American Journal of Pathology, Vol. 146, No. 4, April 1995
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Modulation of JE/MCP-1 Expression in Dermal Wound Repair

Luisa A. DiPietro,*†‡ Peter J. Polverini,§ Sandra M. Rahbe,*† and Elizabeth J. Kovacs*||

From the Shock Trauma Institute and Department of Surgery† and Department of Cell Biology, Neurobiology and Anatomy||, Loyola University Stritch School of Medicine, Maywood, Illinois, and Hines Veterans Administration Hospital,‡ Hines, Illinois, and Laboratory of Molecular Pathology,§ University of Michigan, Ann Arbor, Michigan*

The tissue macrophage plays a prominent role in wound repair, yet the parameters that influence macrophage migration into the wound bed are not well understood. To better understand the process of macrophage recruitment, the production of JE, the murine homologue of monocyte chemoattractant protein 1(JE/MCP-1), was examined in a murine model of dermal wound repair. High levels of JE/MCP-1 mRNA were found in dermal punch wounds at 12 hours and 1 day (24 hours) after wounding; mRNA levels slowly decreased to undetectable by day 21. **In situ** ***hybridization analysis of wounds revealed that JE/MCP-1 was predominantly expressed by monocytic and macrophage-like cells, as well as by occasional fibroblasts and other interstitial cells. To correlate JE/MCP-1 production with macrophage migration, macrophage infiltration into the wound bed was quantitated. The number of macrophages within the wound increased to a maximum at day 3 (11.3 ± 4.5 macrophages per high power field), began to decrease at day 5 (4.8 ± 1.9 macrophages per high power field), and reached near base line at day 10 (3.0 ± 1.1 macrophages per high power field). The results demonstrate that JE/MCP-1 production within wounds is closely linked to the time course and distribution of macrophage infiltration, with maximal JE/MCP-1 mRNA levels occurring 1 to 2 days before maximal macrophage infiltration. The results support a role for JE/MCP-1 in the recruitment of wound macrophages and suggest that macrophages, through the production of JE/MCP-1, may sustain the recruitment of additional monocytes and macrophages into sites of injury.*** ***(Am J Pathol 1995, 146:868–875)***

The tissue repair process is characterized by an orderly pattern of cellular infiltration and regrowth. After an initial influx of neutrophils, macrophages become the predominantly inflammatory cell in the wound within a few days of injury.[1] Previous investigations by Leibovich and Ross[2] have documented the essential role of the macrophage in dermal wound repair, and macrophages within wounds have been shown to produce a wide range of mediators that promote the repair process, including angiogenic and fibrogenic cytokines.[3–6] Despite advances in the understanding of macrophage function within the context of wound repair, the chemoattractants that provide the stimulus for the sustained recruitment of macrophages into the wound bed are not yet known.

Migration of leukocytes into areas of inflammation requires both adherence to and migration through endothelium. Chemoattractants can influence both aspects of the recruitment process. Chemoattractants can augment leukocyte-endothelial cell adhesion by inducing a conformational change in surface adhesion molecules; this change causes an increased adhesiveness in the responding cell.[7–9] In addition, chemoattractants induce cellular migration through the vessel wall toward the chemoattractant gradient. A number of leukocyte chemoattractants are released by platelets during wound hemostasis, including platelet-derived growth factor, transforming growth factor-β, and platelet factor 4.[10–14] Other chemoattractants that are generated in the early wound include inflammatory mediators such as the activated complement component C5a, leukotriene B4, and platelet-activating factor.[15–17] However, the majority

Supported by the Falk Medical Research Trust (LAD), Loyola University BRSG (LAD), National Institutes of Health Grant HL39926 (PJP), and the RGK Foundation (EJK).

Accepted for publication January 12, 1995.

Address reprint requests to Dr. Luisa A. DiPietro, Shock Trauma Institute, Loyola University Medical Center, 2160 S. First Ave., Maywood, IL 60153.

of the chemoattractants that are found in the immediate wound environment attract both neutrophils and macrophages. The shift to a predominantly macrophage infiltrate in the later stages of wound repair is hypothesized to involve the active synthesis of factors that preferentially attract monocytes rather than neutrophils.[18]

Direct evidence for the production of several proteins that are preferentially chemoattractant for macrophages over neutrophils has been provided in rodent wound models. lida and Grotendorst[19] have demonstrated the active transcription of the inflammatory cytokine *gro*, a potent chemoattractant for monocytes and macrophages, in rat dermal wounds. Other powerful monocytic chemokines that have been described to be produced by inflammatory cells derived from dermal wounds include macrophage inflammatory protein 1 and macrophage inflammatory protein-2.[6] One relevant cytokine that has yet to be examined in the healing wound is monocyte chemoattractant protein 1 (MCP-1). JE/MCP-1 is an early response gene, the product of which is potently chemoattractant for monocytes but not neutrophils.[20] In this study, we describe the physiological modulation of JE, the murine homologue of MCP-1, in a murine model of normal wound repair.

Materials and Methods

Animals and Injury

All procedures that were performed on animals were reviewed and approved by the Loyola University Institutional Animal Care and Use Committee. Female adult 3- to 1-month-old Balb/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized by inhalation of Metofane and the hair shaved from the dorsal surface. Four full thickness punch wounds through the panniculus carnosus were prepared on the dorsum of each animal with a standard 3-mm diameter dermal punch biopsy instrument. At various time points after wounding, mice were killed by cervical dislocation and the wounds plus 1 mm of adjacent tissue removed for analysis.

Immunohistochemistry and Macrophage Quantitation

Immunohistochemical staining was performed with F4/80,[21] a rat monoclonal antibody specific for mature murine macrophages. Sections were fixed in acetone at room temperature for 15 minutes, pretreated with 3% H_2O_2 in methanol to block endogenous peroxidase, and then blocked for nonspecific binding with normal mouse serum (1:1000). Incubations in primary antibody (F4/80, 1:10; a generous gift of Dr. Pamela Witte, Loyola University Medical Center) and secondary antibody (biotinylated mouse anti-rat Ig, 1:100; Jackson Laboratories, West Grove, PA) were performed for 30 minutes each, followed by a 30-minute incubation with avidin-biotin-horseradish peroxidase complexes (ABC, Vector Laboratories, Burlingame, CA). Color development was performed with 3,3′-diaminobenzidine and slides counterstained with Gill's hematoxylin. To quantitate macrophages, the number of F4/80-positive cells was counted in 10 random ×200 high power fields (HPFs) within the wound bed. For each time point, at least three individual wounds were counted. The average number of cells per HPF for each time point was determined.

RNA Preparation and Analysis

Total RNA was prepared by the guanidine isothiocyanate method of Davis et al.[22] Probes were pcJE-1, which represents the murine homologue of MCP-1 (American Type Culture Collection, Rockville, MD),[23] and β-actin. Probes were labeled with ^{32}P by random priming to a specific activity of at least 10^8 cpm/μg. mRNA analysis was performed by standard gel electrophoresis, blotting, and aqueous hybridization of Gene Screen Plus membranes (DuPont-NEN, Wilmington, DE) according to the manufacturer's directions. Autoradiography was performed with intensifying screens at −70 C for 6 to 24 hours.

In situ *Hybridization*

In situ hybridization was performed as described by Ausubel et al.[24] Briefly, fresh frozen histological sections were dried for 1 to 2 minutes at 50 C and then fixed for 20 minutes in 4% paraformaldehyde. Riboprobes were prepared by *in vitro* transcription of the plasmid pcJE with [^{35}S]UTP. For antisense riboprobes, T7 polymerase was used; the control sense riboprobe was generated with SP6 polymerase. Sections were subjected to a ribosome disruption (0.2 N HCl for 5 minutes), deproteinization (1 μg/ml proteinase K for 3 minutes), and acetylation (0.1 mol/L triethanolamine-HCl, pH 8.0, 0.25% acetic anhydride for 5 minutes). Slides were hybridized in hybridization solution (50% formamide, 0.6 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L heparin, 50 μg/ml dithiothreitol (DTT), 0.5 mg/ml carrier DNA, and 0.5 mg/ml tRNA) containing 2.5 × 10^7 cpm/ml riboprobe at 50 C for 16 hours. Slides were washed in 2X standard

saline citrate (SSC) (1X SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate) containing 10 mmol/L DTT at 50 C for 30 minutes. Slides were treated with RNAse A (20 µg/ml) at 37 C for 30 minutes and then washed in 2X SSC containing 50% formamide and 10 mmol/L DTT at 50 C for 30 minutes. Slides were then subjected to two 30-minute washes at 50 C in 2X SSC containing 10 mmol/L DTT and 1% sodium pyrophosphate. Slides were dried, dipped in NTB-2-type emulsion, and exposed for 14 to 21 days. After development, slides were counterstained with standard hematoxylin and eosin.

Results

Migration of Macrophages into Wound Sites

An inflammatory infiltrate was prominent in 12-hour, 1-day (24-hour), and 2-day dermal wounds, and inflammatory cells were detected throughout the course of the histological experiment (to day 10). To determine the relative number and sequence of macrophage migration into dermal punch wounds, immunohistochemical staining of wound tissue was performed with F4/80, a rat monoclonal antibody specific for mature murine macrophages.[21] Quantitation of macrophage infiltration into dermal wound sites demonstrated that the number of F4/80-positive cells within the wound increased to a maximum of 11.3 ± 4.5 macrophages/HPF at day 3, declined at day 5 (4.8 ± 1.9 macrophages/HPF), and reached near base line levels at day 10 (3.0 ± 1.1 macrophages/HPF) (Figure 1). Normal skin contained 1.5 ± 1.1 macrophages/HPF. These results are in agreement with the time course of macrophage migration into incisional wounds in other rodent models.[2] Epidermal regeneration was rapid in this model, with complete re-epithelialization occurring in all wounds examined by day 5.

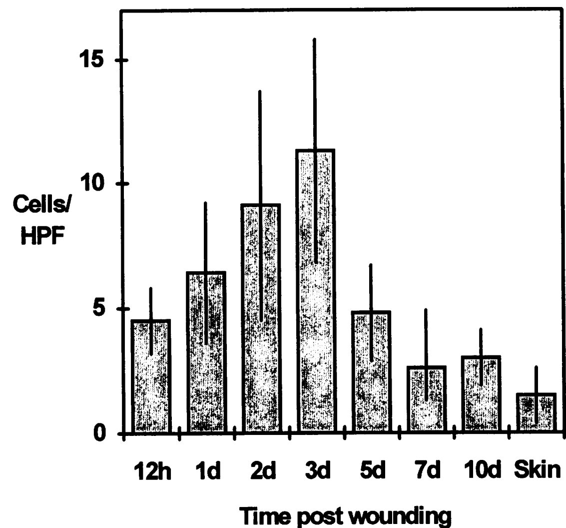

Figure 1. *Macrophage infiltration into wounds. The number of mature macrophages (cells staining positive with F4/80 monoclonal antibody) per HPF was determined in 10 random fields within the wound bed in each of three individual wounds. The calculated mean number of macrophages per HPF and standard deviation (line) are shown.*

Analysis of JE/MCP-1 mRNA in Dermal Wounds

To determine whether JE/MCP-1 is actively transcribed in murine dermal wounds, mRNA from 12-hour and 1-, 2-, 3-, 5-, 10-, 14-, 21-, and 28-day-old wounds was analyzed for JE/MCP-1 mRNA. Northern analysis of mRNA from wounds showed that JE/MCP-1 mRNA was detectable in wounds at time points from 12 hours to 14 days (Figure 2). JE/MCP-1 mRNA levels peaked at 12 hours to 1 day after wounding, diminished significantly by 2 days, and returned to an undetectable level between 14 and 21 days (Figure 2). A similar pattern of expression of JE/MCP-1 was seen in incisional wounds, although, the relative level of steady state JE/MCP-1 mRNA was higher in punch wounds than incisional wounds (data not shown). No JE/MCP-1 mRNA was detectable in normal skin. To assure consistency of the observation, mRNA analysis was repeated in two additional sets of wounds. In each experiment, the greatest expression of JE/MCP-1 was observed at 1 day after wounding, and JE/MCP-1 mRNA levels reached undetectable levels between 14 and 21 days (data not shown).

Localization of JE/MCP-1 mRNA within Wounds by *in situ* Hybridization

To determine the cellular source of JE/MCP-1 in dermal wounds, serial sections of wound tissues were subjected to *in situ* hybridization with antisense JE or control sense riboprobes of equivalent specific activity. Early wounds (12 hours, 1 day, and 2 days after wounding) showed significant hybridization with the antisense JE probe (Figures 3 and 4). At 12 hours, numerous cells within the wound bed were strongly positive for JE/MCP-1 mRNA (Figure 3A, B). Control sections from 12-hour wounds that were hybridized with sense probes showed only background labeling (Figure 3C, D). The JE/MCP-1-positive cells were large, vacuolated mononuclear cells with eccentric

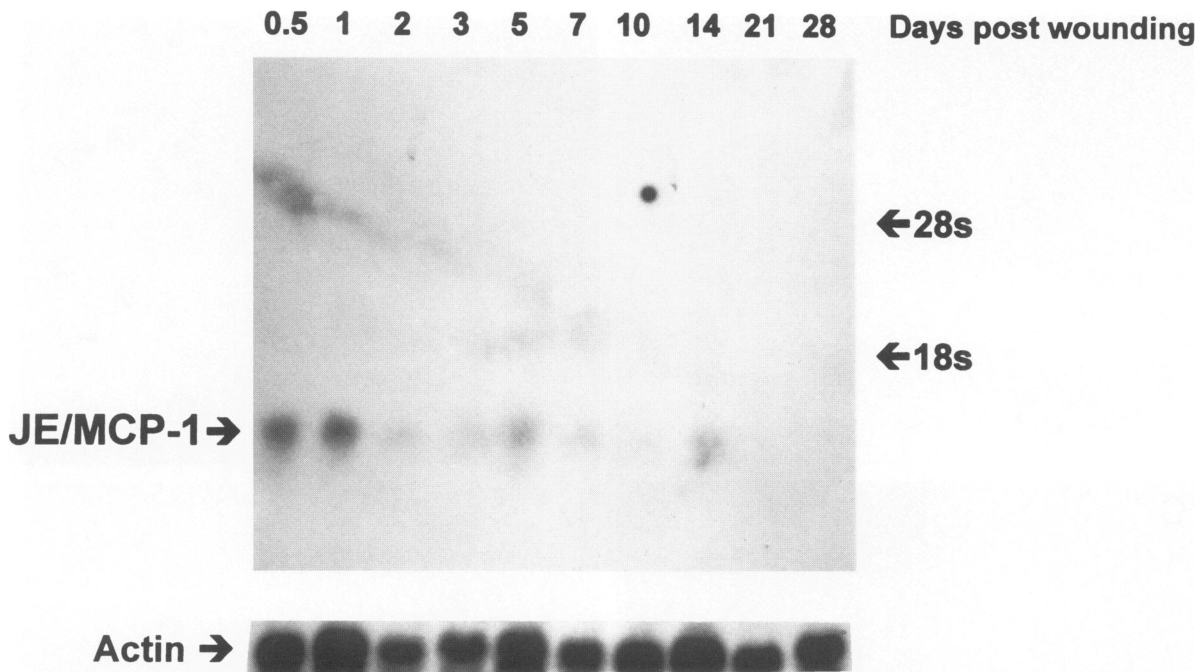

Figure 2. *mRNA analysis of JE/MCP-1 mRNA in punch wound tissue. mRNA blots were hybridized with probes specific for either JE or β-actin, as shown. Both panels depict a blot of mRNA from punch wounds that was probed sequentially; each exposure was performed for 20 hours. Lanes contain 10 μg of total mRNA prepared from days 0.5 (12-hour), 1, 2, 3, 5, 7, 10, 14, 21, and 28 day wounds.*

nuclei and occasionally contained phagocytosed debris (Figure 3A, B). On the basis of these criteria, we judged these cells to be macrophages. Few, if any, polymorphonuclear leukocytes were positive. The reactivity pattern of the 1-day wound bed was similar to that at 12 hours (Figure 4A, B). At day 2, numerous JE/MCP-1-positive cells were still apparent within the wound bed but at diminished numbers as compared with the 12-hour and 1-day wounds (Figure 4C). By day 2, occasional interstitial cells with a fibroblast-like morphology were also positive (Figure 4D). These cells were located within the dermis immediately adjacent to the wound. The *in situ* hybridization results correlated with the mRNA blot analysis, which showed a peak in JE/MCP-1 mRNA levels at 12 hours and 1 day.

To confirm that the cells within the wound that were actively producing JE/MCP-1 were macrophages, serial sections from day 1 wounds were subjected to *in situ* hybridization and immunostaining with F4/80, a rat monoclonal antibody directed against mature murine macrophages.[21] The tissue sections were cut at 12 μ to facilitate the *in situ* hybridization, and, therefore, many individual cells did not appear in both of two sequential sections. However, careful analysis of individual cells at a measurable distance from histological landmarks such as vessels did allow for the identification of cells that were visible in two sequential serial sections. Those cells that were positive for JE/MCP-1 by *in situ* hybridization were also F4/80 positive in the adjacent section. Thus, this experiment established that cells within the wound bed that contained abundant JE/MCP-1 mRNA were also F4/80 positive (Figure 5).

Discussion

In this investigation, early infiltrating monocytes and macrophages within wounds are shown to be a prominent source of the monocyte chemoattractant JE/MCP-1. This observation strongly suggests a role for JE/MCP-1 in the recruitment of monocytes and macrophages to sites of injury and implies that those macrophages that initially infiltrate wounds may actively recruit additional macrophages.[25] Fibroblasts, which have been shown to be a source of JE/MCP-1 in chronic granulomatous tissue,[26] do not appear to be a significant source of this cytokine within dermal wounds. This finding intimates an important mechanistic difference between acute and chronic granulation tissue. In chronic inflammation, in which sustained cytokine production occurs, fibroblasts may be induced to actively secrete JE/MCP-1. In contrast, fibroblasts in the early wound, which are located external to the wound bed during early hemostasis and acute inflammation, may not be exposed to sufficient levels of initiating cytokines. This possibility is sup-

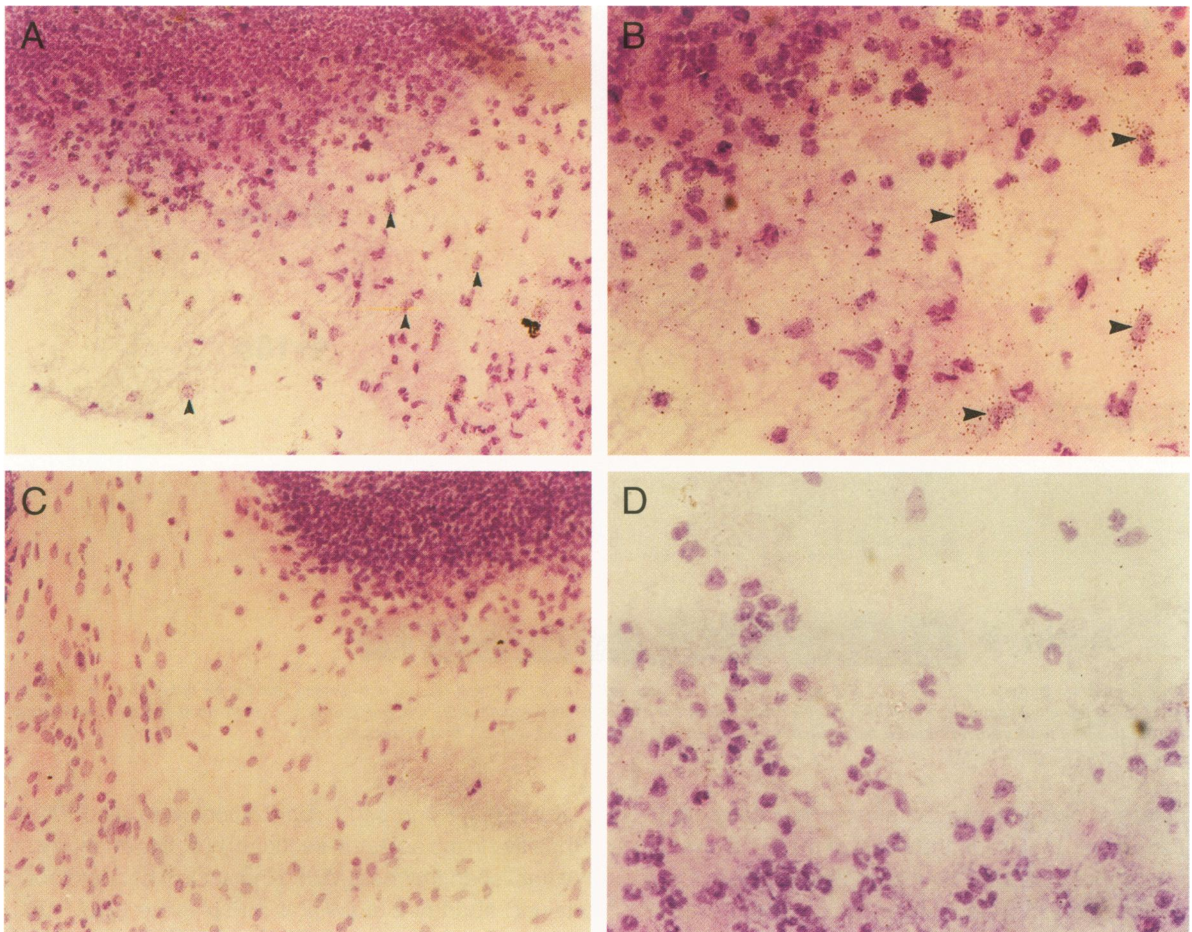

Figure 3. *Photomicrograph of* in situ *hybridization of dermal punch wounds. Hybridization was performed with either antisense* (**A** *and* **B**) *or sense* (**C** *and* **D**) *probes specific for JE. Arrows indicate examples of heavily labeled monocytic and macrophage-like cells.* **A**: *12-hour wound; magnification, ×200.* **B**: *12-hour wound; magnification, ×400.* **C**: *12-hour wound; magnification, ×200.* **D**: *12-hour wound; magnification, ×400.*

ported by the observation that some fibroblasts in close approximation to the early wound bed do express JE/MCP-1.

An increase in the production of JE/MCP-1 has been documented in a variety of inflammatory pathological situations, including pulmonary fibrosis, atherosclerosis, allograft rejection, delayed-type hypersensitivity, and rheumatoid arthritis.[27–33] Therefore, the abundant expression of JE/MCP-1 in the inflammatory setting of acute wounds is perhaps not unexpected. However, as opposed to pathological situations in which sustained JE/MCP-1 expression is observed, JE/MCP-1 mRNA expression is swiftly down-regulated in the normal healing wound. As such, dermal wounds might provide a valuable *in vivo* model in which to further investigate the physiological modulation of JE/MCP-1 gene expression. The rapid increase in JE/MCP-1 expression in the early wound could reflect the monocyte response to early inflammatory mediators that are produced during hemostasis. One likely trigger of JE/MCP-1 expression within wounds is thrombin, as thrombin has been shown to be a potent inducer of JE/MCP-1 expression in monocytes.[34,35] Both thrombin and thrombin receptor-activating peptides have been shown to augment the wound repair process, but the mechanism by which these proteins accelerate the repair process has not yet been investigated.[36] If the addition of thrombin induces increased levels of JE/MCP-1, the subsequent recruitment of increased numbers of macrophages would lead to a more rapid repair. In addition to thrombin, numerous cytokines that up-regulate JE/MCP-1 expression, such as tumor necrosis factor-α, interleukin 1, and platelet-derived growth factor, are present in wounds.[6,37,38] However, the peak production of cytokines within wounds most probably occurs at a later time point than that observed for JE/MCP-1 production, suggesting that the active synthesis of cytokines is not responsible for the observed early induction of JE/MCP-1.

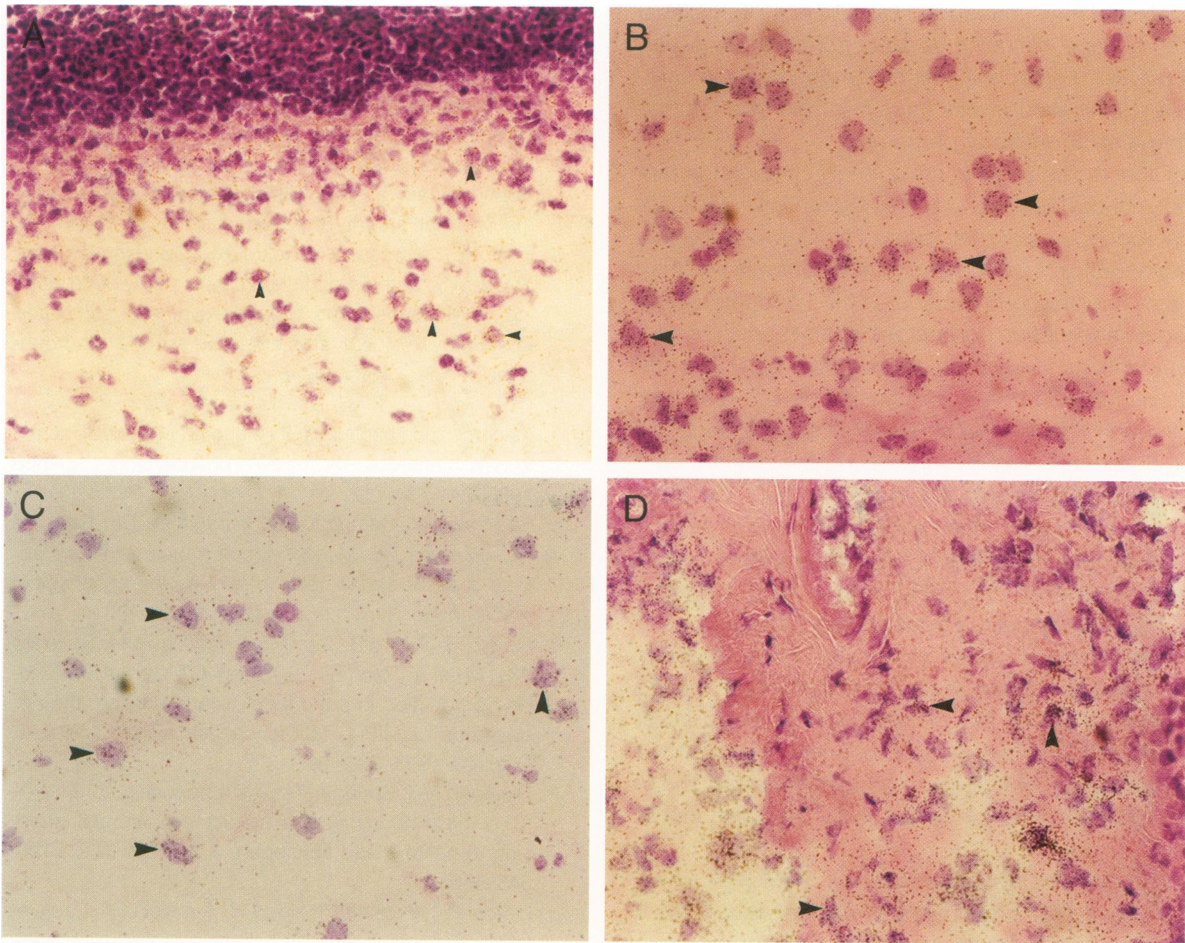

Figure 4. *Photomicrograph of* in situ *hybridization of dermal punch wounds. Hybridization was performed with an antisense probe specific for JE. Arrows indicate examples of heavily labeled monocytic and macrophage-like cells* (**A** *to* **C**) *or fibroblast-like cells* (**D**) **A**: *1-day wound; magnification, ×200.* **B**: *1-day wound; magnification, ×400.* **C**: *2-day wound; magnification, ×400.* **D**: *2-day wound; magnification, ×400.*

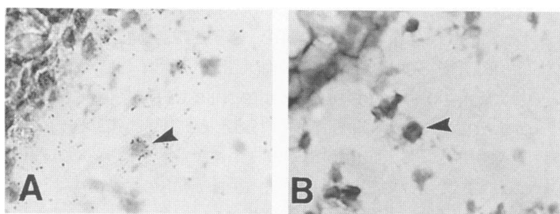

Figure 5. *Photomicrographs of serial sections of 1-day wounds that had been subjected to either* in situ *hybridization with an antisense probe specific for JE* (**A**) *immunostaining with F4/80, a rat monoclonal antibody specific for mature murine macrophages* (**B**). *Arrow indicates cell of identity in serial sections. Note that, as expected, not all cells appearing in one section are found in the sequential section.*

If thrombin is the primary stimulus for JE/MCP-1 production in wounds, the decrease in JE/MCP-1 production in the later phases of wound repair could be indicative of a gradual decrease in the levels of thrombin. Alternatively, the resolution of repair might involve the production of anti-inflammatory cytokines such as interleukin 10 or interleukin 13. The production of such anti-inflammatory cytokines within wounds, and their potential role in the resolution of repair, has not yet been investigated.[39]

In these studies, the production of JE/MCP-1 is linked temporally to maximal macrophage infiltration into the wound. The observed peak in JE/MCP-1 mRNA expression occurs at 12 hours to 1 day after wounding and is followed by a peak in macrophage infiltration at day 3. The lag between maximal JE/MCP-1 mRNA levels and maximal macrophage numbers may reflect the time required for JE/MCP-1 to be synthesized and secreted. However, *in vitro* studies have shown that JE/MCP-1 mRNA levels correlate directly with protein production,[25] suggesting that the detection of mRNA *in situ* will be closely linked to active protein production. One possibility is that murine JE/MCP-1, which is known to be a heavily glycosylated protein,[23] remains within the wound bed for an extended time. Thus, the tissue protein levels of JE/MCP-1 within the wound may peak later than mRNA

levels and may correspond more directly to maximal macrophage infiltration. A direct correlation between JE/MCP-1 protein levels within the wound and macrophage infiltration awaits the availability of a neutralizing anti-JE antibody preparation.

Other molecules that are strongly chemoattractant for monocytes may also preferentially facilitate macrophage egress into sites of active wound repair. Two notable macrophage chemoattractants that may play a role in wound repair are MIP-1 and MIP-2. Reverse transcription polymerase chain reaction analysis of inflammatory cells derived from early wounds has documented the expression of both MIP-1 at days 1 through 7 and MIP-2 at days 1 through 3, suggesting that either or both of these proteins might recruit macrophages to sites of repair.[6] However, because the reverse transcription polymerase chain reaction used to detect MIP-1 and MIP-2 in wound cells was not quantitative, the relative level and temporal course of production of these two chemokines are not known. Another potent monocyte chemoattractant that has been shown to be produced within wounds is *gro*-β.[19] mRNA for *gro* has been detected in rat wound tissue at day 3 after wounding, which, in our model, is the peak of macrophage infiltration. The *in vivo* source of this cytokine has not yet been identified.

The present investigation adds JE/MCP-1 to the growing list of chemoattractants that might favor monocyte migration into wounds. Recent investigations have shown that JE/MCP-1 is also chemoattractant for T lymphocytes, yet few lymphocytes are observed within the dermal wounds described here.[40] This observation might be interpreted as proposed by Springer[41] and others, who suggest that cellular migration is a multi-step process. In wounds, as in other tissues, the sum of several variables most probably dictates which specific cell type will migrate. These variables would include not only which chemoattractants are present but also endothelial activation state and the presence of other influencing cytokines. In the context of the healing wound, the early parameters, including the expression of JE/MCP-1, might preferentially attract monocytes to wounds. Additional study will be necessary to unravel the relevant contributions of each of these potential chemoattractants to the process of macrophage recruitment in wound repair.

Acknowledgments

We thank Ms. Mary Kay Olson for expert technical assistance and Dr. Pam Witte for the generous gift of the F4/80 antibody.

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