Neutrophils Express Tumor Necrosis Factor-α During Mouse Skin Wound Healing

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The expression pattern of tumor necrosis factor-α (TNF-α) mRNA and protein was examined *in vivo* in experimental mouse skin wounds by *in situ* hybridization and immunohistochemistry. TNF-α mRNA and protein is detected in a distinct layer of mainly neutrophils subadjacent to the wound clot. The layer of TNF-α–positive cells extends from the margin of the advancing epithelial outgrowth to the opposing one. By *in situ* hybridization the TNF-α mRNA is detectable 12 h after wounding; the signal peaks after 72 h and remains visible up to at least 120 h after wounding. TNF-α mRNA could not be detected in the normal skin or in 5-hour–old wounds. Immunohistochemical staining for TNF-α and macrophages on adjacent sections confirms that the main part of TNF-α–positive cells are polymorphonuclear neutrophils and shows that most of the cells located just beneath the layer of TNF-α–positive neutrophils are macrophages with weak TNF-α immunoreactivity. The data reported here show that neutrophils serve as an important source of TNF-α during healing of mouse skin wounds. We suggest that this specific expression of TNF-α is related to the process of re-epithelialization. ***Key words: re-epithelialization/in situ hybridization/Mac-2. J Invest Dermatol 105:120–123, 1995***

Tumor necrosis factor alpha (TNF-α), or cachectin, is a multifunctional cytokine with a broad spectrum of activities (reviewed in [1,2]). For example, it is involved in cachexia [3], septic shock [4], acute inflammation [5], the immune response [6], and inhibition of tumor growth [7]. TNF-α has also been shown to activate neutrophils [8], to stimulate angiogenesis [9], and to enhance mitogenesis and invasive migration of normal fibroblasts [10,11]. TNF-α mRNA and protein have been detected in normal mouse kidney, liver, and spleen by *in situ* hybridization and immunohistochemistry, respectively [12].

Healing of cutaneous wounds can be divided into three different phases: inflammation, granulation tissue formation, and matrix formation and remodeling [13]. During these phases a single layer of keratinocytes migrates from the wound margin and penetrates underneath the wound clot, and a regenerated epidermal surface is ultimately formed. The early stage of inflammation is dominated by an influx of neutrophils and is followed by the migration of monocytes to the site of inflammation. The late inflammatory phase is characterized by a decrease in the infiltration of the neutrophils and the conversion of monocytes to activated macrophages. Both neutrophils and macrophages are involved in ingesting debris, clearing of contaminating bacteria, and degradation of the fibrin-fibronectin clot initiated by blood platelets directly after wounding [13].

It has been shown that many of the processes taking place during wound healing are regulated by a wide variety of growth factors and cytokines [13]. Activated macrophages are known to release many of these proteins and are also assumed to be a main source of TNF-α synthesis [2]. Therefore, it is tempting to speculate that TNF-α may function in the wound-healing process, as do other cytokines such as TGF-βs, fibroblast growth factors (FGFs), and platelet-derived growth factor (PDGF) [13]. In a model using subcutaneous implanted wound chambers, expression of TNF-α mRNA was detected in inflammatory cells 1 to 7 d after the implantation [14]. However, experiments examining the effect of exogeneous TNF-α on experimental wounds have produced apparently conflicting data [15–18], and the physiologic role of TNF-α during wound healing therefore needs to be elucidated. In the present study we show by *in situ* hybridization and immunohistochemistry that neutrophils located subadjacent to the wound clot are the main source of *de novo* TNF-α synthesis during mouse skin wound healing.

MATERIALS AND METHODS

Materials T3, T7, Sp6 polymerases, and anti–Mac-1 and anti–Mac-2 monoclonal antibodies directed against the macrophage antigens Mac-1 and Mac-2, were from Boehringer Mannheim, Germany. pBluescript KS(+) was obtained from Stratagene, La Jolla, CA. Rabbit polyclonal anti-mouse TNF-α serum was from Genzyme, MA. Recombinant mouse TNF-α was from R & D Systems, UK.

Animal and Tissue Treatment Procedures Ten-week–old female Balb/C mice were experimentally wounded by surgical incision, and the tissue was processed as described earlier [19] except that the tissue after overnight fixation in 4% paraformaldehyde was embedded in paraffin.

Preparation of RNA Probes Two non-overlapping fragments of the full-length murine TNF-α cDNA [20] were subcloned using standard cloning techniques. The pm TNF-α1 is a 920-bp SacI fragment (709-1629) ligated into pBluescript KS(+). The pm TNF-α2 contains the EcoRI(0)-SacI(709) fragment religated in the pGEM-1 vector. After banding through CsCl-gradients both plasmids were linearized: pmTNF-α1 with SacI or PstI, and pmTNF-α2 with NaeI or SpeI. Sense and antisense RNA probes were

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Abbreviation: uPA, urokinase-type plasminogen activator.

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generated by *in vitro* transcription of 1 μg of the linearized plasmids with the relevant RNA polymerase as described earlier [19].

***In Situ* Hybridization** The *in situ* hybridization was performed as described in detail earlier [21] except that paraffin-embedded tissue was used for the analysis.

Immunohistochemical Staining The sections were deparaffinized in xylene, rehydrated with ethanol, rinsed in water, and incubated for 10 min at 37°C with 0.1% (w/v) trypsin in 0.05 M Tris/HCl and 0.1% (w/v) $CaCl_2$, pH 7.4.

Immunohistochemical staining of TNF-α was performed with rabbit polyclonal anti-mouse TNF-α serum diluted 1:100 or 1:200 by the peroxidase-antiperoxidase method [22]. Staining for macrophages was performed with anti-macrophage monoclonal antibodies (10 μg/ml) against the Mac-1 and Mac-2 antigens by the alkaline-phosphatase–anti-alkaline phosphatase method [23]. Controls for the TNF-α staining omitted specific anti-mouse TNF-α serum or were preabsorbed (1:200 dilution of the specific antibodies with a tenfold excess of recombinant mouse TNF-α). The negative control for the macrophage staining omitted the primary antibody.

RESULTS

TNF-α Is Expressed Primarily by Neutrophils During Skin Wound Healing The expression of TNF-α mRNA during experimental mouse skin wound healing was detected by *in situ* hybridization with antisense mouse TNF-α RNA probes. TNF-α mRNA is present in a discrete layer of cells consisting mainly of neutrophils, which are located subadjacent to the wound clot **(Fig 1*a*,*b*,*d*)**. The TNF-α expressing cells could not be detected in 5-hour–old wounds, but appeared first in 12-hour–old wounds. At later times investigated ranging from 24 to 120 h, the layer of positive cells extends from the margin of the advancing epithelial outgrowth to the opposing one **(Fig 1*a*)**; the strongest signal is observed after 72 h. No signal for TNF-α mRNA could be detected in any other cells in the wound area or in normal skin.

As a confirmation of the specificity of the hybridization, we obtained identical results with two different probes (pmTNF-α1 and pmTNF-α2) generated from two non-overlapping fragments of the TNF-α cDNA (data not shown). Hybridization of adjacent sections with the corresponding sense TNF-α RNA probes gave no specific signals and served as negative controls **(Fig. 1*f*)**. The presence of TNF-α in the wounds was furthermore confirmed with reverse transcriptase–PCR amplification, by which TNF-α mRNA could be detected in poly (A)+ RNA samples isolated from mouse skin wound extracts (data not shown).

The presence of TNF-α protein in the wounds was detected by immunohistochemical staining of 24, 72, 96, and 120-hour–old wounds with a polyclonal rabbit anti-mouse TNF-α serum. The TNF-α immunoreactivity is found in a layer of polymorphonuclear neutrophils corresponding to the TNF-α mRNA expressing layer of neutrophils **(Fig 1*c*,*g*)**. **Figure 2*a*** shows the TNF-α immunoreactivity in primarily polymorphonuclear neutrophils located close to the tip of the migrating epidermal layer. Staining of adjacent sections with a macrophage marker, a rat monoclonal antibody directed against Mac-2 antigen, shows that the macrophages are located just beneath the main layer of TNF-α–positive neutrophils **(Figs 1*e* and 2*b*)**. Some of the weak TNF-α immunoreactivity seen in this area is probably located in these macrophages **(Fig 1*c*,*e*)**. A monoclonal antibody directed against the Mac-1 antigen gave a staining pattern identical to the a–Mac-2 staining (data not shown). No specific staining for TNF-α protein could be detected in control skin, or 5- and 12-hour–old wounds (data not shown). The TNF-α immunoreactivity could be abolished by pre-incubation of the diluted a–TNF-α serum with an approximately tenfold excess of recombinant murine TNF-α (not shown).

DISCUSSION

In this study we have identified wound-infiltrating neutrophils as the main source of TNF-α during healing of incisionally wounded mouse skin. It is noteworthy that the signal for TNF-α mRNA first appears after 12 h by *in situ* hybridization. Thus, either the neutrophils are induced to synthesize TNF-α *in situ* or there is a migration of TNF-α expressing neutrophils to the wound site 12 h after wounding. The specificity of the *in situ* hybridization results is supported by the use of two different antisense RNA probes from non-overlapping TNF-α cDNA clones that give identical results.

The finding of TNF-α mRNA in infiltrating neutrophils by *in situ* hybridization is intriguing, both because in general it has been assumed that activated macrophages are the principal source of TNF-α [2], and in a broader sense because circulating neutrophils have been thought not to synthesize new protein. The influx of inflammatory cells into the wound site is initially dominated by neutrophils, which are present the most during the first 2 d after wounding and then decrease in number, whereas the macrophages become more dominant at the later stages of healing [14]. Our immunostaining with the macrophage markers a–Mac-1 and -2 has shown that the macrophages in the vicinity of the wound clot localized to the area just beneath the neutrophils expressing TNF-α mRNA and protein. However, it cannot be excluded that also some of these macrophages contribute to the synthesis of TNF-α during mouse skin wound healing. A weak but specific staining for the TNF-α protein is detected in some of the macrophages located just beneath the TNF-α–positive neutrophils **(Fig 1*c*,*d*)**, but at present it is unclear whether this macrophage-associated TNF-α is synthesized by the macrophages themselves or is bound to TNF-α receptor. Further experiments are required to answer this question.

The functional role of TNF-α in dermal wound healing is still unclear. Because TNF-α is able to stimulate neutrophils [8], it is possible that these wound-infiltrating neutrophils are stimulated by an autocrine mechanism. Another potential role for TNF-α could be as a signal molecule for the migrating keratinocytes. After 12 h, when the epithelial tongue has begun to migrate, the leading keratinocytes seem to be following the path of TNF-α–positive neutrophils until the epidermal layer is completely regenerated. The possibility of molecular communication involving TNF-α from the layer of TNF-α–positive wound-infiltrating neutrophils and the migrating keratinocytes is sustained by a recent report showing expression of the TNF-α receptor p55 TNF-R in both normal and psoriatic epidermis *in vivo* [24].

The effect of blocking TNF-α activity in mouse-skin wounds could be tested by administration of neutralizing antibodies to TNF-α followed by examination of the effect on the expression of different cytokines and proteolytic enzymes and their specific inhibitors known to be expressed during wound healing. In this respect, the different components of the plasminogen activation system are of great interest. Recently, expression of urokinase-type plasminogen activator (uPA), its receptor, and type-1 plasminogen activator inhibitor has been detected in the migrating keratinocytes at the edge of the wound [19,22,25].

In addition, our finding that extravasated neutrophils are capable of *de novo* synthesis of TNF-α mRNA is in agreement with the results of two recent studies. In one, neutrophils in endotoxin-induced injured mouse lung tissue were found to be a significant source of TNF-α mRNA [26]. In a study of endotoxin-treated mouse bone-marrow neutrophils, TNF-α was detected ultrastructurally in the secretory granules [27]. These and our own results imply a previously unknown functional role of neutrophil-derived TNF-α in tissue-repair processes such as skin wound healing.

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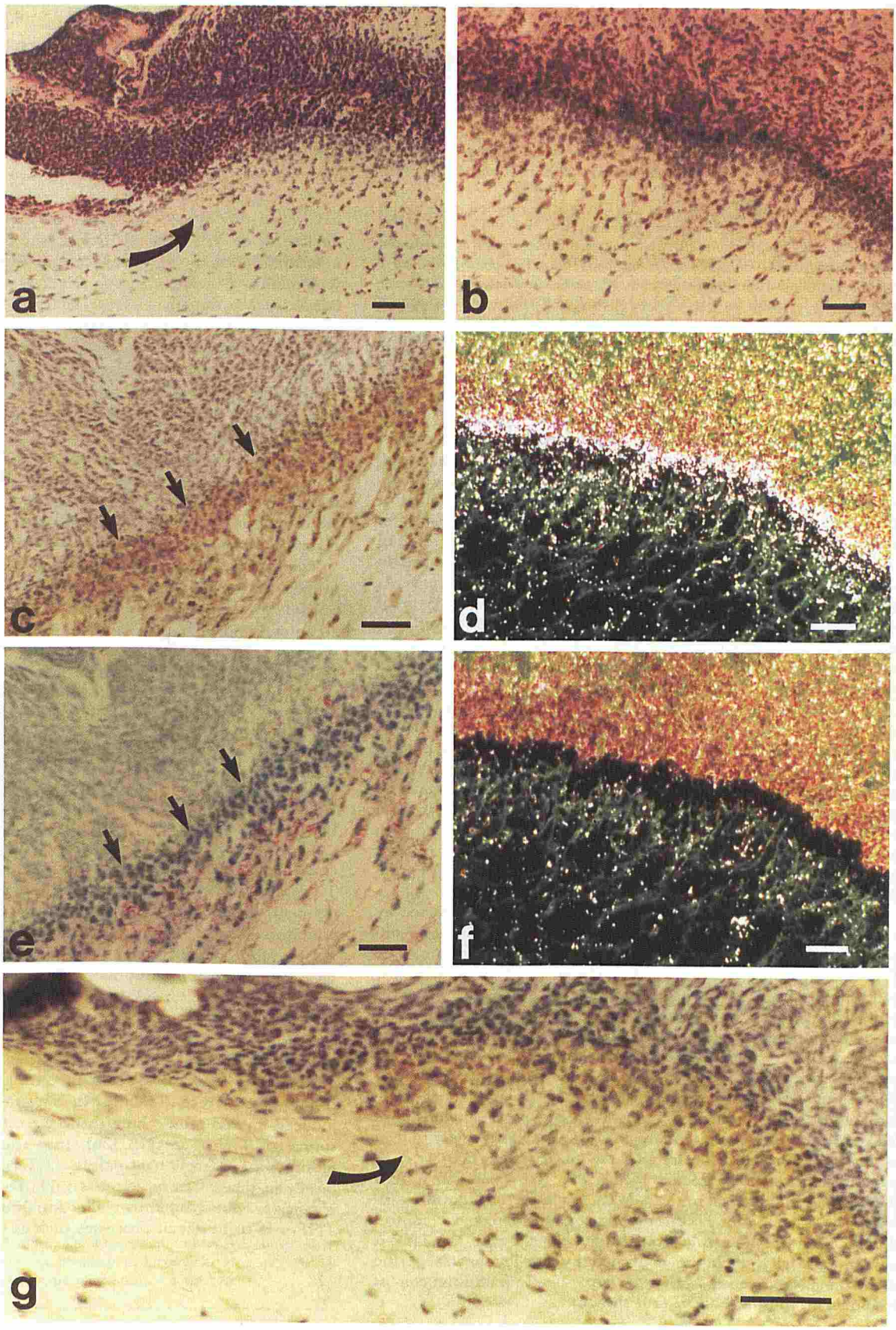

Figure 1. TNF-α is present in a discrete layer of neutrophils subadjacent to the wound clot of a 72-hour–old mouse skin wound. *In situ* hybridization of adjacent paraffin sections of a 72-hour–old mouse skin wound with ^{35}S-labeled antisense RNA probes for mouse TNF-α (*a, b, d*) shows TNF-α mRNA in a layer of neutrophils located subadjacent to the wound clot. No signal above background is detected with the complementary sense RNA probe for TNF-α (*f*). The sections are shown in brightfield (*a* and *b*) or darkfield (*d* and *f*) illumination. Immunohistochemical staining with polyclonal anti-mouse TNF-α IgG (*c* and *g*) demonstrate immunoreactivity in a layer of neutrophils located similarly to the TNF-α mRNA expressing cells. Staining with a macrophage marker (a-Mac-2) on an adjacent section shows that there are no macrophages present in the particular layer of TNF-α mRNA expressing neutrophils, but that the macrophages (staining red) are situated just below this layer (*e*). At the wound margin, the TNF-α–positive neutrophils are seen in close contact with the tip of the epithelial tongue (*g*). The *arrows* in *a* and *g* show the direction of the moving keratinocytes. The *arrows* in *c* and *e* delineate the upper margin of the layer of TNF-α–positive neutrophils. *Bars*, 50 μm.

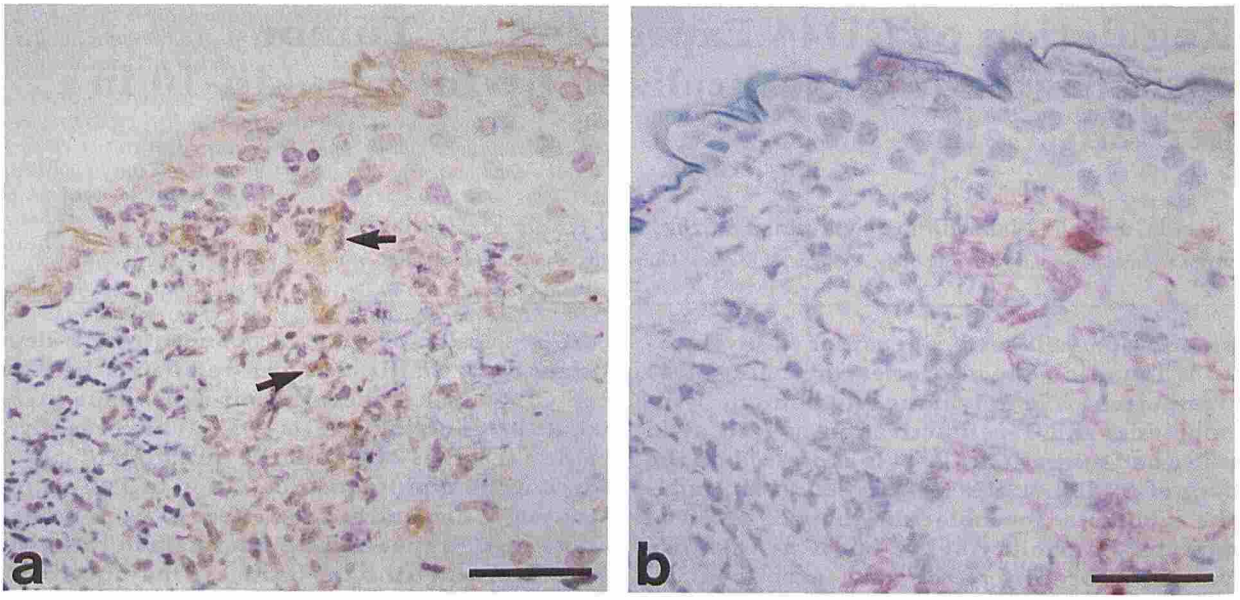

Figure 2. TNF-α is located in polymorphonuclear neutrophils and not in macrophages. High-power magnification of immunostainings for TNF-α (*a*) and the macrophage marker Mac-2 (staining red in *b*) on adjacent sections of a 72-hour–old mouse skin wound. The TNF-α immunoreactivity is located within the polymorphonuclear neutrophils (*arrows* in *a*) and not in the few macrophages present at the margin of the moving keratinocytes. *Bars*, 75 μm.

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