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ORIGINAL ARTICLE

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Continuous recruitment, co-expression of tumour necrosis factor-α and matrix metalloproteinases, and apoptosis of macrophages in gout tophi

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Abstract Gout tophi are characterised by foreign body granulomas consisting of mono- and multinucleated macrophages surrounding deposits of monosodium urate microcrystals. After primary formation, granulomas grow associated with degradation of the extracellular matrix. Based on this background, we have sought (1) to investigate whether during granuloma's growth new macrophages are recruited into the tophi, (2) to find in situ evidence for macrophages' active role in matrix degradation and (3) to examine whether shrunk cells seen within gout tophi are apoptotic. Immunohistochemistry showed that perivascular localised mononuclear cells are CD68+, S100A8+, S100A9+, 25F9–, representing freshly migrated monocytes/macrophages. In contrast, almost all CD68+ mono- and multinucleated cells arranged within granulomas were S100A8–, S100A9–, 25F9+, representing mature (non-migrating) macrophages. Serial sections revealed that macrophages co-express tumour necrosis factor (TNF)-α and matrix metalloproteinases (MMPs) 2 and 9. In situ end-labelling of fragmented DNA demonstrated that CD68+ macrophages undergo apoptosis within gout tophi. Our data show that macrophages are continuously recruited into the gout tophi. These macrophages co-produce the proinflammatory cytokine TNF-α and two TNF-α inducible lytic enzymes, MMP-2 and MMP-9, suggesting that TNF-α may induce MMP production followed by matrix degradation within foreign body granulomas. In parallel, macrophages undergo apoptosis, a phenomenon that may restrict the destructive potential of inflammatory macrophages.

Keywords Gout · Recruitment · Apoptosis · Macrophages

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Introduction

Of the untreated patients with chronic hyperuricemia, 20–40% develop gout tophi typically localised in the subcutaneous tissue overlying cartilage (e.g. ear), joints (e.g. olecranon) or tendons (e.g. patellar bursae) [22]. At the beginning, focal monosodium urate (MSU) microcrystals and intercrystalline matrix are formed, which fuse and get larger in size [13]. If gout is left untreated at this stage, tophaceous deposits can erode into the bone, cartilage and tendons, causing significant structural damage [9, 21, 23].

Foreign body granulomas composed of mono- and multinucleated macrophages enclosing deposits of MSU microcrystals represent the histological hallmark of gout tophi. Macrophages are pivotal effectors of inflammation, the activity of which may result in phagocytosis and degradation of foreign bodies, synthesis of proinflammatory cytokines [e.g. tumour necrosis factor (TNF)-α], and production of lytic enzymes [e.g. matrix metalloproteinases (MMPs)] [3, 26]. Furthermore, activation of macrophages may cause extensive local damage, systemic symptoms and ultimately, death of the host. One mechanism regulating homeostasis of macrophages is programmed cell death (apoptosis) [15]. Recently, it was demonstrated that macrophages undergo apoptosis in foreign body granulomas and in granulomas with a central necrosis, such as those seen in cases with tuberculosis, rheumatoid nodules and granuloma annulare [5, 6, 8].

In this study, we show that the recruitment of macrophages in gout tophi is a dynamic process continuing even after primary formation of foreign body granulomas, macrophages express lytic enzymes which may digest the extracellular matrix within the lesions and apoptosis of macrophages occurs in parallel to macrophage recruitment.

Materials and methods

Tissue samples

Skin biopsy specimens of patients with gout tophi (n=15) were studied. Patients did not receive any uricostatic therapy at the time of diagnosis. Normal skin biopsy specimens served as a control (n=4). Tissue samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (5- to 10-µm thick) were mounted on slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma, Munich/Germany) dissolved in acetone. After deparaffinisation, sections were stained histochemically (haematoxylin and eosin, Giemsa). Immunohistochemistry and in situ end-labelling (ISEL) of fragmented DNA was also performed.

Immunohistochemistry

Antibodies

The polyclonal antibody against bax and the monoclonal antibody (mAb) against bcl-2 (clone 124) were obtained from Dako (Hamburg/Germany), the mAb recognising MMP-2 and MMP-9 were obtained from Calbiochem (Bad Soden/Germany), the mAb against S100A8 (MRP8, clone 8–5C2), S100A9 (MRP14, clone S32.2) and 25F9 were obtained from Dianova (Hamburg/Germany) and the mAb against TNF-α (clone 1E8-G6) was obtained from Santa Cruz (Heidelberg/Germany). The mAb CD68 (clone Ki-M1P), recognising all subpopulations of monocytes/macrophages, was kindly provided by Professor R. Parwaresch, Department of Pathology, University of Kiel, Germany [20]. The antibodies were used at a working dilution of 1:50 (bax, bcl-2, MMP-2, MMP-9, S100A8, S100A9, and 25F9), 1:100 (TNF-α) or 1:2000 (CD68).

Immunohistochemistry using biotin-streptavidin amplified system

For immunohistochemical staining with the antibody against CD68 and MMP-9, deparaffinised sections were digested with proteinase K (10 mg/ml, 10 min; Roche, Mannheim/Germany). For bax, bcl-2 and MMP-2 staining, sections were incubated three times for 5 min in a bath of 0.01 mol/l citrate buffer (pH 6.0) in a microwave set on high power (600–700 W). For S100A8 and S100A9, no pre-treatment was necessary. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. Thereafter, endogenous biotin was blocked with an avidin/biotin blocking system (Dako) as recommended by the manufacturer. Sections were incubated overnight with the primary antibodies at 4°C. To visualise bound primary antibodies, the following detector components were applied at working dilutions recommended by the manufacturer (Biogenex, Hamburg/Germany): biotinylated secondary antibody (linker; 60 min incubation), peroxidase-conjugated streptavidin (label; 60 min incubation) and 3-amino-9-ethylcarbazole (AEC; chromogen).

Immunohistochemistry using a catalysed signal amplification system

For the immunohistochemical staining with the antibody against TNF-α, deparaffinised and rehydrated sections were digested with proteinase K (see above), and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. After incubation with the primary antibody for 15 min, the catalysed signal amplification (CSA) system was used to detect and visualise the bound antibodies as described by the manufacturer (Dako): biotinylated $F(ab)_2$ secondary antibody (15 min incubation), biotinyl tyramide (15 min incubation), peroxidase-conjugated streptavidin-biotin complex (15 min incubation) and AEC as the chromogen.

The samples were counterstained with Meyer's haematoxylin, mounted in SuperMount Medium (Biogenex) and analysed using light microscopy. As a negative control, sections from all samples were stained using the above procedures but omitting the primary or secondary antibodies. As a positive control, sections from lymph nodes with tuberculosis were proved to contain TNF-α-expressing cells.

ISEL

Following deparaffinisation, sections were digested with proteinase K (see above) before incubation for 60 min at 37°C with 50 µl of the labelling mix [150 U/ml terminal transferase, 20 µl/ml digoxigenin (Dig)-DNA labelling mix at 10× concentration and 1 mmol/l $CoCl_2$ in reaction buffer for terminal transferase; Roche]. After rinsing in tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5), sections were blocked with fetal calf serum (Roche) for 15 min. Sections were then incubated for 60 min with a sheep alkaline phosphatase-conjugated $F(ab)_2$ fragment against Dig (Roche). The alkaline phosphatase-conjugated $F(ab)_2$ fragment was applied at a working dilution of 1:250. The nuclear black signals were detected using 5-bromo-4-chloro-3-indolyl phosphate as a substrate and nitro blue tetrazolium as a coupler (Roche) as described previously [4]. Control sections were stained using the above methods, but terminal transferase was omitted. Lymph nodes with reactive follicular hyperplasia were used as positive controls.

To determine which cell populations undergo DNA fragmentation, a combined ISEL and immunofluorescence method was carried out. ISEL was performed using a tetramethylrhodamine isothiocyanate (TRITC)-conjugated $F(ab)_2$ fragment against Dig at a working dilution of 1:20 (Roche). Immediately after detection of red nuclear signals, an indirect immunofluorescence against CD68 was performed (working dilution 1:2000; incubation 60 min). Binding of CD68 was detected with a fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-mouse immunoglobulin (Ig) G antibody for 60 min (working dilution 1:25; Dako). Cells with DNA fragmentation (ISEL positivity) and intact cellular shape (CD68 positivity) were considered to be apoptotic macrophages. Sections were evaluated using a digital fluorescence microscopy (Zeiss, Jena/Germany).

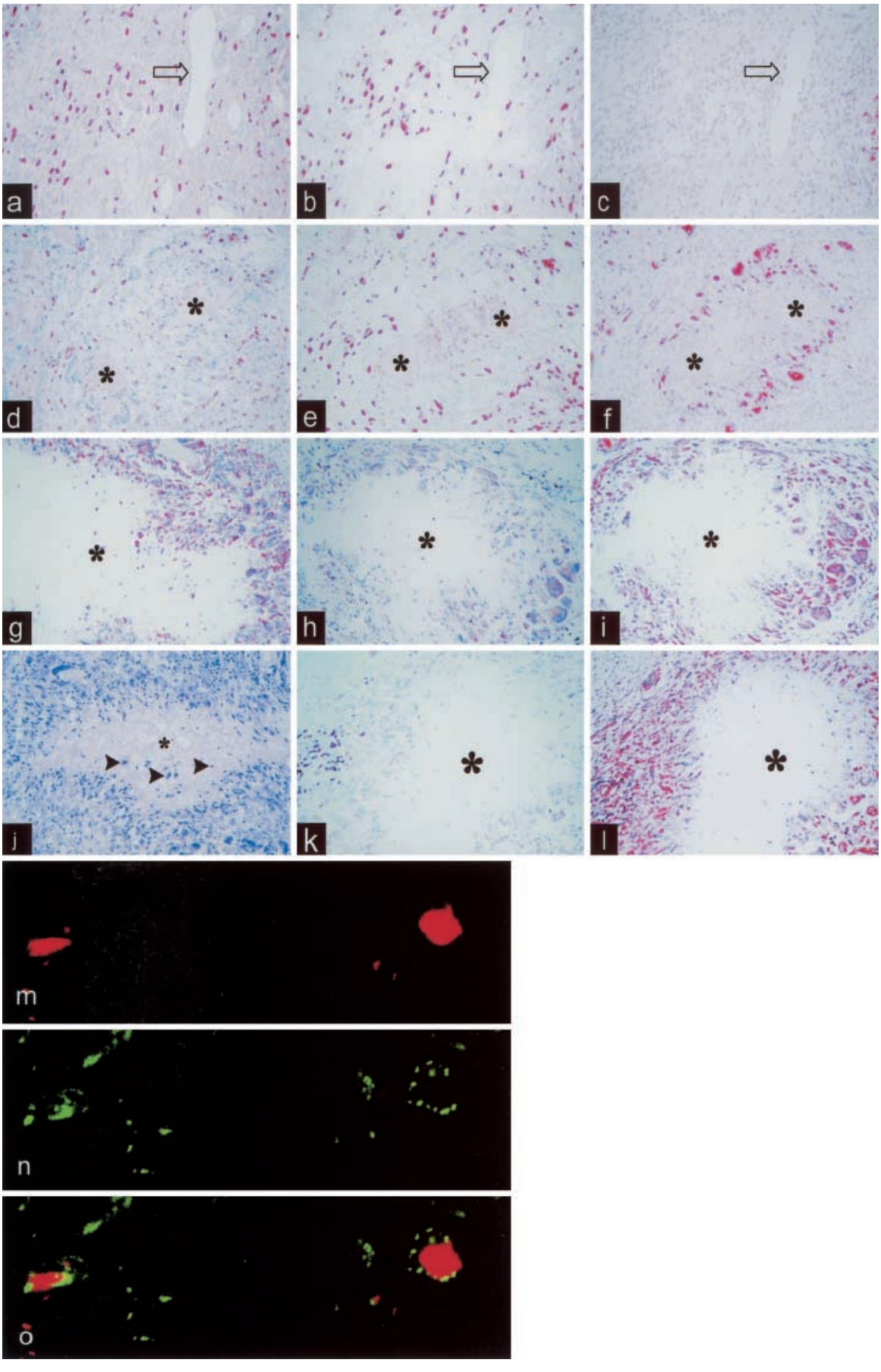
Semiquantitative evaluation

The number of positively stained cells were counted in three perivascular and three gout tophi regions of each of the cases at high magnification [high-power field (HPF); ×400]. The extent of labelled cells was scored semiquantitatively as follows: no labelled cells; small number of labelled cells (<10 cells/HPF); moderate number of labelled cells (10–50 cells/HPF); large number of labelled cells (≥50 cells/HPF).

Results

Characterisation of macrophages and detection of apoptotic cells in gout tophi

Skin biopsy specimens with gout tophi (n=15) were examined according to the histological criteria described by Palmer et al. [19]. In the perivascular compartment of the affected connective tissue, a moderate number of CD68+ mononucleated monocytes/macrophages were seen. The vast majority of these cells showed a strong expression of S100A8 and/or S100A9 but were negative for 25F9 (Fig. 1a–c). In this compartment, a moderate number of cells with positive immunoreaction for TNF-α, MMP-2, MMP-9, bcl2 and bax were seen. On serial sections, it was technically not possible to determine whether these proteins are expressed in the same cell population. Morphologically and/or by applying ISEL no apoptotic cells could be detected adjacent to vessels.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In gout tophi, the numerous mono- and multinucleated macrophages were strongly positive for CD68 and 25F9 but negative or only weakly positive for S100A8 and/or S100A9 (Fig. 1d–f). Further immunohistochemical examinations revealed that macrophages arranged in gout tophi are strongly positive for TNF-a and MMP-9 and weakly to moderately positive for MMP-2 (Fig. 1g–i).

Morphologically, small to moderate numbers of cells showed chromatin condensation and nuclear and cellular shrinkage in this compartment (Fig. 1j). Immunohistochemistry demonstrated that only a small number of macrophages arranged in gout tophi express bcl2, whereas almost all of them produce bax (Fig. 1k,l). ISEL showed small to moderate numbers of apoptotic cells with fragmented DNA. The combination of ISEL with immunofluorescence revealed that apoptotic cells within gout tophi represent CD68+ macrophages (Fig. 1m–o).

Characterisation of macrophages and detection of apoptotic cells in the normal skin

In the normal skin, a small number of CD68+, S100A8–, S100A9– and 25F9+ mononuclear macrophages were noted within the subepithelial connective tissue or in the epidermis. No or only a small number of these resident mononuclear cells within the normal skin showed a positive immunoreaction for TNF-α, MMP-2, MMP-9, bcl2 or bax. They were mainly localised around subepidermal vessels. On serial sections of the normal skin, it was not technically possible to determine which cell population(s) express TNF-α, MMP-2, MMP-9, bcl2 or bax within the normal skin (data not shown). Upon application of ISEL, a small number of apoptotic cells were detected in sebaceous glands and within the granular keratinocyte layer of the epidermis as described elsewhere [25]. Apoptotic macrophages could not be found within the normal skin (data not shown).

◀ **Fig. 1 a–i** Immunohistochemical characterisation of macrophages in chronic gout tophi. In the perivascular compartment, freshly recruited monocytes/macrophages show a strong expression of S100A8 (**a**) and S100A9 (**b**), but are negative for 25F9 (**c**). In contrast, sessile mono- and multinucleated macrophages surrounding cores of amorphous material are negative or only weakly positive for S100A8 (**d**) and/or S100A9 (**e**), but strongly positive for 25F9 (**f**). Macrophages arranged in gout tophi also express TNF-α (**g**), MMP-2 (**h**) and MMP-9 (**i**). Immunohistochemistry (×400). **j–o** Characterisation of apoptotic cells in chronic gout tophi. Morphological evaluation reveals a small number of cells with chromatin condensation and nuclear and cellular shrinkage within gout tophi (**j**). Serial sections illustrate that a small number of macrophages localised in gout tophi express bcl2 (**k**), whereas nearly all of them are positive for bax (**l**). In situ end-labelling (ISEL) reveals a small number of apoptotic cells with DNA fragmentation in the tophi (**m**; *red* nuclear signals). Indirect immunofluorescence of the same area shows several CD68+ macrophages (**n**; *green* signals). Digital fluorescence microscopy indicates apoptotic CD68+ macrophages (**o**; *red* nuclear and *green* cytoplasmic signals). *Arrows* denote blood vessels, *arrowheads* indicate apoptotic cells with chromatin condensation and *asterisks* indicate centres of gout tophi. Giemsa staining (**j**; ×200); immunohistochemistry (**k, l**; ×400); ISEL and immunofluorescence, (**m–o**; ×1000)

Discussion

Mono- and multinucleated macrophages represent the main inflammatory cell population involved in the development of gout tophi. In the present study, it was shown that CD68+ macrophages in the perivascular compartment of the affected connective tissue express S100A8 and/or S100A9 but are negative for 25F9. In contrast, both mono- and multinucleated CD68+ macrophages surrounding amorphous cores of gout tophi are negative for S100A8 and S100A9 but strongly positive for 25F9. S100A8 and S100A9 are calcium-binding proteins belonging to the S100 family [11]. Several studies demonstrated that S100A8 and S100A9 are mainly expressed by freshly recruited (migrating) monocytes/macrophages [7, 17, 24, 31]. In contrast, mature (sessile) macrophages express neither S100A8 nor S100A9 but are strongly positive for 25F9 [29, 30]. Hence, the late differentiation antigen 25F9 is expressed by macrophages found in chronic inflammatory diseases such as sarcoidoisis and multiple sclerosis and by macrophages infiltrating tumours such as primary and/or metastatic melanomas [1, 2, 10] but not by macrophages seen in acute inflammatory reactions [7, 17, 24, 31]. Based on this background, one may argue that the S100A8+ and S100A9+ freshly recruited monocytes/macrophages observed in perivascular regions represent precursors of 25F9+ macrophages surrounding amorphous cores of gout tophi.

We next investigated the expression of the proinflammatory cytokine TNF-α and the lytic enzymes MMP-2 and MMP-9. Immunohistochemistry revealed that mono- and multinucleated macrophages in gout tophi co-express TNF-α, MMP-2, and MMP-9. Recently, it was shown that MSU microcrystals stimulate macrophages to produce a number of cytokines such as TNF-α [3]. In addition to proinflammatory effects, TNF-α induces the production of MMPs [28]. MMPs are lytic enzymes which destroy connective tissue throughout extensive degradation of collagen and elastic fibres [16]. Several studies have already shown that MMPs are responsible for matrix degradation in inflammatory diseases such as granuloma annulare, necrobiosis lipoidica and rheumatoid arthritis, whereby macrophages function as effector cells [26, 27]. Based on this background MSU microcrystal-induced TNF-α seems to lead to MMP production in macrophages followed by matrix degradation in gout tophi.

Within the degraded matrix, small to moderate numbers of cells with chromatin condensation and nuclear and cellular shrinkage were found. ISEL showed DNA fragmentation in these cells. Combined ISEL and immunofluorescence demonstrated that DNA-fragmented cells posses an intact cellular shape and are positive for CD68, thus representing apoptotic macrophages. Characterisation of vital macrophages localised within gout tophi revealed that the vast majority of them is completely negative for bcl2 but strongly positive for bax. This finding was surprising because macrophages are capable of expressing bcl2, an anti-apoptotic protein

that protects cells from apoptosis and augments their survival [12, 14]. It has been well documented, however, that bax is a pro-apoptotic protein. An excess of the protein leads to the formation of bax homodimers and therefore cell susceptibility to apoptosis [18]. It may be speculated that the lack of bcl2 and the abundance of bax in macrophages render them sensitive to apoptosis in gout tophi. Considering the continuous migration of macrophages into the gout tophi, apoptosis may contribute to the homeostasis of macrophages in gout tophi. However, a major out-standing question remains concerning the mechanisms executing apoptosis in infiltrating macrophages.

Taken together, our data suggest that (1) CD68+ S100A8+ and/or CD68+S100A9+ mononuclear cells represent migrating monocytes/macrophages which are permanently recruited into the gout tophi, (2) CD68+ 25F9+ cells surrounding amorphous areas of gout tophi are sessile macrophages co-expressing the proinflammatory cytokine TNF-α and the TNF-α-inducible lytic enzymes MMP-2 and MMP-9, (3) both MMPs may be involved in matrix degradation within the tophi and (4) apoptosis of bcl2–bax+ macrophages may control the haemostasis of inflammatory cells at the site of lesions. This concept, however, seems not to be specific for gout tophi, because some pathological changes were also observed in rheumatoid nodules, granuloma annulare and tuberculosis-associated granulomas with caseous necrosis (5, 6). Hence, it is possible that the sequence of events described for these entities also occur in other granulomas, thus representing a general principle.

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