DPBS for 5 min on ice. After washing, DNA was digested with 2U/μl DNase (Worthington Biochemicals, Freehold, NJ) solution containing 0.1 M sodium acetate and 0.005 M $MgSO_4$ for 4 hr at 37°C and again in fresh DNase overnight at 37°C. After washing, cDNA was synthesized at 42°C for 60 min. On each slide, one section was incubated in a cDNA reaction mixture without reverse transcriptase and served as a negative control. PCR was performed using a 50 μl reaction mixture containing 1x IS PCR reaction buffer, 4.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphates, 50 μl IS Taq polymerase (PE Applied Biosystems), 10 μM digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN), and 0.8 μM each of the respective primers (MCP-1 and MIP-1). Amplification consisted of 2 min at 95°C, then 30 cycles of denaturation at 95°C for 1 min and annealing at 60°C for 1 min with a final extension at 72°C for 7 min. After washing, incorporation of digoxigenin-11-dUTP was demonstrated by incubation in alkaline phosphatase conjugated anti-digoxigenin (Boehringer Mannheim) overnight at 4°C, followed by visualization using Vector Red substrate (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. Five samples representing three different time periods were analyzed for each chemokine.

Immunohistochemistry. Skin tissue was snap-frozen in liquid nitrogen and stored at -70°C. Cryosections (10 μm) were fixed in acetone for 5 min at room temperature and then stored at -20°C until used. All staining was performed at room temperature in a humidified chamber. On the day of staining, sections were incubated in 10% normal goat or hamster serum, followed by avidin-biotin blocking solution (Vector, Burlingame, CA). After washing, they were reacted with primary antibody directed to MCP-1 (hamster anti-mouse MCP-1, Pharmingen, San Diego, CA) or MIP-1 (goat anti-mouse MIP-1α, Sigma, St. Louis, MO), then with biotin-conjugated anti-species IgG. An avidin: biotinylated horseradish peroxidase complex (Vectastain ABC Reagent, Vector) was used with 3,3'-diaminobenzidine and hematoxylin counterstain to visualize cells. Tissue sections treated with PBS instead of the primary antibody served as control samples and gave negative results. Two samples representing two different time periods were analyzed for each chemokine.

Statistical analysis. Wounded and nonwounded skin were compared to control (normal) skin for chemokine and cytokine expression. Some groups were not normally distributed and nonparametric methods were therefore used. Groups were compared using the Kruskal-Wallis one-way ANOVA test (SigmaStat 2.0 program). Significance was defined as $p < 0.05$.

Results

Detection of chemokine mRNA. We recently demonstrated the expression of monocyte-attracting chemokine mRNAs during experimental mouse wound healing, using RT-PCR [10]. Because induction of chemokines appears exquisitely sensitive to variations in the tissue environment, we wished, in addition, to determine the expression of certain chemokines in normal control skin. In this study, we have confirmed our findings for the chemokines, MCP-1 and MIP-1, and the cytokine, TGF-β, and we have compared their expression in wounded and nonwounded skin with control (normal) skin, that is, skin neither wounded nor prepared for surgery. Table 1 shows data for gene expression of MCP-1, MIP-1 and TGF-β at specific time periods after wounding. Chemokine or cytokine values are standardized to β-actin for each specimen (eg, MCP-1/β-actin ratio) and the data are reported as relative levels.

MCP-1 mRNA, while undetectable in control skin, was observed in some animals as early as 6 hr after wounding. By 24 hr, MCP-1 message was found in all animals and the levels were significantly higher in wounded versus control skin at this time and at 48 hr after wounding ($p < 0.05$). Whereas MCP-1 expression was also observed in nonwounded skin, the levels were not significantly different from those found in control skin at any time point.

In contrast, the expression of MIP-1 mRNA, which is constitutively expressed in control (normal) skin, was dramatically reduced at 6 hr and appeared to be the result of preparing the skin for surgery, in that nonwounded and wounded skin both showed this effect. At 24 hr, MIP-1 message was observed in

Table 1. Relative levels of chemokines and cytokines in control, nonwounded, and wounded skin samples[a]

Skin sample & time	N	MCP-1 mean±SD [median]	N	MIP-1 mean±SD [median]	N	TGF-β mean±SD [median]
Control	3	0.00[b] [0.00]	3	0.37±0.16 [0.44]	3	1.61±0.10 [1.56]
Nonwounded						
6 hr	3	0.30±0.52 [0.00]	3	0.00[c] [0.00]	4	0.98±1.67 [0.23]
24 hr	5	0.08±0.05 [0.09]	5	0.00[c] [0.00]	5	0.61±0.14[c] [0.61]
48 hr	4	0.08±0.12 [0.04]	4	0.00[c] [0.00]	4	0.76±0.47[c] [0.58]
72 hr	4	0.08±.0.14 [0.01]	4	0.40±0.43 [0.37]	4	0.81±0.32[c] [0.94]
Wounded						
6 hr	5	0.59±0.56 [0.82]	5	0.00[c] [0.00]	5	0.72±0.35 [0.53]
24 hr	6	0.38±0.28[c] [0.36]	6	0.16±0.13[c] [0.14]	6	0.64±0.32[c] [0.53]
48 hr	5	0.30±0.19[c] [0.21]	5	0.37±0.22 [0.28]	5	0.55±0.23[c] [0.57]
72 hr	6	0.49±0.50 [0.40]	6	0.67±0.31 [0.68]	6	0.88±0.39[c] [0.93]

[a] N = number of specimens analyzed. [b] Chemokine/cytokine levels below the detection limits are given a value of 0.00. [c] $p < 0.05$ vs control skin samples, computed by Kruskal-Wallis one-way ANOVA..

wounded skin, albeit at low levels, and it recovered to control skin levels by 48 hr. In comparison, MIP-1 was not detected in nonwounded skin until 72 hr, at which time it had returned to control skin levels.

TGF-β transcripts were also examined and comparable levels were found in both wounded and nonwounded skin, but were significantly decreased relative to control skin ($p<0.05$), with the exception of samples obtained at 6 hr after wounding.

In situ localization of MCP-1 and MIP-1. In situ RT-PCR was used to study the distribution of MCP-1 and MIP-1 mRNA in wounded skin (Fig. 1A, 1B). Sections were first treated with DNase to remove genomic DNA and then reverse transcribed to generate cDNA for all mRNAs. This was followed by amplification with specific primers. For both MCP-1 and MIP-1, intensely positive single cells were found in a scattered pattern in the epidermis. Other cells positive for both chemokines were detected mainly in the lower dermis. MIP-1 mRNA was also consistently observed in follicular epithelium and sebaceous gland cells, while MCP-1 mRNA was only occasionally found in this location. Specificity of the messages was confirmed by lack of signal in sections incubated without reverse transcriptase (Fig. 1C).

The presence of MCP-1 and MIP-1α protein in wounded skin was detected by immunohistochemical

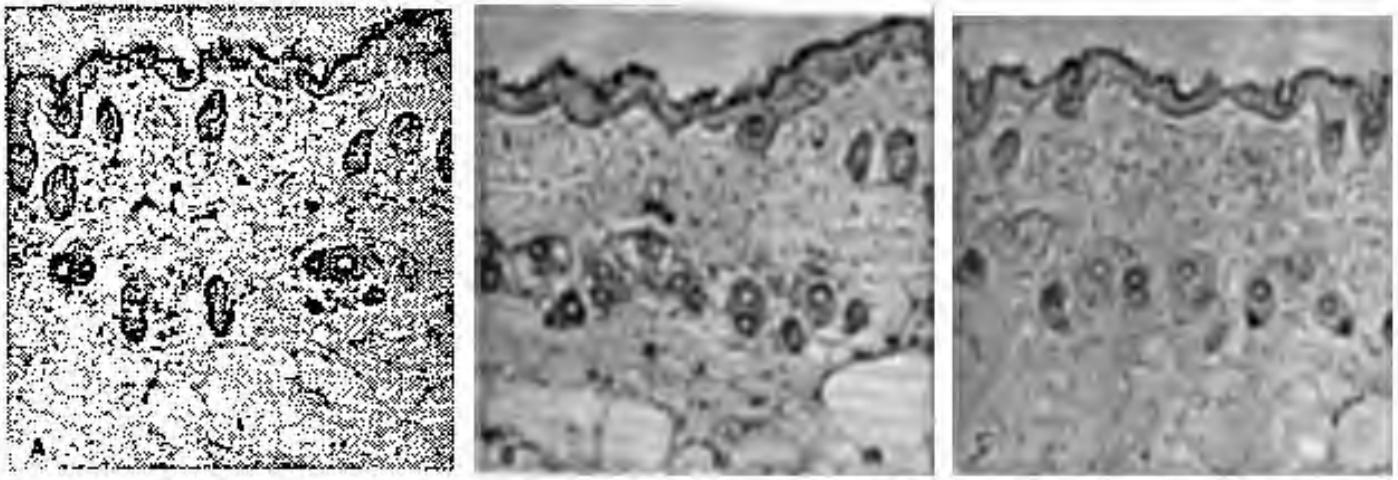

Fig. 1. Localization of MCP-1 and MIP-1 mRNA by in situ RT-PCR. Tissue sections from wounded skin, treated with DNase to remove genomic DNA, were reverse transcribed and then amplified with specific primers and labeled nucleotide. PCR products were detected by antibody to the nucleotide label. At 72 hr after wounding, MCP-1 (A) and MIP-1 (B) messages were found within the epidermis, hair follicles, and sebaceous glands, and in cells in the lower dermis. (C) A negative control section, treated as described but not reverse transcribed prior to PCR amplification, demonstrates a negative reaction; section shown is for MCP-1, with similar results for MIP-1. The photograph depicts tissue approximately 1 mm from the incision site. (Vector Red chromogen with hematoxylin; original magnification x150).

staining. MCP-1 immunoreactivity was found predominantly within the epidermis (Fig. 2A) corresponding to MCP-1 mRNA expression. Likewise, MIP-1α protein paralleled its mRNA localization, with positive staining in the epidermis and follicular epithelium and in association with sebaceous glands (Fig. 2B). Weak but specific MIP-1α reactivity was also observed in dermal cells (Fig. 2B). No staining for either protein could be detected in control sections in which the primary antibody was not used (Fig. 2C).

For both techniques, the staining patterns were independently assessed by two of the authors and were confirmed by a third person with expertise in microanatomy.

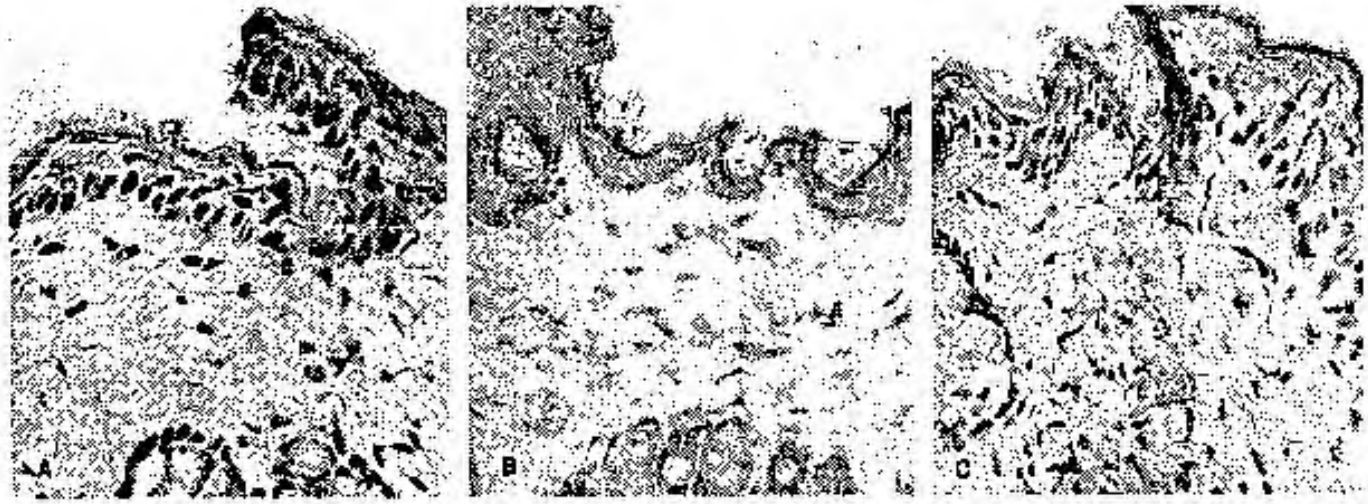

Fig. 2. Localization of MCP-1 and MIP-1α protein by immunohistochemistry. Tissue sections from skin 6 hr after wounding for MCP-1 (A) or 72 hr after wounding for MIP-1α (B) reveal specific signal within the epidermis and in cells in the dermis. MIP-1α is also found in hair follicles and sebaceous glands (B). (C) Sections in which the primary antibody was not used demonstrate a negative reaction; section shown for MCP-1 with comparable results for MIP-1α. The photograph depicts tissue approximately 1 mm from the incision site. (DAB chromogen with hematoxylin; original magnification x 400).

Discussion

Elucidating the mechanism for recruitment and activation of macrophages at a wound site is essential for a complete understanding of this cell's role in the repair process. In the present study, we employed a surgically wounded mouse model to examine the pattern and localization of monocyte-attracting chemokine gene expression in vivo.

Prior studies in mice and humans show that MCP-1 [5-7] and MIP-1 [6-9] are both associated with wounding. Whereas in previous investigations, each chemokine was studied either individually or at only one time period, in this study we tested both MCP-1 and MIP-1 at several times after wounding. MCP-1 appeared before MIP-1, with expression at 6 hr after wounding [5], while MIP-1 did not appear until 24 hr, with increasing expression through 72 hr [8,9]. These present results confirmed previous findings.

Our experimental design, however, revealed several unexpected observations in regard to MCP-1 expression. First, by making a distinction between nonwounded versus control (normal) skin, we found MCP-1, albeit at very low levels, in nonwounded but not control skin. While this difference may reflect the biopsy of nonwounded skin too close to the wound, careful efforts were made to delineate the two samples. Alternatively, our observation suggests that induction of MCP-1 gene expression at cutaneous sites is highly sensitive to mechanical or chemical stress and that the clipping of hair and/or treatment of the site with iodine in preparation for surgery may have initiated the slight expression of MCP-1 detected in nonwounded skin. Indeed, induction of cytokine expression is reported in mild epidermal barrier disruption in humans [11], lending support to this hypothesis. In addition, the appearance of MCP-1, even within wounded skin, was at relatively low levels, an observation that is consistent with reports that tissue-specific low levels result in accumulation of monocytes, while high levels of expression do not [12]. This suggests that inducing the optimal amount of MCP-1, rather than eliciting expression per se, may be necessary for the recruitment of monocytes/macrophages to a wound.

For MIP-1 mRNA, we observed constitutive expression in normal skin, supporting most [15-17] but not all [9] investigations. Based on these levels in control skin, the striking lack of MIP-1 at 6 hr post wounding, with a gradual increase to normal skin levels, implies an alternative function for this chemokine. Rather than recruiting macrophages to a wound site, MIP-1 may instead fine-tune the macrophages' activities [19] and promote other wound repair processes [9]. The increase in MIP-1 message after the appearance of MCP-1 in our model suggested that MIP-1 was modulating the inflammatory response rather than initiating it.

Cytokines are seldom expressed in isolation from other cytokines, so functionality must be assessed in conjunction with other molecules in the local milieu. TGF-β, a pleotrophic molecule involved in promoting the repair process [13], has also been shown to have an inhibitory effect on MCP-1 expression in macrophages [14]. Decrease in TGF-β in our model, concomitant with increased MCP-1 expression, suggests that physiologic concentrations in concert with timed expression may determine the function of a cytokine at a local site.

In our study, we also investigated the site of chemokine production by in situ RT-PCR and immunohistochemistry. Other reports localized MCP-1 to monocytes/macrophages [5-7,20] and to cells within the epidermis [7,20] and dermis [5,20]. Likewise, MIP-1 has also been identified in macrophages [6,9] as well as within the epidermis [16,17]. While we clearly found both MCP-1 and MIP-1 message and protein within the epidermis and at lower dermal locations, we also consistently observed both chemokines associated with hair follicles and sebaceous glands. It is interesting that the strongest expression was in cells of epithelial lineage. The implication of this finding with respect to function warrants further study.

Although the exact role of chemokines in the sequence of events and cellular interactions in the inflammatory phase of wound healing is still under investigation, it has been established that they are key molecules in the development of the repair process. By examining the differential expression of several chemokines/cytokines, our study suggested that to determine adequately the function of any particular cytokine in wound healing, its expression must be examined, not only with respect to time and place, but also in the context of the local milieu of other

cytokines. Such information may provide new strategies for enhancing repair processes by manipulating, directly or indirectly, macrophage activities at a wound site.

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