Proteolytic Cleavage and Activation of pro–Macrophage–Stimulating Protein and Upregulation of its Receptor in Tissue Injury

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Macrophage stimulating protein (MSP) exists in blood as inactive pro–MSP. Cleavage yields active MSP, the ligand for a membrane receptor (RON) that is expressed on keratinocytes as well as macrophages. Because both cells have roles in tissue injury, we looked for active MSP and expressed RON in wounds. Concentration of pro–MSP + MSP in wound exudates was in the range for optimal activity. Western blot showed that MSP comprised about half the total, in contrast to less than 10% of the total in blood plasma. The presence of MSP was attributed to an exudate pro–MSP convertase that had an inhibitor profile consistent with a trypsin-like serine protease. Exudate evoked morphologic changes in macrophages *in vitro* like that of MSP. Removal of this activity by an anti–MSP column shows that exudate stimulation of macrophages is due to MSP. RON was infrequently detected in normal skin. RON protein was markedly upregulated in burn wound epidermis and accessory structures, in proliferating cells or differentiated cells, or both. RON was also detected on macrophages and capillaries. Tissue injury leads to cleavage of pro–MSP to MSP, which has potential to act on keratinocytes, macrophages, and capillaries, all components of the wound healing response. *Key words: enzyme activation/keratinocytes/receptor tyrosine kinase/wound. J Invest Dermatol 111:573–581, 1998*

Macrophage stimulating protein (MSP) was originally identified as a mammalian serum protein that caused shape changes in murine peritoneal resident macrophages and made them responsive to chemoattractants (Leonard and Skeel, 1976, 1978). MSP also acts directly as a chemoattractant for resident macrophages (Skeel and Leonard, 1994), stimulates macrophage ingestion of C3 bi-coated erythrocytes via the CR3 integrin receptor (Skeel *et al*, 1991), inhibits expression of inducible nitric oxide synthase mRNA in endotoxin or cytokine-stimulated macrophages (Wang *et al*, 1994a), and stimulates proliferation or motility in selected epithelial cell lines (Wang *et al*, 1996a, b).

Purification and cloning MSP showed that it belongs to a family of proteins that have evolved from an ancient serine protease precursor (Donate *et al*, 1994) and that have retained a highly conserved triple disulfide loop structure (kringle). Typical family members, including prothrombin and plasminogen, circulate in the blood as serine protease zymogens, which are activated by proteolytic cleavage to participate in coagulation or fibrinolysis. Amino acid substitutions in the serine protease catalytic triad have resulted in loss of enzymatic activity of two family members, MSP and hepatocyte growth factor/scatter factor (Matsumoto and Nakamura, 1992). Both proteins have retained the activation mechanism, however, in that they are secreted as biologically inactive single chain precursors, which become active after proteolytic cleavage at a single site to yield disulfide-linked αβ-chain heterodimers. It is reasonable to speculate that the common functional denominator for this kringle protein family is host defense response to wounding: blood coagulation, fibrinolysis, and stimulation of cells for debridement and repair.

In contrast to the coagulation and fibrinolytic kringle proteins, relatively little is known about the identity and locus of pro–MSP convertases, the enzymes that cleave pro–MSP to biologically active mature MSP. If MSP has a role in host response to wounding, pro–MSP would be expected to diffuse with other serum proteins into the wound exudate where a convertase would cleave it to MSP. Although several pure enzymes of the coagulation cascade, including kallikrein, factor XIIa, and factor XIa, cleave and activate pro–MSP (Wang *et al*, 1994), this does not occur when blood clots (Wang *et al*, 1996b), indicating that pro–MSP is not a preferred substrate for these enzymes. We recently showed that murine peritoneal macrophages have a cell surface pro–MSP convertase activity (Wang *et al*, 1996b). This *in vitro* finding suggests that resident dermal macrophages, potential target cells, might also have the capacity to generate the active ligand from the inactive precursor. This study is the first attempt to detect pro–MSP conversion *in vivo*, by testing for the presence of mature MSP as well as pro–MSP convertase activity in wound fluid.

The other part of our study relates to RON, the MSP receptor. The RON gene was cloned from a human foreskin keratinocyte cDNA library (Ronsin *et al*, 1993). RON belongs to a family of receptor protein tyrosine kinases that includes Met (Park *et al*, 1987),
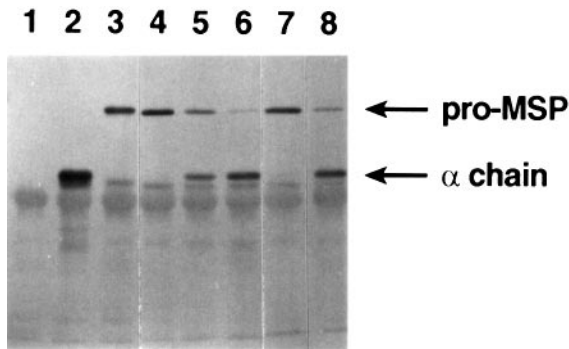
Figure 1. Detection of mature MSP in wound fluids. Immunoprecipitation with immobilized monoclonal anti-MSP and western blot with polyclonal antibody to MSP α-chain. *Lane 1*, medium control; *lane 2*, recombinant mature MSP; *lane 3*, recombinant pro-MSP; *lane 4*, patient BC, blood plasma; *lane 5*, patient BC, wound fluid, day of surgery; *lane 6*, patient BC, wound fluid, first postoperative day; *lane 7*, patient SK, blood plasma; *lane 8*, patient SK, wound fluid, first postoperative day. An α-chain band characteristic of mature MSP is present in *lanes 2, 5, 6,* and *8*.

Table I. Presence of mature MSP in surgical and burn wound fluids

	[a]A_{280}[a]	[pro − MSP + MSP] (nM)[b]	MSP, % of total[c]
Breast reduction fluid sampled at 24 h (n = 8)	46 ± 9	5.7 ± 0.6	56 ± 2
Burn wound fluid sampled in first 24 h (n = 5)	53 ± 14	6.1 ± 1.4	56 ± 7
Normal blood plasma (n = 4)	65 ± 5	4.9 ± 0.7	8 ± 5

[a]Values of fluids diluted 1:50 in 0.15 M NaCl were multiplied by the dilution factor to give the numbers shown.
[b]Determined by a sandwich enzyme-linked immunosorbent assay that detects both pro-MSP and MSP.
[c]Fluids were immunoprecipitated with monoclonal anti-MSP covalently bound to Sepharose-4B. After SDS-PAGE under reducing conditions, proteins were transferred to Immobilon and immunoblotted with antibody to MSP α-chain, which detects both MSP and pro-MSP. Relative intensities of the pro-MSP and MSP bands were determined by densitometry of negative transparencies.

the receptor for hepatocyte growth factor/scatter factor (Bottaro *et al*, 1991), a ligand with 45% sequence similarity to MSP (Yoshimura *et al*, 1993). Studies of cells transfected with the RON cDNA showed that MSP is a ligand for RON, because it bound specifically and saturably to these cells and caused receptor phosphorylation (Wang *et al*, 1994c). Cloning of RON from a keratinocyte cDNA library suggested that MSP might be a modulator of keratinocyte activites. This led to two reports showing that MSP induced the migration and proliferation of a number of murine keratinocyte cell lines (Wang *et al*, 1996a) and migration of human keratinocyte cell lines (Wang *et al*, 1996b). The capacity of MSP to stimulate both macrophages and keratinocytes suggested that this protein might play a role in wound repair, a possibility evaluated in this study.

MATERIALS AND METHODS

Reagents Recombinant human pro-MSP and MSP were from Toyobo (Osaka, Japan). Mouse monoclonal antibody to the extracellular domain of RON was a gift from Dr. Felix Montero-Julian (Immunotech, Marseille, France). As determined by flow cytometry, the antibody binds to Madin-Darby canine kidney epithelial cells transfected with RON cDNA. There is no detectable binding to nontransfected cells (Danilkovitch and Leondard, unpublished observations). Monoclonal anti-MSP was as described (Skeel *et al*, 1991). Rabbit antibodies to MSP and to MSP α-chain were as described (Wang *et al*, 1993). Monoclonal antibody AM-3K, which is specific for human macrophages (Zeng *et al*, 1996), was a gift from Motohiro Takeya (Kumamoto University Medical School, Kumamoto, Japan). Secondary antibodies for immunohisto-chemistry were from Jackson ImmunoResearch (West Grove, PA). Leupeptin
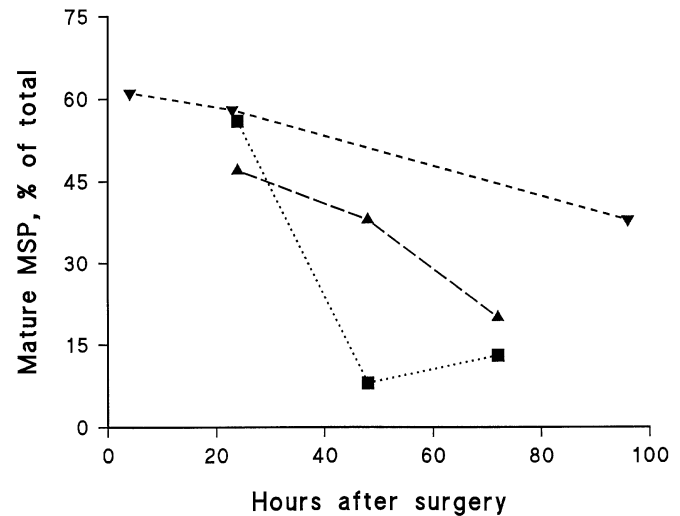

Figure 2. MSP in wound fluids collected from three patients at different times after reduction mammoplasty. Fluids were immunoprecipitated and analyzed as described in **Table I**.

and aprotinin were from Boehringer (Indianapolis, IN). Human serum kallikrein, C-1 esterase inhibitor, α-2-macroglobulin, and α-1-antichymotrypsin were from Athens Research and Technology (Athens, GA). Coagulation factors XIa and XIIa were from Enzyme Research Laboratories (South Bend, IN). Protease-free bovine serum albumin, 4-(1-aminoethyl)-benzenesulfonylfluoride and Phe-Pro-Arg-CH2 CL were from Calbiochem (La Jolla, CA). Alkaline phosphatase conjugated anti-rabbit IgG and soybean trypsin inhibitor were from Sigma (Cleveland, OH). Peroxidase conjugated anti-rabbit IgG was from Bio-Rad (Richmond, CA).

Sample collection Human partial thickness or edges of full thickness burn samples (n = 19) were harvested from various body locations in patients undergoing routine burn excision and autografting procedures at the Vanderbilt University Burn Center. Normal skin samples (n = 6) were collected from various body regions of patients undergoing elective surgery. Tissue samples were obtained in accordance with procedures approved by the Institutional Review Board. Cutaneous specimens were either frozen, sectioned, and immunostained or were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and immunostained. Venous blood was drawn into syringes containing ethylenediamine tetraacetic acid (EDTA), aprotinin, and leupeptin, making final concentrations ≈10 mM, 6 μM, and 85 μM, respectively. Exudates were collected from human burn wound blisters and also from breast reduction surgical drains. Samples of the latter were obtained by syringe in 1–2 ml volumes at various times after surgery.

Cells Peritoneal resident macrophages were obtained from C3H/HeN mice by lavage with RPMI 1640 medium (Skeel *et al*, 1991). Cells were washed once in RPMI 1640 and were then resuspended in Dulbecco's modified Eagle medium (DMEM) at a concentration of 5×10^5 cells per ml. The cell suspension was distributed in 0.5 ml aliquots into wells of a 48 well tissue culture plate and incubated with or without stimulating agents at 37°C in a humidified incubator containing 5% CO_2 in air.

Labeling of human pro-MSP with [125]I Fifteen micrograms of pure recombinant human pro-MSP in 30 μl of 0.1 M borate buffer, pH 8.5, were added to 250 μCi of Bolton-Hunter reagent (Bolton and Hunter, 1973) and equilibrated on ice for 150 min. The reaction was terminated by addition of 300 μl of borate buffer containing 0.5 M glycine. After 10 min on ice the reaction mixture was applied to an Excellulose GF-5 desalting column (Pierce, Rockford, IL) that had been equilibrated with phosphate-buffered saline containing 0.25% gelatin. The iodinated protein was eluted with 1 ml of phosphate-buffered saline-gelatin buffer, and counted in gamma-counter (Gamma 5500, Beckman). The specific activity of the labeled pro-MSP was ≈400 Ci per mmol.

Sandwich enzyme-linked immunosorbent assay for pro-MSP and MSP The assay utilized antibodies developed in the authors' laboratory (Skeel *et al*, 1991; Wang *et al*, 1993). The capture antibody was murine IgG monoclonal anti-MSP, lot 2S (0.1 μg per assay well); the detection antibody was polyclonal rabbit IgG anti-MSP. Details of the assay have been described (Wang *et al*, 1993). Dose–response curves for purified human plasma MSP, recombinant
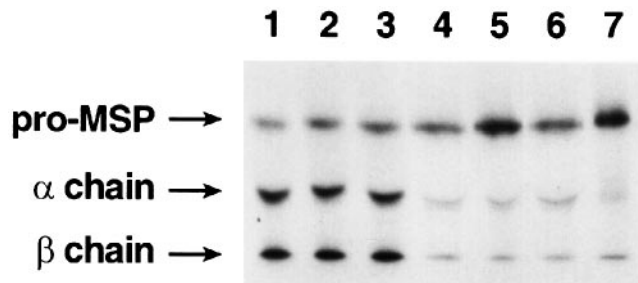
Figure 3. Pro-MSP convertase activity is detected in wound fluids, but not in normal human serum. ^{125}I-pro-MSP was incubated for 1 h at 37°C with wound fluids or undiluted sera. Samples were analyzed by radioautography after SDS-PAGE under reducing conditions. *Lanes 1–3,* fluids from reduction mammoplasty drains (*1, 2*) or burn wound (*3*); *lanes 4–6,* sera from three normal human subjects; *lane 7,* medium control (1 mg bovine serum albumin per ml in 20 mM, pH 7.4 phosphate buffer). The three wound fluids cleaved pro-MSP, as shown by a diminution in the intensity of the pro-MSP line, and the appearance of lines corresponding to the α- and β-chains of mature MSP.

pro-MSP, and recombinant MSP are comparable (Leonard and Skeel, unpublished data).

Immunoprecipitation and western blot of human plasma and wound fluids Monoclonal IgG anti-MSP was covalently linked to Sepharose-4B. One milliliter of a 1:2 dilution of plasma or wound fluid was equilibrated with 30 μl of settled gel on a rocker at 4°C for 2 h. After centrifugation, the supernatant was discarded and the gel was washed three times. It was then mixed with 30 μl of 2 × sample buffer containing 2-mercaptoethanol and placed in a boiling water bath for 10 min. Twenty microliter volumes were added to wells of an 8% polyacrylamide gel in a BioRad Mini-Protean II chamber, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was placed in a BioRad TransBlot cell for transfer of proteins to Immobilon P. After transfer, the membrane was blocked with a solution of 5% dried milk, rinsed, and equilibrated overnight with polyclonal rabbit antibody to the α-chain of MSP. The last step antibody was peroxidase conjugated goat anti-rabbit IgG; protein bands were detected after addition of 4-chloro-1-naphthol substrate and H_2O_2. The membrane was photographed, and the negative was photographed again to make a positive transparency in which the protein bands appeared as dark lines. An estimate of the relative amounts of pro-MSP and MSP was made by densitometry of the two bands, using an LKB densitometer.

Pro-MSP convertase activity of serum or wound fluids The assay was based on cleavage of labeled pro-MSP and detection of free MSP α-chain by radioautography after SDS-PAGE under reducing conditions. ^{125}I-pro-MSP was added to human serum or wound fluids and incubated at 37°C for different times. Samples were then mixed with sample buffer containing 2-mercaptoethanol. After SDS-PAGE as described above, the gel was dried and placed in an intensifying screen cassette with X-ray film (XAR-5; Eastman Kodak, Rochester, NY). The film was developed after 1–4 d at −80°C.

MSP activity of wound fluids The assay was based on the capacity of MSP to induce shape change of murine resident peritoneal macrophages in tissue culture wells. Cells were prepared as described above. They were then incubated for 1–2 h with DMEM alone, dilutions of wound fluid in DMEM, and MSP as a positive control. Cells were then fixed and photographed. After we found that wound fluids had shape change activity, wound fluids were passed down Sepharose columns with either anti-MSP or a control IgG; eluates were tested for shape change activity.

Localization of immunoreactive RON receptor Paraffin sections were dewaxed and microwaved in citrate buffer (Biogenex, San Ramon, CA) in accordance with antigen retrieval techniques. For both frozen sections and paraffin-embedded sections, endogenous peroxidase activity was quenched for 20 min in a 3% H_2O_2/methanol solution. All sections were then equilibrated in normal 10% porcine serum for 20 min and in primary anti-serum (ID-2 Ron anti-serum) (1:2000) for 18 h in a humidified chamber at 4°C. Serial sections were incubated in irrelevant anti-sera to distinguish between possible brown background and specific immunoprecipitates. Tissues were rinsed in phosphate-buffered saline for 10 min and equilibrated with reagents in a mouse peroxidase Vectastain ABC kit (Burlingame, CA). Immunoreactive sites were visualized with 3,3-diaminobenzidine as the chromagen (Biogenex, San Ramon, CA). Sections were counterstained in hematoxylin and rinsed in water, dehydrated, coverslipped, and photographed with an Olympus AH Vanox light microscope.
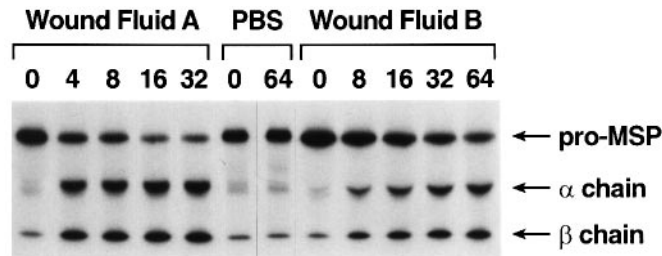

Figure 4. Time course of ^{125}I-pro-MSP cleavage by wound fluids. Experimental conditions were as described for **Fig 3**, except for different incubation times, shown in minutes.

Localization by immunofluorescence To confirm the presence of the RON receptor in a macrophage population in normal and burned human skin, double immunolabeling studies were undertaken. After an antigen retrieval treatment, sections were equilibrated in anti-RON antibody (1:400 dilution) for 18 h. Sections were rinsed and equilibrated in biotinylated goat anti-mouse IgG (1:1000) for 1 h and fluoroscein isothiocyanate-conjugated streptavidin (1:1000) for 1 h. Sections were then rinsed, blocked with 5% goat serum, and macrophages were selectively labeled as follows: sections were equilibrated for 3 h in a 1:1000 dilution of a murine monoclonal macrophage specific antibody (AM-3K), followed by Cy3 conjugated donkey anti-mouse IgG (1:1000 for 1 h).

An additional RON receptor anti-serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), to confirm RON receptor distribution in normal and burned human skin. In these experiments, sections were equilibrated for 18 h in the Santa Cruz polyclonal anti-RON receptor serum (1:50) and reacted with Cy3-goat anti-rabbit IgG (1:1000 for 1 h). Sections were blocked in 5% goat serum, followed by equilibration in monoclonal AM-3K antibody for 3 h, followed by sequential equilibration in biotinylated donkey anti-mouse IgG (1:1000 for 1 h) and fluoroscein isothiocyanate conjugated streptavidin (1:1000 for 1 h). Unless otherwise noted, all reagents used for immunofluorescent staining were purchased from Jackson ImmunoResearch. Coverslips were mounted on slides with Vectashield (Burlingame, CA). Images were viewed on a Zeiss Axioplan II Microscope, captured using Image ProPlus Software, formatted for presentation with Adobe Photoshop and printed on a TekTRONic dye-sublimation printer.

Localization by *in situ* hybridization Deparaffinized sections were acid treated to denature RNA at 25°C for 15 min in 0.2 M HCl, followed by 7.5 min of proteinase K treatment 20 μg per ml in 50 mM Tris pH 7.5, 5 mM EDTA (Sigma, St. Louis, MO) to permeabilize the cells. Sections were postfixed for 15 min in 4% paraformaldehyde. Samples were briefly acetylated and air dried. The RON receptor probe was generated by subcloning a BamH1-Sal1400 bp fragment into a pB/bluescript SK vector (Stratagene, La Jolla, CA). The plasmid was linearized by BamH1 or Sal1 for anti-sense or sense riboprobe labeling with T7 or T3 RNA polymerase (Amersham, Arlington Heights, IL) and ^{35}S-UTP. Hybridization was performed overnight at 50°C and for 4 h at 42°C with 2 × sodium citrate/chloride buffer/50% formamide for humidification using 200 μl of hybridization mixture to treat the slides at a final probe concentration of 2 × 10^4 cpm per μl. The hybridization mixture contained 300 mM NaCl, 10 mM Tris HCl pH 7.4, 10 mM NaH_2PO_4 pH 6.8, 5 mM EDTA pH 8.0, 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone, 50 mM dithiothreitol, 10% Dextran sulfate, 50% deionized formamide, and 10 μg yeast tRNA per ml. After hybridization, sections were washed in 2 × sodium citrate/chloride buffer, 20 mM β-mercaptoethanol at 50°C for 30 min. After two washes with 4 × sodium citrate/chloride buffer, 50% formamide, 20 mM β-mercaptoethanol at 55°C for 30 min each, slides were washed in a buffer containing 4 × sodium citrate/chloride buffer, 20 mM TrisHCl pH 7.5, 2 mM EDTA pH 8.0 at 37°C for 10 min each. Slides were digested with Rnase A (20 μg per ml) in this same buffer for 30 min. This wash procedure was then repeated. Slides were rinsed in water, air dried, dipped in Kodak NTB2 Emulsion, exposed for 3 d, and developed with D-19.

RESULTS

Detection of pro-MSP and MSP in wound fluids Using an enzyme-linked immunosorbent assay that detects both pro-MSP and MSP, we measured the concentration of [pro-MSP + MSP] in fluids collected from burn wounds and from surgical drains placed after reduction mammoplasty. As shown in **Table I**, the concentration was comparable with that of normal human blood plasma. This was the expected result, inasmuch as the A_{280} shows that the fluids are exudates, with protein concentrations similar to that of blood plasma.
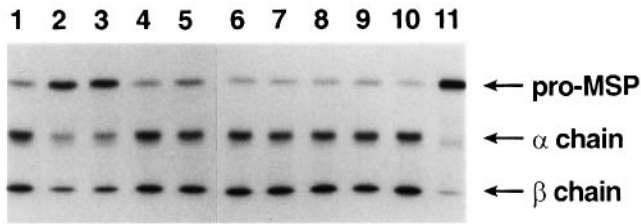
Figure 5. Effect of different protease inhibitors on wound fluid pro-MSP convertase activity. Wound fluid from a breast reduction patient was incubated at 37°C for 20 min with protease inhibitors. ^{125}I-pro-MSP was then added, and after 3 h solutions were analyzed for pro-MSP cleavage. *Lane 1,* 4-(2-aminoethyl)benzesulfonylfluoride, 1 mM; *lane 2,* aprotinin, 50 μg per ml; *lane 3,* leupeptin, 50 μg per ml; *lane 4,* soybean trypsin inhibitor, 50 μg per ml; *lane 5,* Phe-Pro-Arg-chloromethylketone, 10 μM; *lane 6,* EDTA, 10 mM; *lane 7,* α1-antichymotrypsin, 400 μM; *lane 8,* α2-macroglobulin, 0.3 μM; *lane 9,* C1-inhbitor, 50 μg per ml; *lane 10,* wound fluid without inhibitor; *lane 11,* medium control (cf. **Fig 3**, *lane 7*).

Biologically inactive pro-MSP is the predominant form in normal human circulating blood. Cleavage of pro-MSP at a single site creates active MSP, a disulfide-linked αβ-chain heterodimer. Pro-MSP is readily distinguished from MSP by SDS-PAGE under reducing conditions and western blotting with a polyclonal antibody that can interact with α-chain epitopes in both pro-MSP and MSP. Uncleaved pro-MSP appears as an 80 kDa band, whereas MSP is detected by its reduced free 53 kDa α-chain. This assay was used to determine if pro-MSP was proteolytically cleaved to mature MSP in wound fluids. A representative result for drainage fluids obtained from two reduction mammoplasty patients is shown in **Fig 1**. The western blot of the blood plasmas of these two patients shows a pro-MSP band (*lanes 4* and *7*), without a detectable MSP band. In contrast, free MSP α-chain is seen in the blots of wound drainage fluid (*lanes 5, 6,* and *8*). An estimate of the relative amounts of pro-MSP and MSP was made by densitometry of the western blot bands. As shown in **Table I** for the series of drain fluids collected 24 h post-surgery and for burn fluids obtained within the first 24 h post-burn, MSP comprised about half the total pro-MSP + MSP, in contrast to less than 10% for blood plasma. **Figure 2** shows that for three patients from whom multiple samples were obtained, the relative amount of MSP diminished with time after surgery, suggesting a decrease in conversion of pro-MSP to MSP as postoperative inflammation subsided.

Pro-MSP convertase activity of wound fluid   The detection of MSP in wound fluid suggested the presence of a cell-bound or fluid phase pro-MSP convertase in the wound bed. We therefore tested the capacity of wound fluids from six patients (five reduction mammoplasty, one burn) to cleave ^{125}I-pro-MSP to MSP, and found pro-MSP convertase activity in all. Representative radioautographs for three different wound fluids after SDS-PAGE under reducing conditions show MSP α- and β-chains, indicative of pro-MSP cleavage (**Fig 3**). In contrast, three different normal human sera had no pro-MSP convertase activity. **Figure 4** illustrates the time course of ^{125}I-pro-MSP cleavage by two undiluted wound fluids containing ≈3 nM pro-MSP. The data indicate that there is sufficient enzymatic activity in these fluids to generate detectable MSP within 10 min. The site of pro-MSP cleavage is R_{483}-V_{484} (Yoshimura *et al*, 1993), a typical scissile bond for trypsin-like proteases. To obtain preliminary information about the identity of the wound fluid pro-MSP convertase, we generated a protease inhibitor profile, shown in **Fig 5**. Inhibition by aprotinin and leupeptin is consistent with a trypsin-like serine protease (Barrett and Salvesen, 1986). Among serum trypsin-like enzymes, purified kallikrein, as well as coagulation factors XIa and XIIa, can cleave pro-MSP to MSP (Wang *et al*, 1994b). Cleavage of pro-MSP by each of these enzymes is inhibited by C1-inhibitor, and cleavage by serum kallikrein is also inhibited by α2-macroglobulin (data not shown). Therefore, it is unlikely that these enzymes account for the wound fluid activity, which was not inhibited by α2-macroglobulin or C1-inhibitor (**Fig 5**, *lanes 8* and *9*). Lack of inhibition by α1-antichymotrypsin rules

out chymotrypsin-like enzymes, and the EDTA result suggests that metalloproteases do not have a predominant role.

Biologic activity of wound fluid MSP   The concentration of MSP in wound fluids (**Table I**) is in the range for optimal biologic activity on target cells. We therefore determined if MSP in wound fluid is biologically active, by adding the fluid to murine resident peritoneal macrophages adherent to tissue culture wells. The wound fluid caused the macrophages to put out cytoplasmic extensions, a shape change characteristic of the action of MSP (Leonard and Skeel, 1976). To determine if the effect of the wound fluid was due to MSP, we tested for activity after passage of wound fluid down a column of immobilized monoclonal antibody that binds MSP. **Figure 6** shows that activity was absorbed out by the anti-MSP column (**Fig 6D**), whereas wound fluid effluent from the control column (**Fig 6C**) had an effect comparable with that of the MSP positive control (**Fig 6B**). This result shows that the macrophage shape change action of wound fluid was mediated predominantly or exclusively by MSP.

RON expression in normal human skin   Detectable immmuno-reactivity for the RON receptor was not a prominent feature of normal human skin (**Tables II** and **III**, **Fig 7A**). All cell layers comprising the interfollicular epidermal surface were generally negative in either frozen or paraffin sections ($N = 6$); however, sections from two of six normal subjects did show staining in epidermis and hair follicles; and, as noted below, high RON expression was also observed in macrophages and capillary endothelium in specimens from one of these two subjects. The occasional detection of RON receptor protein in epidermal cells of normal skin indicates that large differences in steady state expression of RON may occur in the absence of observable pathology.

In the underlying normal human dermis, RON was not detected in the endothelial cells lining veins or arteries in the three specimens that contained these structures; however, RON was detected in the capillary endothelium in sections from the same two subjects who exhibited RON immunostaining in the epidermis (**Table II**). Immuno-staining for RON was not observed in the major dermal cell population, presumed to be fibroblasts.

RON immunoreactivity was noted in resident macrophages in normal human dermis (**Fig 7A**). By quantitative morphometric analysis, the incidence of RON positive resident macrophages was 3.4 macrophages per 100 μ^2. To confirm that these cells were macrophages and to determine if RON was expressed on all dermal macrophages, tissues were evaluated by immunofluorescence double labeling with antibodies to RON and to a macrophage-specific marker (see *Materials and Methods*). In skin samples from six normal subjects, RON receptor immunoreactivity appeared perfectly matched to cells identified as macrophages (**Fig 8A, B**). Because no singly labeled cells were found, the results show that all macrophages had detectable RON protein, and all RON-positive cells scattered throughout the dermis are macrophages.

RON receptor distribution in burn wounds

Epidermal resurfacing   Re-epithelialization, one of the initial events in wound repair, involves migration and proliferation of epidermal keratinocytes. Because MSP has been shown to stimulate migration and proliferation of murine keratinocyte cell lines, we expected that RON, the receptor for this ligand, might be detected on the actively healing epidermal elements within human burn wounds. Therefore wounds from the acute burn period as well as later stages of repair were evaluated for RON expression.

One of the first visible events of wound resurfacing is migration of surviving keratinocytes. Surprisingly, migrating keratinocytes either at the edges of the wounds (**Fig 7D**) or from surviving keratinocytes within the deeply positioned sweat ducts (**Fig 7C**) or hair follicles expressed little or no detectable RON receptor protein at any of the time points examined (post-burn days 1–10). At wound edges and in surviving epidermal islands, the proliferative population of keratinocytes is immediately adjacent to the migratory population. In this proliferative population, RON receptor protein was frequently not detectable in the early burn wound period (**Fig 7E**); however, in the more established
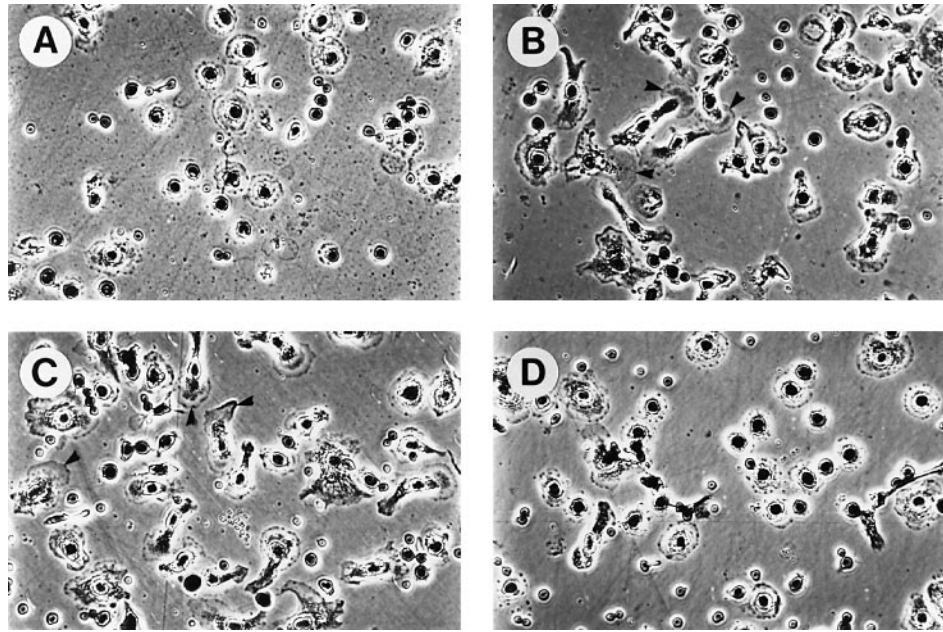
Figure 6. Murine resident peritoneal macrophage activation assay. Peritoneal cells were harvested from normal mice, and suspensions in DMEM were added to tissue culture wells. After 30–45 min, DMEM was aspirated, and fluid samples to be assayed were added to the adherent cells. One hour later, cells were washed with phosphate-buffered saline, stained with Diff-Quick, and photographed. (*A*) DMEM control; (*B*) 2 nM MSP; (*C*) wound fluid eluted from a control mouse IgG column and diluted 1:30 in DMEM for assay; (*D*) wound fluid eluted from a monclonal anti-MSP immunoaffinity column and diluted 1:30 for assay. Numbers of macrophages with lamellipodia (*arrows*) or elongated shapes: (*A*) 0; (*B*) 19; (*C*) 20; (*D*) 3.

Table II. Detection of RON receptor protein in normal skin

	Epidermis			Hair follicle		Sweat gland		
Sample	Basal	Spinous	Granulosum	Outer	Inner	Secretory	Ductal	Capillary
1p[a]	−	−	−	+/−	+/−	−	+	−
2p	−	−	−	−	+	×[b]	+	−
3p	+/−	+++	+++	+/−	++	×	−	+++
4f	−	−	−	−	−	×	×	−
5f	++	++	++	+	+	×	×	+
6f	+	−	−	×	×	×	×	−

[a]p, paraffin sections; f, frozen sections.
[b]×, cell or structure was not present in the section; +/−, patchy staining distribution.

Table III. Increased frequency of RON detection in burn wounds[a]

	Epidermis[b]	Hair follicle[c]	Capillary[d]
Normal skin	2/6	4/6	2/6
Burn wound	14/14	12/12	7/16

[a]Frequencies derived from **Tables II** and **IV**.
[b]RON staining intensity of 1+ or more in spinous keratinocytes in normal skin or in burn wound hypertrophic epithelium
[c]RON staining of 1+ or more in differentiated cells.
[d]RON staining of 1+ or more in endothelial cells.

proliferative populations such as those comprising upwardly growing sweat ducts (**Fig 7C**), or the multistratified epidermis in later phases of resurfacing, or the hyperproliferative epidermis adjacent to the wound bed (**Fig 7F**), prominent immunoreactivity for the RON receptor was evident. Abundant RON protein was also frequently apparent in the differentiated population of keratinocytes (**Fig 7C–E**). A tabular summary of these data is shown to illustrate spatial distribution of RON within each specimen in the series (**Table IV**).

Six burn wound specimens were re-examined for RON gene expression, using *in situ* hybridization techniques. **Figure 9(A–D)** illustrate that RON gene expression is more widespread than indicated by the distribution of immunoreactive RON receptor protein. RON

receptor transcripts were prominent in advancing epithelial tips at the wound edge (**Fig 9A**, **B**).

Granulation tissue formation (dermal repair) Immunoreactive RON receptor protein was not detected in neutrophils, which accumulate in early stages of burn wounds. As healing proceeds during days 2–5 after burn injury, tissue macrophages increasingly infiltrate into the overlying eschar as well as granulation tissue within the wound bed. RON receptor protein was readily apparent in these macrophages within the overlying eschar (**Fig 7G**) and in focal regions of inflammation within deeper areas of the burn wound (**Fig 8C**). Double-labeling with a macrophage marker confirmed the identity of these RON-positive cells (**Fig 8C**, **D**). Nearly 100% of the cells that were identified by the Am-3K macrophage marker appeared to be positive for the RON receptor protein. By *in situ* hybridization, transcripts for the RON receptor were prominently displayed on the infiltrating population of macrophages (**Fig 9A–D**), thus confirming expression of the RON receptor by macrophages within cutaneous sites of inflammation.

An unexpected finding of this study was the distribution of the RON receptor protein on endothelial cells lining many but not all capillaries in two of six sections of normal skin and eight of 16 sections of burn wound skin (**Tables II–IV, Fig 7H**). The staining appeared to be most prominent within areas of neovascularization but was by no means a uniform finding. Expression of RON by endothelial cells
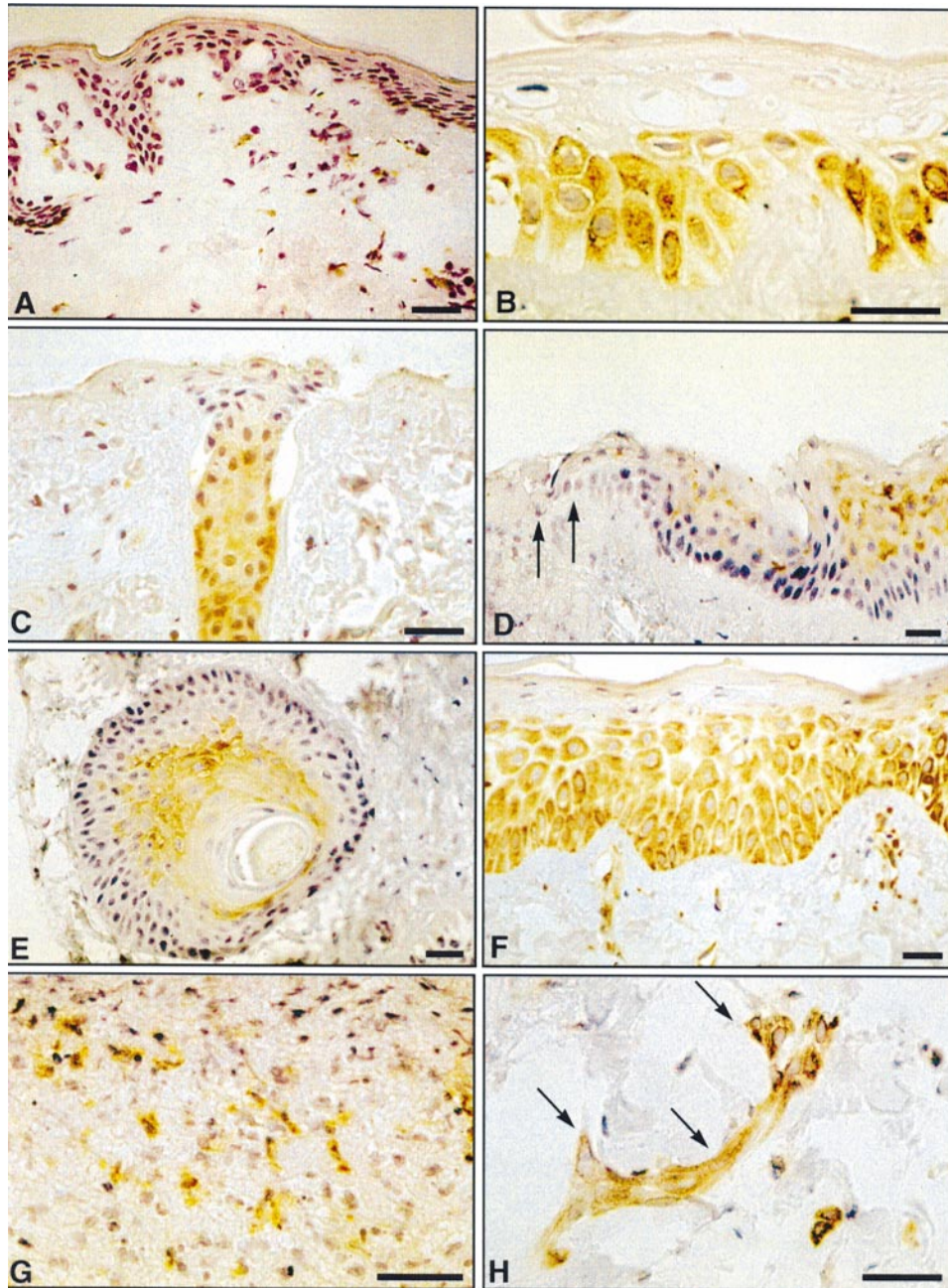
Figure 7. Immunolocalization of the RON receptor using the ID-2 anti-serum. (*A*) Normal human skin shows no epidermal staining but RON protein is present in some dermal cells, presumably macrophages. (*B*) In this newly resurfaced region of the epidermis that has not become fully stratified, RON receptor protein is prominently displayed in the basal population but not in the suprabasal keratinocytes. (*C*) A surviving epithelial appendage growing toward the surface at post-burn day 4. The migrating epidermal tip at the surface is unstained, but prominent immunoreactivity is present in the deeper (proliferative) keratinocytes that are contributing cells upward to the surface layers. (*D*) At the epidermal edge (post-burn day 5), migrating cells (*arrows*) and the immediately adjacent proliferating cells at the tip are unstained, but staining is observed in the more differentiated (upper) epidermal layers. (*E*) This noninjured hair follicle located beneath the burned region shows RON receptor immunoreactivity only in the more differentiated cells of the inner root sheath. (*F*) This hyperproliferative epidermis immediately adjacent to the burn shows strongest RON receptor staining in the proliferative layers but diminished staining in the differentiated layers of outer stratum spinosum and granulosum layers. Staining is also present in capillaries in the papillary region of the dermis macrophages. (*G*) RON-positive cells (presumably macrophages) in the exudate/eschar. (*H*) Staining for RON is present in capillary endothelium (*arrows*) and adjacent macrophages, but is absent in fibroblasts. *Scale bars*: 25 μm.

lining capillaries was confirmed by detection of RON transcripts by *in situ* hybridization (data not shown).

DISCUSSION

Members of the kringle protein family have in common the fact that they are constitutively secreted as biologically inactive precursors, which can be rapidly activated at the appropriate time and locus by proteolytic cleavage. Pro-MSP is secreted by the liver (Bezerra *et al*,

1993; Yoshimura *et al*, 1993). Minimal or absent MSP in human serum (Wang *et al*, 1994b) or plasma (**Fig 1**) indicates that pro-MSP is the predominant form in circulating blood. We anticipated that if MSP has a role in tissue injury, diffusion of pro-MSP from the circulating blood into wound beds would result in proteolytic cleavage to active MSP by a fluid phase or cell-associated pro-MSP convertase. Detection of MSP by western blot in all tested exudates from both burn wounds and surgical drains fulfilled this prediction. Furthermore, the
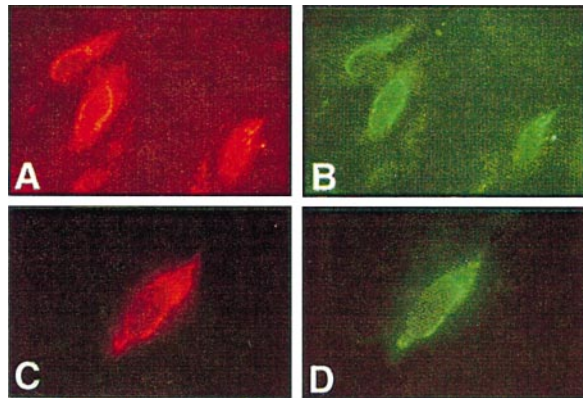
Figure 8. Immunofluorescence colocalization using ID-2 antibody to the RON receptor (green) and AM-3K macrophage–specific antibody (red). (*A, B*) Colabeling of a resident macrophage in normal (nonwounded) human dermis. (*C, D*) Colabeling of infiltrating macrophages in granulation tissue at post-burn day 6.
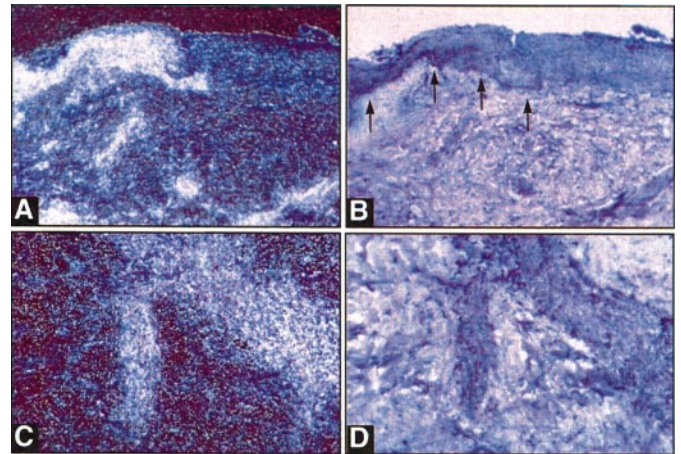

Figure 9. Transcripts for the RON receptor at post-burn day 6 as detected by *in situ* hybridization. (*A, B*) In these matching dark field and bright field photomicrographs, silver grains [bright in (*A*), dark in (*B*)] are concentrated over the migrating and proliferating epidermal sheet in the left upper edge of the wound (*arrows*). In the right upper corner, no labeling is present in the eschar covering the surface of the wound bed. Focal labeling within inflammatory foci around dermal blood vessels is also present within the deeper areas of this wound (×65). (*C, D*) In these matching dark and bright field photomicrographs, silver grains are clustered over the heavily vascularized areas within the wound bed. Labeling is highest over inflammatory cells (macrophages) that immediately surround blood vessels (×110).

Table IV. Immunostaining for RON protein in tissues from 19 human burn wounds

PBD[a]	Resurfacing epithelium			Hypertrophic epithelium			Hair follicle		Sweat gland		Cap.	Mac.
	Migr.	Prolif.	Diff.	Basal	Spinous	Gran	Outer	Inner	Secr.	Ductal		
1	×[b]	×	×	×	×	×	×	×	×	×	−	−
1	×	−	−	×	+	×	×	×	×	×	×	×
2	−	−	++	+/−[c]	++	++	−	+	+	±	+	++/−
3	×	×	×	×	×	×	×	×	×	×	−	−
4	−	−	+	−	+	−	×	×	+	+	−	+
4	−	++/−	++/+	−	++	−	×	×	−	+	+	+
4	×	×	×	−	++	−	+/−	++	×	−	+	++
5	×	×	×	×	×	×	×	×	×	×	+	++
5	+/−	+/−	++	−	++	−	−	++	×	+	−	++
5	−	×	×	×	+	+	×	×	×	×	×	×
5	−	−	++	−	++	−	−	++	×	×	×	×
5	+/−	+/−	++	+/−	++	+	−	++	×	+	+	+/−
6	×	×	×	×	×	×	+	+	×	×	−	+
6	×	×	×	×	×	×	−	+	+/−	+	−	?
6	−	+	++	+/−	++	++	+/−	++	×	+/−	++	++
7	−	−	++	−	++	−	−	++	×	+	−	−
7	+/−	−	++	−	++	−	−	++	×	+	−	+
10	−	−	++	−	++	+++	−	+++	×	+	++/−	++
11	×	+/−	++	+/−	+++	−	+	++	×	×	−	+++

[a]PBD, post-burn day.
[b]×, cell or structure was not present in the section.
[c]+/−, patchy staining.

concentration of MSP as determined by enzyme-linked immunosorbent assay is in the optimal range for biologic activity.

In addition to immunochemical evidence for MSP in wound exudates, we found that exudates induced morphologic changes in adherent murine macrophages comparable with those induced by pure recombinant MSP (**Fig 6**). The exudate activity was absorbed out almost completely by an anti-MSP immunoaffinity column, but not by a control column, indicating that the observed activity was accounted for predominantly by MSP. Considering the host of mediators present in wound exudates (Gailit and Clark, 1994), it is remarkable that MSP appears to be almost exclusively responsible for induction of the changes illustrated in **Fig 6**. Thus, in addition to our previous findings that MSP induces phagocytosis via the CR3 receptor (Skeel *et al*, 1991), the time lapse study provides evidence for global membrane activation.

RON expression is a relatively late event in mononuclear phagocyte maturation, and also appears to be restricted to select phagocyte populations: it is not present on human monocytes, and in mice it occurs in resident peritoneal macrophages, but not in acute peritoneal exudates or in splenic or alveolar lavage macrophages (Iwama *et al*, 1995). An important aspect of this work is the immunohistochemical detection of the MSP receptor on all dermal macrophages of both

normal skin and burn wound skin. This finding makes it reasonable to extrapolate the *in vitro* data on murine macrophage pinocytosis and phagocytosis to human dermal macrophages, and to suggest that MSP may stimulate macrophage dependent wound debridement. The importance of macrophages in wound debridement was first shown by Leibovich and Ross in macrophage-depleted guinea pigs (Leibovich and Ross, 1975). Electron micrographs of macrophages in wounds of control animals showed cells filled with erythrocytes, neutrophils, fibrin, and unidentifiable debris. In wounds of macrophage-depleted animals, this debris was not ingested, and wound healing was delayed.

The concentration of mature MSP in extracellular fluid (ECF) will depend on the rate of diffusion of pro-MSP from circulating blood into tissues, the activity of ECF pro-MSP convertases, and degradation of MSP in the ECF or after ligand-receptor cellular internalization. We reported that murine peritoneal macrophages have a membrane-bound pro-MSP convertase, and also an enzyme that cleaves and inactivates pro-MSP (Wang *et al*, 1996b). A serum inhibitor, recently identified as α1-antichymotrypsin (Skeel and Leonard, unpublished data), blocks the inactivating enzyme. Thus a tissue macrophage target cell might generate MSP when pro-MSP and α1-antichymotrypsin diffuse into the tissue from the circulation. Additionally, we have now shown that wound fluid exudates have pro-MSP convertase activity (**Figs 3**, **4**). The time course of pro-MSP cleavage by wound fluid convertase (**Fig 4**) suggests that this enzymatic activity is sufficient to account for the presence of mature MSP in wound fluids.

In addition to providing the first *in vivo* evidence of proteolytic cleavage of pro-MSP to active MSP following tissue injury, our study also shows that the RON receptor for MSP is highly upregulated during the complex events that occur during human wound repair (**Table III**). Numerous studies have shown upregulation of several growth factors and their receptors in human or experimental animal wounds (Antoniades *et al*, 1991, 1993; Wenczak *et al*, 1992; Danilenko *et al*, 1995; Takenaka *et al*, 1997). Our methods for detection of MSP receptor message or protein are similar, but differ with respect to the MSP ligand, which is generated in injured tissue by cleavage of preformed pro-MSP. In contrast, published studies focus on de novo synthesis of growth factor ligands by cells in the damaged locus. It would be of interest to identify the putative mediators in the wound environment that stimulate synthesis of these growth factors and upregulation of their receptors.

Table IV shows that upregulation of RON expression by epithelial cells in human burn wounds occurs in proliferating or differentiated populations or both. It is possible that MSP could promote proliferation in some keratinocytes and differentitation in others. There is abundant evidence in the literature that effects of ligand binding to receptor tyrosine kinases can be determined by the type and state of the target cell (Schlessinger and Ullrich, 1992). For example, occupancy by NGF of the trk receptor tyrosine kinase caused differentiation of neurons, but proliferation of trk-transfected fibroblasts (Glass *et al*, 1991). In cell lines transfected with STK, the murine receptor for MSP, addition of the ligand caused proliferation of the pro-B cell line, but apoptotic death of the mouse erythroleukemia (MEL) cell line Iwama *et al* (1996). Although an identical group of signal transduction proteins became associated with the STK multifunctional docking site in both transfectants, two interacting phosphorylated proteins were found only in the MEL line. Thus, the functional result of receptor occupancy appears to depend on the state and availability of a broad spectrum of transducing proteins. In the case of the MSP receptor, already documented outcomes include proliferation, apoptosis, stimulation of membrane and cytoplasmic motility, and activation of integrin receptors that result in increased adherence to substrate (Danilkovitch and Leonard, unpublished data) or phagocytosis. Alternatively, the prominence of RON receptor transcripts in advancing epithelial tips and the paucity of RON protein in these same cell populations may relate more to RON receptor production and turnover. Wounded epidermis at tips may be high turnover, but the equilibrium may be tipped in favor of RON accumulation in the more differentiated stable cell populations.

In conclusion, this study provides the first *in vivo* evidence for a role for MSP in tissue injury: upregulation of the RON receptor on keratinocytes, as well as the presence of pro-MSP convertase activity and mature MSP in wound exudates. Because not all mononuclear phagocyte populations express the receptor for MSP (Iwama *et al*, 1995), it was also important to find that dermal macrophages express RON, which makes it likely that mature MSP can stimulate wound debridement by these cells. Another novel finding of this study was the expression of RON message and protein on capillary endothelial cells. Perhaps the presence of yet another receptor protein tyrosine kinase on capillary endothelial cells might have been anticipated. These cells, with their assortment of tyrosine kinase receptors, are targets for various ligands that are thought to mediate proliferation and migration of new capillaries, the hallmark of granulation tissue. Experiments to determine if MSP is angiogenic are in progress.

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