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REGULAR ARTICLE

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Hepatocyte growth factor and macrophage-stimulating protein are upregulated during excisional wound repair in rats

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Abstract Hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP) are structurally related molecules that stimulate epithelial cell proliferation and migration. MSP also acts directly as a chemoattractant for resident macrophages. These activities are integral to the wound repair processes of inflammation, epithelialization and tissue remodelling. To begin to examine the involvement of HGF and MSP in healing of cutaneous wounds we have mapped the temporal expression of these two molecules and their receptors, MET and RON respectively, in adult rat excisional wounds. Four 2×2-cm full-thickness excisional wounds were created on the dorsum of 18 rats, and biopsies were taken through the wounds at 3, 5, 7, 14, 21, and 28 days postwounding. These biopsies were analyzed using immunofluorescent staining and in situ hybridization (ISH). The number of cells staining positively for HGF and MET significantly increased in response to wounding. HGF staining and mRNA peaked at 7 days postwounding whereas MET was upregulated earlier, peaking after 3 days. Both HGF and MET protein were observed in fibroblasts of the dermis and in the newly forming granulation tissue. ISH studies also revealed that fibroblasts at the wound edges and within the newly forming granulation tissue also expressed HGF and c-met mRNA. Immunofluorescent staining revealed both MSP and RON within the wound, with maximum staining occurring between 7 and 21 days for both the ligand and receptor. In addition, MSP co-localized with a small subset of ED1-positive cells (monocytes). In contrast, ED2-positive cells (macrophages) did not co-localize with MSP. Thus, increased expression of HGF, MSP and their receptors MET and RON respectively was observed in response to wounding. Furthermore, MSP co-localization with a subset of monocytes may confirm a role for MSP in the activation of mature macrophages, which may be important in tissue remodelling.

Keywords HGF · MET · MSP · RON · Inflammation · Wound healing · Rat (Sprague Dawley)

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Introduction

Growth factors play a key role in directing the integrated phases of wound repair, which consists of inflammation, epithelialization, formation of granulation tissue and tissue remodelling. In particular, two structurally related proteins, hepatocyte growth factor/scatter factor (HGF/SF), and macrophage-stimulating protein (MSP) have been identified as having specific biological properties pertinent to wound healing (Stoker et al. 1987; Gheradi et al. 1989) and reviewed by Trusolino et al. (1998). Both HGF and MSP are characterized by the presence of highly conserved triple disulphide loops or kringle regions within their amino terminus and significant carboxy terminal homology to the catalytic domain of the serine-protease family of proteins (Matsumoto and Nakamura 1996; Tamagnone and Comoglio 1997). In addition, both are secreted as larger inactive precursors, which are proteolytically cleaved to produce active disulphide, linked αβ-heterodimers. Both pro-MSP and pro-HGF are cleaved to their active form by serine protease-like enzymes of the blood fibrinolytic cascade (Shimomura et al. 1995). Notably, HGF is processed by urokinase-type (uPA) and tissue-type (tPA) plasminogen activators and has recently been found to have a specific serum-derived convertase, hepatocyte growth factor activator, which generates HGF at the tissue site of injury (Naldini et al. 1992; Mars et al. 1993; Miyazawa et al. 1996; Wang et al. 1996). Finally, both ligands activate membrane-span-

ning tyrosine-kinase-containing receptors (MET and RON respectively) to elicit their biological actions (Naldini et al. 1991; Gaudino et al. 1994).

HGF is mesenchymally derived and exerts powerful mitogenic, morphogenic and motogenic effects on a number of epithelial cells in vitro, via its high-affinity receptor, MET (Bottaro et al. 1991; Naldini et al. 1991). MET is the gene product of the oncogene c-met and is expressed on the surface of a variety of epithelial cells, including those of the skin, gastrointestinal tract, lungs, kidney and liver (Park et al. 1987; DiRenzo et al. 1991). In addition, MET has recently been found to be expressed in fibroblasts of the dental papilla and in the fibroblast-like mesangial cells of the kidney (Kallincos et al. 1998; Kajihara et al. 1999), suggesting possible autocrine actions of HGF. HGF has been shown to bind heparin and is thought to associate with heparin-sulphated proteoglycans of the extracellular matrix (Lyon et al. 1994). In vitro, HGF has also been observed to bind strongly to thrombospondin and fibronectin (Lamszus et al. 1996). Although HGF was found to be essential for mammalian placental and hepatic development by gene knockout experiments, HGF-null mice died in utero, preventing analysis of subsequent wound healing in these animals (Schmidt et al. 1995; Uehara et al. 1995). As well as its ability to stimulate proliferation and migration of keratinocytes, HGF has also been shown to be a potent promoter of restitution in wounded epithelial cell monolayers (McCawley et al. 1998). Furthermore, HGF has also been shown to be a potent angiogenic factor (Bussolino et al. 1992; Laterra et al. 1997). Together these findings suggest HGF's potential for a role in wound repair but to date little direct evidence supports HGF's wound healing function in vivo.

Macrophage-stimulating protein (MSP) is a liver-derived serum glycoprotein involved in cell proliferation and differentiation (Leonard and Skeel 1976). Although it acts directly as a chemoattractant for resident macrophages, its primary biological role is not as recruiter of blood monocytes to sites of inflammation but as an activator of mature macrophages (Skeel and Leonard 1994). It also stimulates the proliferation of mammary duct epithelial cells and keratinocytes, and influences motility of keratinocytes acting through its tyrosine-kinase-containing receptor, RON. MSP has been detected in nanomolar concentrations in the serum, but physiologically active MSP is only generated at the surface of macrophages (or other target cells), resulting in locally restricted levels of the cytokine (Wang et al. 1996b). Pro-MSP is the predominant form in the circulating blood; however, proteolytic cleavage of pro-MSP to active MSP by pro-MSP convertase occurs in wound fluid from burn wounds and surgical drains (Nanney et al. 1998). Additionally, unlike blood monocytes and acute exudate macrophages (derived from blood monocytes) (Iwama et al. 1995), dermal macrophages express the RON receptor for MSP, which therefore makes it likely that MSP can stimulate macrophage-dependent wound debridement (Nanney et al. 1998). Finally the RON receptor for MSP is upregulated on keratinocytes in burn wounds (Nanney et al. 1998).

The potent mitogenic and motogenic properties of both HGF and MSP suggest their potential as exogenous therapeutics in wound repair. Therefore, the aim of this study was to determine the temporal and spatial localization of HGF, the MSP, and their receptors MET and RON during excisional wound repair in rats.

Materials and methods

Animal surgery

Eighteen male Sprague-Dawley rats were anaesthetized, the hair of the dorsum clipped and the skin aseptically prepared with povidone-iodine (Betadine, Mundipharm AG, Switzerland) and 70% (v/v) ethanol. Using a template, four full-thickness excisions 2×2 cm were created on the dorsum of the rats. The wounds extended through the panniculus carnosus to the deep fascia. Haemostasis was controlled before the rats were allowed to recover and returned to their cages. The wounds were left exposed throughout the study. Biopsy samples were taken of selected wounds on days 3, 5, 7, 14, 21, and 28 postwounding. These biopsies were fixed in 10% buffered formalin and embedded in paraffin wax. All experiments were approved by the Women's and Children's Hospital Animal Care and Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes.

Immunofluorescent staining of proteins

Rabbit anti-rat primary antibodies to HGF (C-20), MSP (T-19), and MET (SP260) were obtained from Santa Cruz Biotechnology Inc., CA. Mouse anti-rat RON was obtained from Transduction Laboratories, KY, and mouse anti-rat ED1 and mouse anti-rat ED2 were obtained from Serotec Ltd., Oxford, UK. Biotinylated secondary antibodies (anti-rabbit IgG and anti-mouse IgG) and CY3-conjugated streptavidin were all obtained from Sigma-Aldrich, St. Louis. Goat anti-mouse directly conjugated to fluorescein isothiocyanate (FITC) was obtained from Serotec Ltd., Oxford, UK.

Indirect immunofluorescent localization was used with a biotin-streptavidin amplification step for increased sensitivity. Serial 4-µm sections of the paraffin-wax-embedded biopsies were cut using a microtome, deparaffinized in xylene for 10 min and rehydrated. The sections were washed with phosphate-buffered saline (PBS) and incubated for 1 h at room temperature with the primary antibodies (all at 1:50 dilution). The sections were then washed 3 times in PBS and incubated with the biotinylated secondary antibodies (1:500) for 1 h at room temperature. The sections were again washed 3 times with PBS before being incubated with CY3-conjugated streptavidin (1:400) for 40 min at room temperature. The sections were washed 3 times in PBS and mounted in Immumount (Shandon, Pittsburgh, USA). The stained sections were visualized by fluorescence microscopy and pictures of the sections were captured using Image-Pro Plus for image analysis. For verification of staining, negative controls included preadsorption of the anti-HGF antibody by an excess amount of human HGF (100 ng/ml) for 1 h at room temperature and replacement of the primary antibodies by either normal rabbit IgG or normal mouse IgG for HGF, MET, MSP and RON, ED1, ED2, respectively. On additional control sections, the primary and secondary antibodies were omitted to determine non-specific binding. Staining was also performed on the skin of unwounded control animals to derive baseline data for HGF, MET, MSP and RON.

Immunofluorescent dual staining of MSP with ED1 or ED2

Sections were processed as described above with the primary antibody (raised in rabbit) against MSP before a second primary antibody (raised in mouse) against either ED1 or ED2 was then added to the sections at 1:50 and left for a further 1 h at room temperature. After another three washes with PBS, goat anti-mouse-FITC secondary antibody (1:200) was added for a further 1 h at room temperature. The sections were finally rinsed 3 times with PBS before being mounted with Immumount as before and examined using a fluorescent microscope. Separate images were captured for each antibody and then overlaid to identify cells staining positively for both peptides.

Image analysis

MET, MSP and RON staining was assessed by counting positively stained cells within three (random) fields of view within the wounds of three different animals at each time point and expressed as cells/unit area (mm^2). Student's *t*-tests were performed to determine the significance of the results and considered significant if $P<0.05$.

In situ hybridization of HGF and c-met in excisional wounds

In situ hybridization of HGF and c-met was performed on 4-µm paraffin-embedded sections of the excisional wounds at 3, 5, 7, 14, and 21 days postwounding using a non-radioactive in situ protocol based on the method of Braissant and Wahli (1998).

Generation of rat HGF and rat c-met-specific probes

The rat HGF antisense riboprobe was prepared by subcloning an *Eco*RI fragment of the rat HGF cDNA (kindly provided by Professor T. Nakamura, Osaka, Japan) into pSP73 vector (Geneworks, Adelaide, Australia). Subsequent transcription of the *Hin*cII linearized construct was performed with T7 polymerase using an Ambion Maxiscript kit (Austin, TX).

Reverse transcription (RT)-polymerase chain reaction (PCR) was carried out using the following nested primers based on the published rat c-met sequence (Liu et al. 1996): forward primer (a) ^{52}AACAACGTACGGTGTCTCCAG^{72}, reverse primers (b) $_{211}$GTCAAGAGTACATGGTTGAG$_{230}$, and (c) $_{346}$CAGGATAGGAATCCAGGAGGA$_{368}$. RNA was extracted from adult rat liver using RNAzol B (Biotecx, Houston, TX) and poly A+ RNA purified using the Oligotex mRNA purification kit (Qiagen Inc., Chatsworth, CA). Reverse transcription was performed on polyA$^+$ RNA (1 µg) using AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany) in conjunction with primer (c). Subsequent PCR amplification of the first strand of cDNA was performed using primers (a) and (b) under the following thermocycling regime: 1 cycle at 94°C/5 min, then 30 cycles at 94°C/2 min, 60°C/30 s, 72°C/1 min and a final cycle of 94°C/2 min, 60°C/30 s, 72°C/10 min. This PCR generated the predicted 179-bp and 317-bp products as determined by agarose electrophoresis. The PCR product was ligated into the pGEM-T vector (Promega, Madison, WI), with the candidate-positive clones screened using c-met and vector-specific primers. Positive clones (containing the 317-bp insert) were then sequenced to confirm their identity. The resultant c-met pGEM clone was linearized with *Sal*I and riboprobes were synthesized as detailed below.

Digoxygenin (DIG)-UTP-labeled sense and antisense riboprobes for both c-met and HGF were synthesized from the linearized plasmids using the DIG-RNA-labeling kit (Roche Diagnostics, Mannheim, Germany) and T7 or SP6 RNA polymerase (Promega, Madison, WI). Ribroprobes were quantitated by a spot labeling test according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The riboprobes were run on a 1.0% agarose, 0.5×TBE gel together with control-labeled RNA and DNA molecular weight markers to confirm probe size.

Sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and washed in diethyl pyrocarbonate (DEPC)-H_2O. After further DEPC-PBS washes, sections were fixed in 4% buffered paraformaldehyde and subsequently washed in PBS containing 0.1% active DEPC (2×15) min, before being left in 5×SSC. The sections were prehybridized at 52°C in a solution of 50% formamide, 5×SSC and 40 µg/ml sheared herring sperm DNA for 60 min. The DIG-UTP-labeled probes were diluted to 400 ng/ml in the prehybridization solution and hybridization was carried out overnight at 52°C in a sealed humidified chamber. Stringency washes were performed as follows to remove unbound probe: 2×SSC at room temperature for 30 min, then 2×SSC at 65°C for 1 h and finally 0.1×SSC at 65°C for 1 h. After the stringency washes, the sections were washed in TBS (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl), before being incubated with the alkaline phosphatase-conjugated DIG antibody in TBS plus 1% bovine serum albumin (Sigma-Aldrich, St. Louis, OH) for 2 h at room temperature. After removal of unbound antibody, alkaline phosphatase was detected using the NBT-BCIP detection system (Roche Diagnostics, Mannheim, Germany) such that a positive purple signal was produced. Sections were counterstained using methyl green to reveal cell morphology, mounted using Immumount and images captured using Image-Pro Plus software.

Results

The excisional wounds were healed to approximately 50% of their original wound area by day 7 postwounding, were fully closed by days 14–21 and resembled unwounded skin by day 28. All the control treatments produced no positive staining with any of the antibodies used in the immunofluoresence studies. Furthermore, no staining was observed in the in situ hybridization studies when sense probes against HGF and c-met were added to control sections.

HGF expression in excisional wounds

Intense immunofluorescent staining of HGF was observed 3 days postwounding in the extracellular matrix of the dermis immediately adjacent to the wounds and in fibroblasts directly under the epidermis at the wound margins although no staining was observed within the epidermis (Fig. 1a). In contrast, mRNA was observed predominantly in the epidermis and dermal appendages of the unwounded adjacent tissue, and this was markedly increased at the wound edge at 3 days postwounding (Fig. 1b). By day 5, dermal staining of the HGF protein was again observed directly under the epidermis (Fig. 1c). Additionally, epidermal cells throughout the thickened epidermis, close to the wound edges, were now staining positive for HGF protein and this staining pattern showed a marked gradation away from the wound edge (Fig. 1c). Similarly, increased mRNA expression was seen in the epidermis at the wound margins compared to the epidermis of normal adjacent skin. Dermal fibroblasts adjacent to and within the wound also showed marked expression of the HGF gene (Fig. 1d). By 7 days, intense staining of the HGF protein was clearly seen in the migrating tip of the epidermis with particularly strong staining observed in the basal epider-

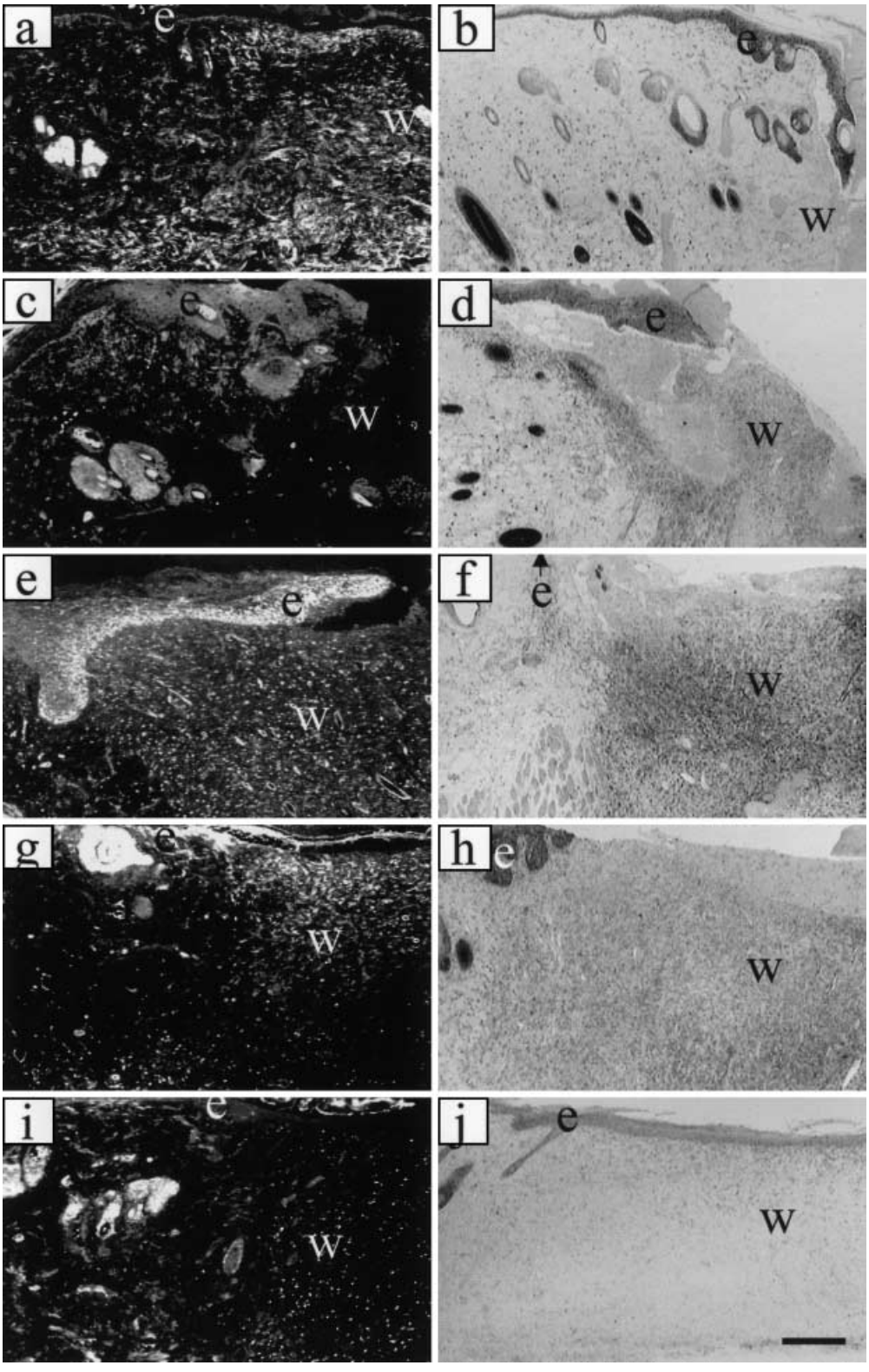

Fig. 1a–j Immunofluorescent staining and expression of HGF in excisional wounds. HGF protein immunolocalization is shown at 3, 5, 7, 14, and 21 days postwounding (**a, c, e, g,** and **i,** respectively). In situ hybridization of HGF mRNA expression is shown at 3, 5, 7, 14, and 21 days postwounding (**b, d, f, h,** and **j**, respectively). The positions of the wounds are marked by a *w* while the epidermis is shown by *e*. *Scale bar on* **j** refers to all sections and = 500 µm

mal cells (Fig. 1e). Additionally, cells within the wound, notably fibroblasts, endothelial cells of newly regenerating blood vessels and inflammatory cells stained positive for HGF (Fig. 1e). A concomitant increase in HGF mRNA was seen within the excisional wound, with fibroblasts staining positive for HGF in the wound (Fig. 1f). By 14 days postwounding, the staining of both protein and mRNA was less intense although staining was still apparent in fibroblasts within the wound and in epidermal cells (Fig. 1g, h, respectively). By 21 days

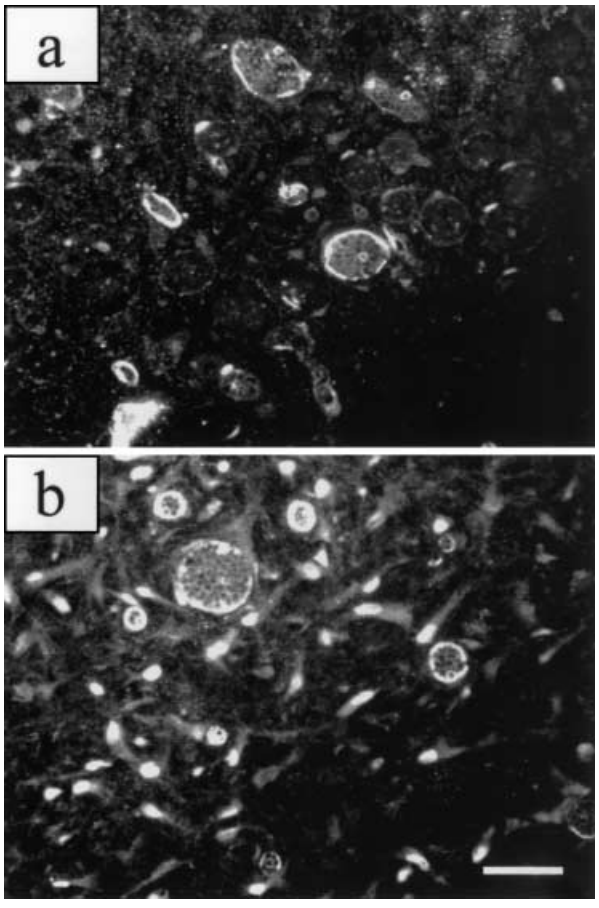

Fig. 2a, b HGF immunofluorescent staining of new blood vessels in excisional wounds. HGF immunofluorescence is shown in endothelial cells around the perimeter of blood vessels within the wounded tissue at **a** 7 and **b** 14 days postwounding. *Scale bar in* **b** refers to both sections and = 50 μm

postwounding (Fig. 1i, j), the wound had completely re-epithelialized and HGF expression had returned to levels observed in the unwounded adjacent skin. The localization of HGF in budding endothelial cells surrounding new blood vessels was particularly striking (Fig. 2). At 7 days postwounding, blood vessels within the wounds were highlighted with HGF-positive staining (Fig. 2a). By 14 days the numbers of blood vessels staining positive for HGF had increased (Fig. 2b), but by 21 days endothelial cell staining had disappeared.

MET expression in excisional wound healing

A similar expression profile for both the protein and mRNA was observed for the HGF receptor MET. Discrete staining for the MET protein was observed 3 days postwounding in large numbers of cells in the wounds (Fig. 3a). At this time, increased c-met mRNA was observed in epidermal cells and dermal appendages at the wound margins (Fig. 3b), but, interestingly, no protein expression was observed within the epidermis at any time point postwounding. Increased numbers of MET-positive cells were observed in the wounds at day 7 (Fig. 3d), but it was not until day 7 that increased mRNA expression was observed in the fibroblasts and inflammatory cells within the wound itself and in the epidermis of the wound margins (Fig. 3e, f). The numbers of positive cells reduced by 14 days (Fig. 3g–i) and by day 21 only basal levels of expression could be observed throughout the skin (Fig. 3j–l). Due to the discrete cellular staining of MET, it was possible to quantitate the numbers of cells staining positive in the wound and wound margins. A significant increase in MET-positive cells was observed in the wounds compared to the wound margins up to 21 days postwounding. The maximum number of positive cells was observed 3 days after wounding, with the numbers gradually decreasing over the subsequent timepoints until 28 days, when the numbers of MET-positive cells were minimal in both the wounds and wound margins (Fig. 4a).

MSP localization in excisional wound repair

MSP-positive cells were observed in the wound margins at 3 days postwounding (Fig. 5a), although it was not until day 5 that MSP-positive cells were observed within the wounds themselves (Fig. 5d). Increased MSP staining was observed at 14 days postwounding in the wounds (Fig. 5f). The number of cells staining positive for MSP was counted within the wound and wound margins (Fig. 4b). These results again highlighted the increased numbers of MSP-positive cells in the wound after 7–14 days, which were significantly greater in number when compared with the wound margins.

RON localization in excisional wound repair

No RON-positive cells were observed in the wound margins at any of the time points investigated (Fig. 6a, c, e, g, i, k). Positive staining for RON did not appear in the wound until 7 days postwounding, when the characteristic staining of the cell surface was observed (Fig. 6h). Staining was most intense in the wound at day 7, with numerous cells directly below the epidermis staining positive. Dermal staining was also observed in the wound tissue at 14 and 21 days postwounding (Fig. 6h, j), but was gone by day 28 (Fig. 6l). The number of cells staining positive for RON was counted within the wound (Fig. 4c). No positive cells were observed in the wound margins. These results showed the increase in RON-positive cells in the wound compared with the wound margins at 7, 14 and 21 days.

Dual localization of MSP with inflammatory cell markers

To determine which inflammatory cells were staining positive for MSP, dual immunolocalization of MSP was

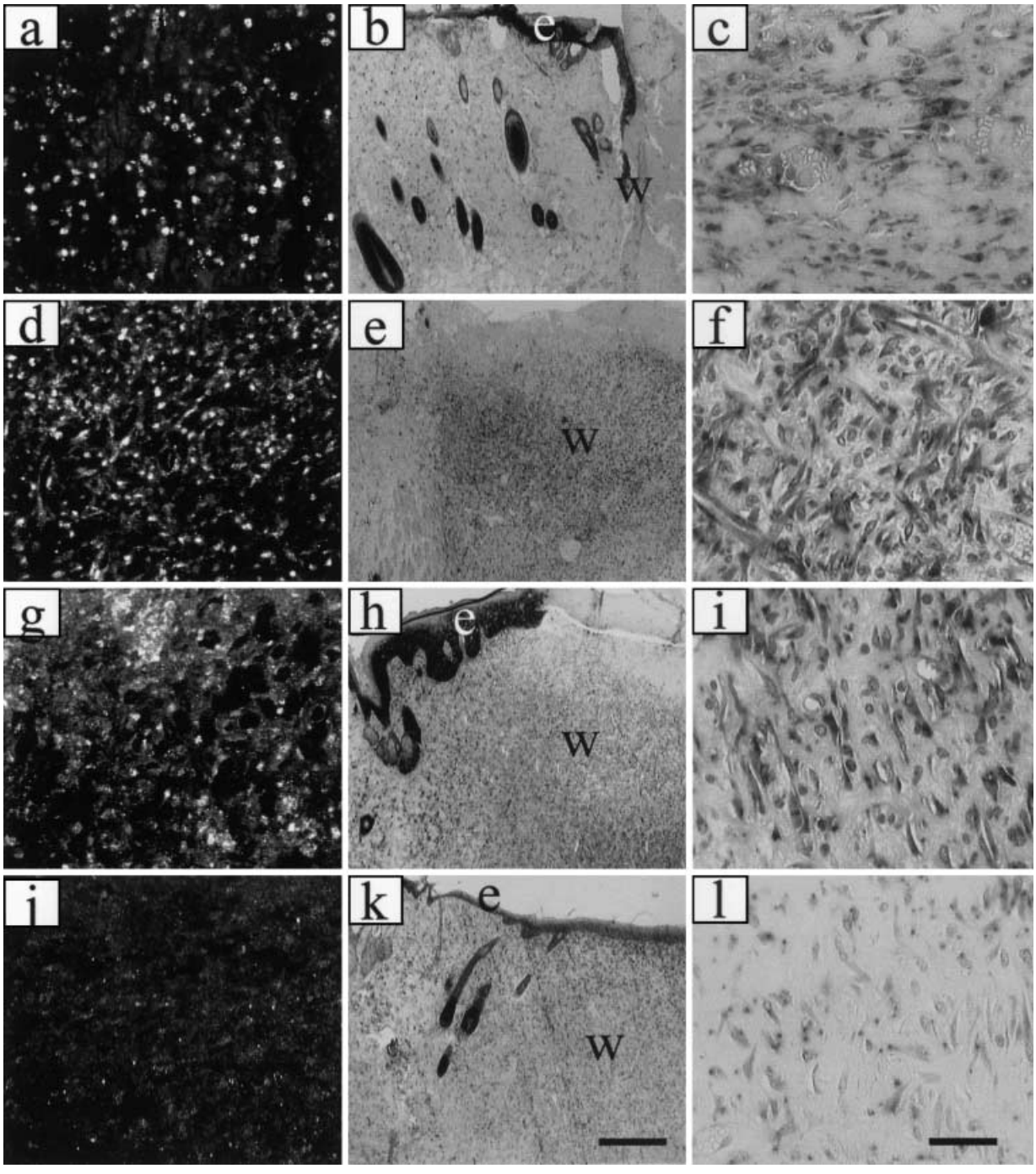

Fig. 3a–l Immunofluorescent staining and expression of MET in excisional wounds. MET protein was immunolocalized within the wounds at 3, 7, 14, and 21 days postwounding (**a, d, g,** and **j,** respectively). In situ hybridization of c-met mRNA expression is shown within the wounds at 3, 7, 14, and 21 days postwounding at low magnification (**b, e, h,** and **k,** respectively) and at high magnification (**c, f, i** and **l**). The positions of the wounds are marked by a *w* while the epidermis is shown by *e*. *Scale bar on* **k** also refers to **b, e** and **h** and = 500 µm. *Scale bar on* **l** refers to remaining sections and = 125 µm

performed with either ED1, which recognizes a single-chain glycoprotein of 90–100 kDa that is expressed predominantly on the lysosomal membrane of undifferentiated monocytes, or ED2, which recognizes a membrane antigen of activated macrophages. Figure 7a, b shows ED1- and ED2-positive cells respectively staining in the wounds at 7 days postinjury. ED1-positive cells were observed particularly in the wound margins at 3 days postwounding and in high numbers within the wound at 5, 7, and 14 days postwounding. By 21 days postwounding the positive cells had disappeared. Similarly ED2-positive cells were observed present in the wound margins

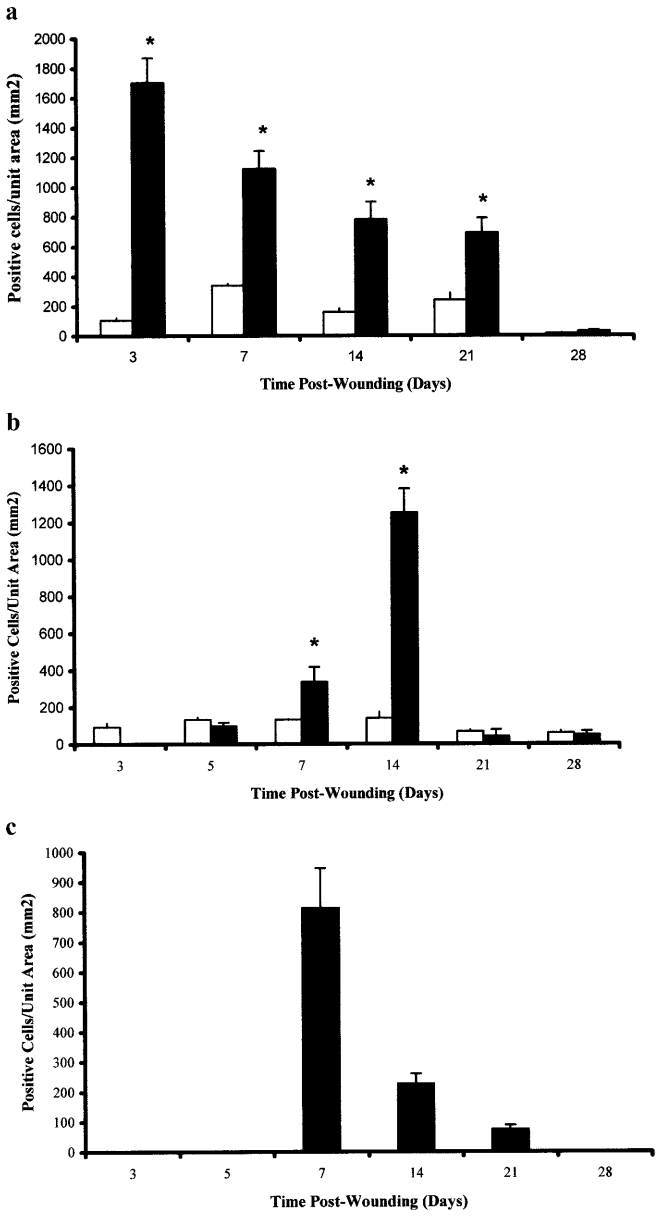

Fig. 4A–C Temporal changes in the number of cells expressing MSP, MET and RON after excisional wounding. Positive cells immunofluorescing for **A** MET, **B** MSP, and **C** RON within excisional wounds at 3, 7, 14, 21, and 28 days postwounding were counted using Image-Pro Plus software. Three fields of view were assessed per wound, three animals per time point (■ wound, □ wound margin). *Error bars* are means ± SEM, n=3. *Wound vs wound margin, P<0.05

3 days postwounding, peaking in the wound at 5–7 days postinjury and disappearing by 21 days.

When MSP was co-localized with ED1, dual-stained cells were observed particularly in the wounds. ED1-positive cells fluoresce red (open arrow in Fig. 7c), MSP-positive cells fluoresce green (Fig. 7c), and cells expressing both ED1 and MSP fluoresce yellow. Co-localization of MSP with ED1 (undifferentiated monocytes) was observed (solid arrow in Fig. 7c). In addition, MSP-positive cells were also observed, indicating that the monocytes might only account for a fraction of the MSP-producing cells in the wound. When ED2 was stained with MSP, although both red-fluorescing cells (ED2 positive) and green-fluorescing cells (MSP positive) were observed (Fig. 7d), no yellow-fluorescing cells, indicative of co-localization, could be seen. This indicates that these activated macrophages are not producing MSP in the wounds.

Discussion

Our studies have revealed that HGF and its receptor MET are both expressed following excisional wounding. Elevated HGF staining was observed 3–5 days postwounding, but did not appear to be associated with any particular cell type at these early time points. As HGF binds to heparin and is thought to associate with heparin-sulphated proteoglycans of the extracellular matrix (Lyon et al. 1994), thrombospondin and fibronectin (Lamszus et al. 1996), our initial staining may represent extracellular HGF at the edges of the wound. As the antibody used in this study does not distinguish precursor from mature HGF, it is possible that the matrix-bound HGF is in an inactive precursor form, providing a reservoir of growth factor on the surface of the cells. Activation of precursor-HGF could be accomplished by proteases of the fibrinolytic cascade prevalent immediately after injury or by the specific action of HGF activator released in zymogen form from the liver and activated at the site of injury (Miyazawa et al. 1996). Thus, upon activation, this extracellular matrix-bound HGF would be immediately available to instigate the early cellular responses to wounding. In support of this finding, we observed a concomitant increase in staining for the HGF receptor, MET, in response to wounding, with high numbers of MET-positive cells observed particularly between 3 and 7 days postwounding, decreasing over the following 14–21 days. Our results are in accordance with a recent study on an epithelial wound showing increased expression of HGF and c-met mRNA 7 days after the scrape wounding of mouse corneas (Wilson et al. 1999). It has also been shown that HGF is able to induce the expression of c-met (Boccaccio et al. 1994), suggesting a mechanism whereby HGF responsiveness might be auto-amplified following excisional wounding. Therefore it is clear that both HGF and its receptor MET are both present in abundance immediately postinjury and likely to be involved in the regulation of cells involved in proliferation and migration into and across the wound bed.

Previous in vitro studies have revealed that HGF is a stimulator of keratinocyte migration and proliferation (Matsumoto et al. 1991). Furthermore HGF strongly promotes restitutive responses in wounded monolayers (Watanabe et al. 1994; Takahashi et al. 1995) and influences epithelial cell adhesion by altering expression of integrins (Nebe et al. 1998). HGF-stimulated keratinocyte migration in vitro is coincident with the induction of

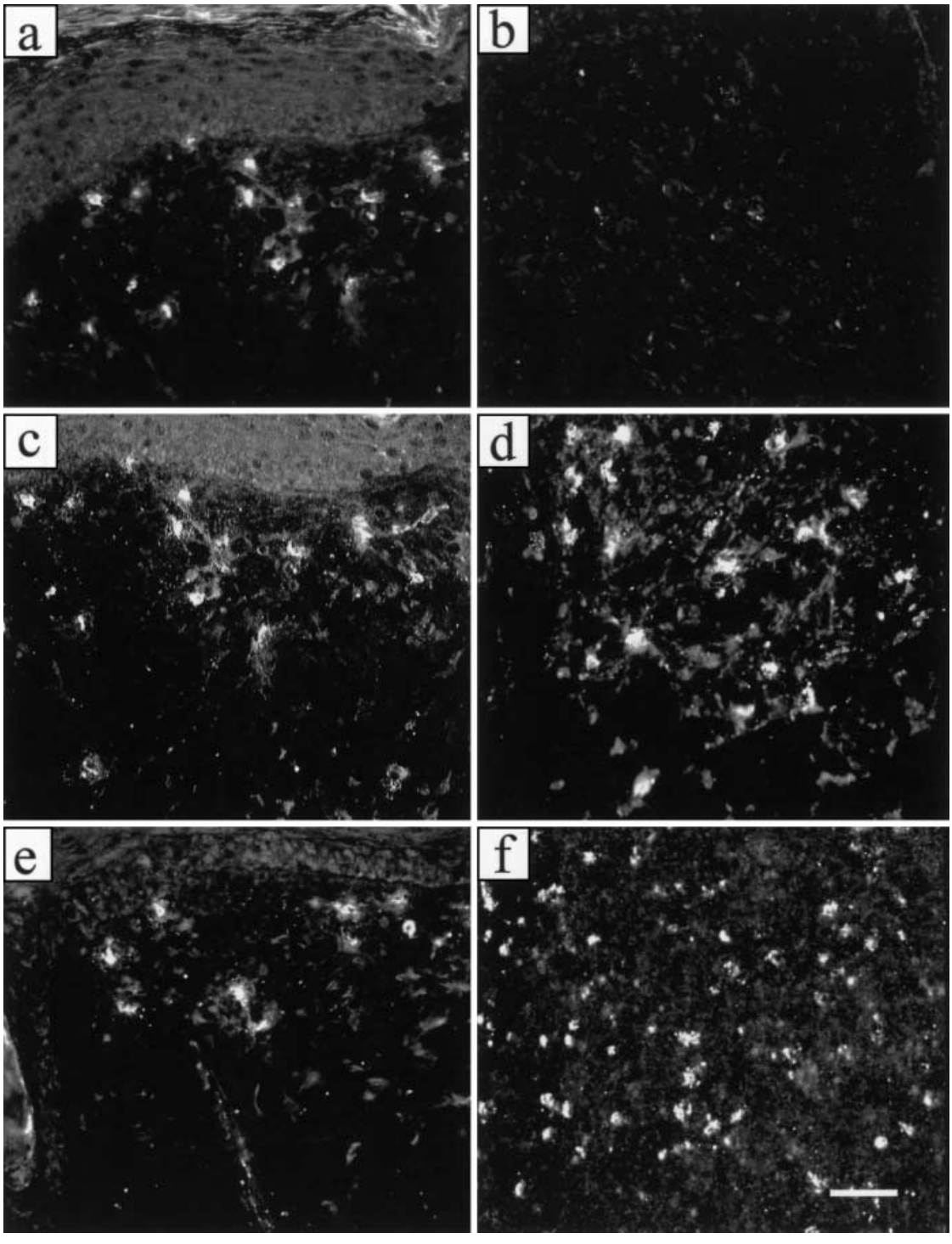

Fig. 5a–f Immunofluorescent staining of MSP in excisional wounds. Immunolocalization of MSP protein at 3, 5, and 14 days postwounding in the wound margin (**a, c** and **e,** respectively) and in the wound (**b, d** and **f**). *Scale bar on* **f** refers to all sections and = 125 µm

MMP-9, a matrix metalloproteinase (McCawley et al. 1998), and HGF has also recently been shown to stimulate collagenase I and stromelysin I production (Dunsmore et al. 1996). Several studies have identified the role of the MMPs in wound repair, indicating the requirement of matrix degradation for cell migration to occur. Thus, the intense staining of HGF protein and mRNA in the migrating tips of the epithelium at 7 days postwounding may suggest that HGF is stimulating these epidermal cells to proliferate and migrate across the wound bed. Additionally, the co-localization of HGF and c-met mRNA in the migrating keratinocytes may suggest a role of HGF in c-met activation. Previous studies have proposed that HGF may act as a paracrine mediator of stro-

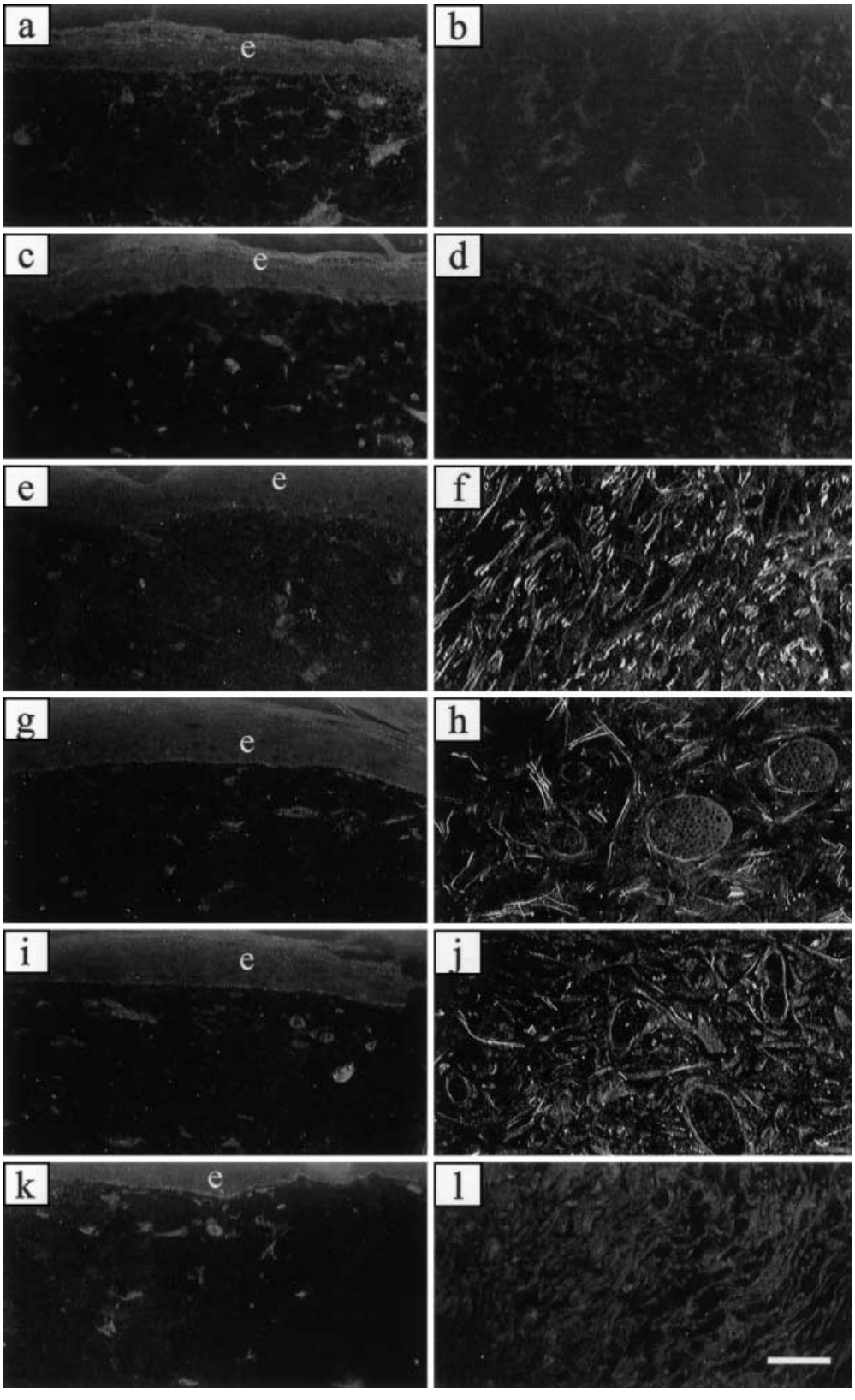

Fig. 6a–l Immunofluorescent staining of RON in excisional wounds. Immunofluorescence of RON protein at 3, 5, 7, 14, 21 and 28 days postwounding in excisional wound margins (**a, c, e, g, i, k,** respectively) and in the wounds at 3, 5, 7, 14, 21 and 28 days (**b, d, f, h, j, l,** respectively). *Scale bar on* **l** refers to all sections and = 125 µm

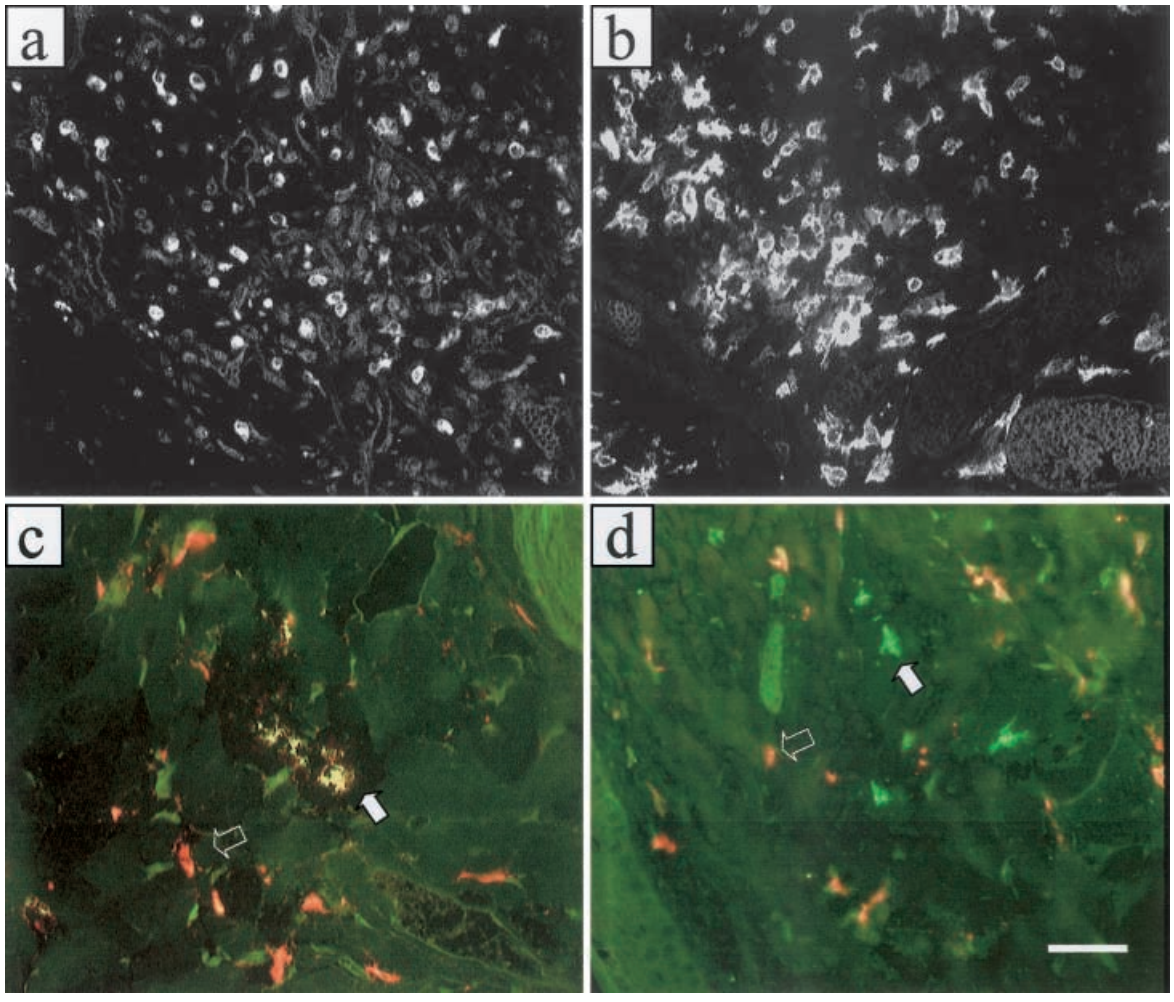

Fig. 7 Dual localization of MSP with inflammatory cell markers. ED1 immunofluorescent monocytes **(a)** and ED2 immunofluorescent macrophages **(b)** are shown at 7 days postwounding. Dual-stained day 7 wounds, for MSP and either ED1 or ED2, are shown in **c** and **d,** respectively. Cells costaining for ED1 and MSP fluoresce yellow (*solid arrow in* **c**) while ED1-positive cells alone fluoresce red (*open arrow in* **c**). Cells staining for MSP alone fluoresce green (*solid arrow in* **d**) while cells staining positively for ED2 alone fluoresce red (*open arrow in* **d**). *Scale bar in* **d** refers to all sections and = 125 μm

mal-epithelial interactions (Kasai et al. 1996); however, our results clearly show that HGF may also have an autocrine role in epidermal cell proliferation and migration particularly in response to wounding. Coexpression of HGF and c-met has also been observed in epithelial cells in human prostate cancer (Kurimoto et al. 1998). In addition, HGF has been shown to induce a variety of intrinsic tissue-specific morphogenic programs in epithelial cells grown in three-dimensional collagen matrices (Brinkmann et al. 1995) and is a prime candidate to enable instructive remodelling of disrupted tissues.

Moreover, HGF can induce the proliferation and migration of endothelial cells and is a potent angiogenic factor (Bussolino et al. 1992; Zioncheck et al. 1995). In part, HGF's angiogenic actions may be due to its stimulation of vascular endothelial growth factor production (Wojta et al. 1999). We observed strong staining of HGF around the perimeters of blood vessels within the wound bed at 7–14 days postwounding, which diminished with healing. This pattern suggests an important role for HGF in the generation of new capillaries during wound repair.

Previous studies have revealed a role for MSP in tissue injury (Nanney et al. 1998). We also observed a temporal upregulation of MSP during wound repair in rat excisional wounds. Immediately postwounding we observed MSP activity to be low within the wound and wound margins; however, this significantly increased over time, peaking at 14 days postinjury. It has previously been speculated that under inflammatory conditions or within injured sites, increased vascular permeability may allow diffusion of pro-MSP from the circulation into the tissues where enzymes involved in coagulation can cleave pro-MSP to the biologically active form (Wang et al. 1994). Indeed, Nanney et al. (1998) have shown that pro-MSP is cleaved by the pro-MSP convertase to active MSP in wound fluid of burn injuries. We observed similar staining patterns for both MSP and its receptor RON in the excisional wounds. Increased dermal staining was noted within the wounds for both the ligand and its re-

ceptor between 7 and 21 days postwounding, which was diminished by 28 days. Previous studies have observed RON expression within normal skin (Gaudino et al. 1994) and human burn wound epidermal cells. We observed minimal immunofluorescence within the skin surrounding the wounds and only saw the characteristic cell surface staining for RON in the dermis of the wound. Cell morphology indicated that some of the cells staining positive for MSP were inflammatory cells. Two markers were used to identify which populations of inflammatory cells were staining for MSP. ED1 recognizes an antigen expressed by the majority of tissue macrophages, whereas ED2 also recognizes an antigen expressed by macrophages but will not stain monocytes. As acute wound exudate macrophages (derived from blood monocytes) do not express RON receptor for MSP (Iwama et al. 1995), it is possible that as ED2 did not co-localize with MSP, these macrophages are derived from blood monocytes and as such do not have the MSP receptor. In contrast a subpopulation of ED1-positive cells also stained positive for MSP in the early stages of wound repair, suggesting that not all ED1-positive cells have the RON receptor. Additionally, cells that were not monocytes or macrophages stained positive for MSP, indicating that inflammatory cells might only account for a fraction of the MSP-producing cells in the wound. Although the original function of MSP was thought to make resident peritoneal macrophages responsive to chemoattractants (Skeel and Leonard 1994), it is now known that MSP is actually capable of inducing multiple biological effects similar to those described for HGF (Trusolino et al. 1998). Therefore the peak of MSP activity, which we observed in the later stages of the wound repair process, could indicate a role for MSP in activating macrophages or in the tissue-remodelling process. The latter may be supported by the dermal staining observed for RON, indicating a receptor-mediated response for MSP in the dermis of the excisional wounds; in support of this dermal macrophages express the RON receptor for MSP (Nanney et al. 1998). Despite our findings, a recent study found no evidence of impaired wound healing in mice carrying a targeted inactivation of the MSP gene (Bezerra et al. 1998) although only responses to incisional wounding were assessed. This study did, however, find a delayed activation of macrophages in the mutant animals. Thus while MSP may not be essential for wound healing responses it undoubtedly plays a cooperative role within the milieu of the wound repair machinery.

In conclusion, increased expression of both HGF and its receptor MET was observed in response to wounding. Furthermore, MSP co-localized with a subset of monocytes, perhaps indicating its role in stimulating these cells to become phagocytic macrophages. These results point to the importance of two members of the HGF family in excisional wound repair.

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