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Macrophage subtyping in the determination of age of injection sites

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Abstract Determination of the age of injection marks in skin may be of particular interest in the investigation of drug abuse-related fatalities. The aim of our study was to assess the value of macrophage subtyping by antibody-based markers in the determination of the age of injection marks. Immunohistochemical investigations were performed with the antibodies Ki-M1P, 27E10, MRP14, MRP8 and 25F9. Monocytes/macrophages in acute lesions (several hours to 2 days old) expressed proteins detectable with the antibodies 27E10 and MRP14 and showed acute erythrophagia. An additional reaction with the antibody MRP8 was seen in lesions a few days old. An antigen recognized by the antibody 25F9 was found in tissue macrophages, multinucleated giant cells of active granulomas and siderophages. The expression of the 25F9 detectable antigen was absent in inactive granulomas and siderophages, whereas the macrophages were always detectable with the pan-macrophage marker Ki-M1P.

Key words Monocyte/macrophage system · Wound age · Macrophage subtyping · Macrophage differentiation antigen · Drug abuse · Injection lesions

Introduction

Conventional histological and histochemical investigations on injection marks have often been described. The time-dependent micromorphological course of this special kind of mechanical injury and the phases of wound healing are well known (Boltz 1951; Schollmeyer 1965; Friebel and Woohsmann 1968; Kellner and Feucht 1969; Gerlach 1977; Oehmichen 1990). These findings are of special interest for the elucidation of drug abuse-related fatalities (Hirsch 1972; Gerlach 1978; Kringsholm and Christophersen 1989; Althoff and Schäfer 1992; Janssen 1993; Karch 1993; Kringsholm 1993)

Monocytes/macrophages play an essential role in wound healing. Activated by mediator-associated release from capillaries and endothelial/macrophage interactions, the monocyte/macrophage system has proteolytic and phagocytic functions which are directly involved in the mechanisms of repair. Morphologically, the initial appearance of monocytes/macrophages in a lesion as well as the presence of erythrophages, siderophages, hematoidin and hemosiderin deposits, phagocytosis around hematomas and foreign-body reactions with multinucleated giant cells, are all determinants which can be utilized to age wounds. If stimulation of monocytes/macrophages leads to a gradual and sequential activation and differentiation of these cells, a characterization of distinct phenotypes by antigen determinants whose expression is time-dependent may be expected to produce valuable results. Various antigens have previously been characterized and their time-dependent expression was shown in cultured monocytes and by immunohistochemical staining of tissue sections (O'Laughlin et al. 1992; Betz et al. 1995). For example, the activity of demyelinating lesions of multiple sclerosis in the CNS can be determined with these antibodies (Ozawa et al. 1994).

The aim of our study was to assess the value of a characterization of macrophage subtypes for the determination of the age of drug abuse-related injection marks.

Material and methods

Postmortem skin samples from the cubital vein area were taken from 17 drug-related fatalities and 5 control cases (hospitalized patients with no indications of drug abuse) fixed in paraformaldehyde, embedded in paraffin and stained immunohistochemically with the macrophage antibodies Ki-M1P, 27E10, MRP14, MRP8 and 25F9. Immunohistochemical detection was carried out using the alkaline phosphatase/anti-alkaline phosphatase (APAAP)

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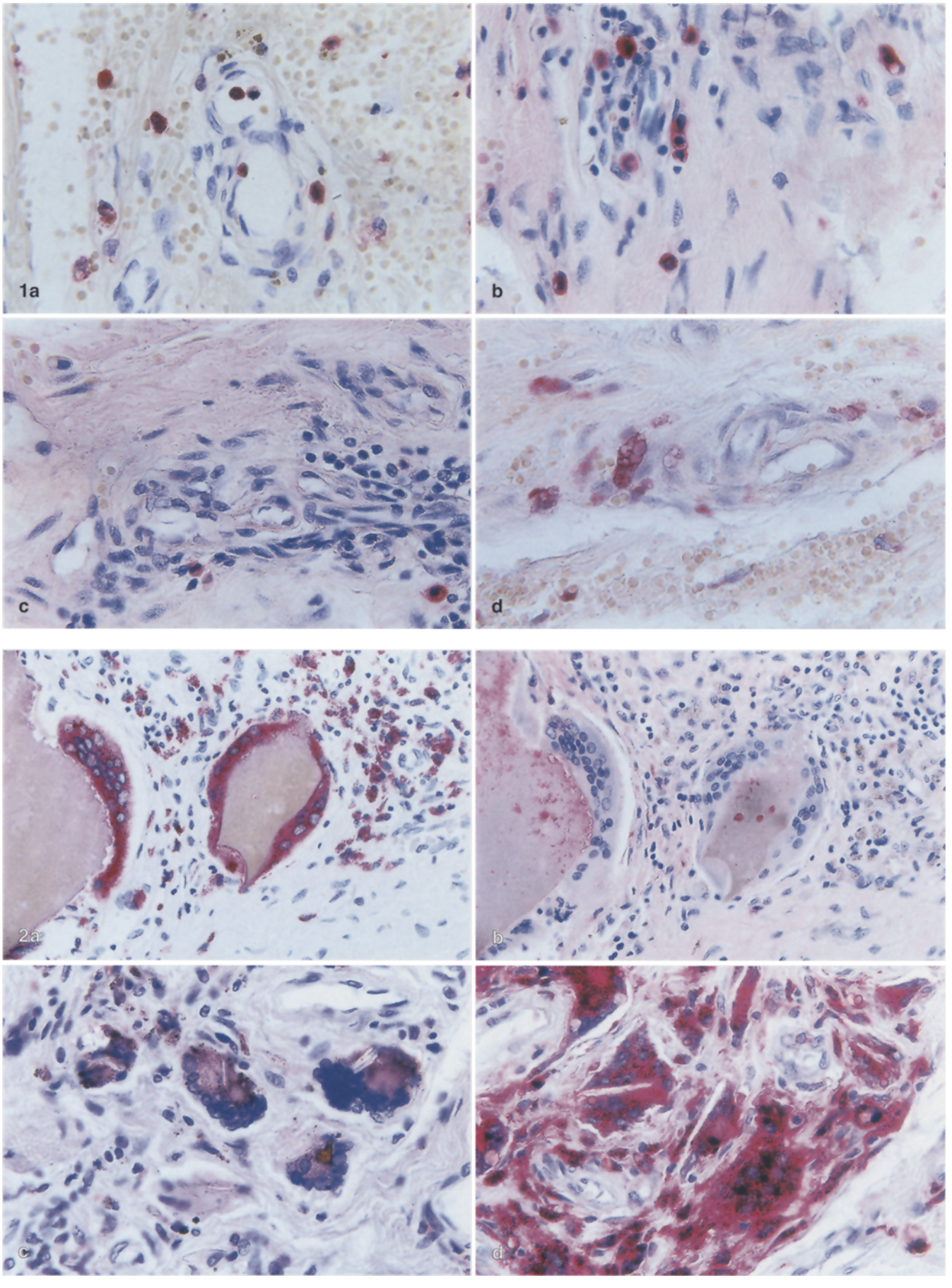
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◀ **Fig. 1 a–d** Antigen expression of macrophages in acute needle marks from drug abuse-related fatalities (paraffin, APAAP, 630 ×) (**a**) short-term activated monocytes/macrophages, erythrophages and granulocytes (internal standard) expressing the antigen 27E10; (**b**) the antibody MRP14 showing the same reaction pattern as 27E10; (**c**) the antibody MRP8 reacting with granulocytes and only a few macrophages in an acute lesion. An erythrophage stained negative. (**d**) antibody Ki-M1P reacting with macrophages of different activation stages and demonstrating erythrocyte aggregation on macrophages and erythrophagia

Fig. 2 a–d Antigen expression on macrophages of foreign body reactions and macrophages activated for weeks (**a**) hematoma-phagocytosing multinucleated giant cells and hemosiderophages detected with antibody 25F9; (**b**) antibody MRP8 does not react with hematoma-phagocytosing macrophages or hemosiderophages (a and b paraffin, APAAP, 400 ×); (**c**) multinucleated giant cells and hemosiderin/hematoidin-containing macrophages in inactive granulomas have lost their 25F9-antigen expression; (**d**) the pan-macrophage marker Ki-M1P detected macrophages of all activation stages, and inactive granulomas (c and d paraffin, APAAP, 630 ×)

method. Sections 2–3 μm thick were prepared on silanized slides and the paraffin was removed. The sections utilized for 27E10 and 25F9 antibody incubation were predigested for 10 min in 50 μg/ml protease type XXIV. After washing twice with PBS the sections were incubated for 1.5 h with the primary antibody. In the same manner the sections were incubated with the secondary rabbit anti-mouse-antibody (Dako Z 259) and the APAAP complex (Dako D 651). The staining reaction was performed with a neufuchsin developer solution (Boenisch 1989) and counter-stained with Mayer's Hemalum (Merck).

The age of the injection marks in the cutaneous and subcutaneous tissue sections investigated was unknown in drug abuse-related fatalities since the previous history was not available and multiple lesions were often present. The classification of the age of the lesions was determined by standard morphological criteria and compared with the immunohistochemical expression pattern of the five macrophages antibodies. These data were compared with the findings in five control cases, where the time of injection was known from medical protocols (a negative control and four fatalities with injection sites which were some hours, 2, 10 and 16 days old). Macrophage subtyping was performed on serial sections to ensure that comparable regions were stained with the different antibodies. The areas to be evaluated were chosen by morphological criteria allowing a designation of the postinfliction age of the lesion. The sections for morphological assessment were stained with the hematoxylin and eosin (HE) and Giemsa methods.

The migration inhibitory factor-related proteins 14 and 8 (MRP14 and MRP8) detect subunits from calcium binding, non-covalently bound heterodimers of the S-100 protein family. MRP14 and MRP8 are components of various heterodimers and monomers (Odnik et al. 1987; Teigelkamp et al. 1991; Edgeworth et al. 1991; Goebler et al. 1994). The antibody 27E10 detects a 17 kDa surface-bound antigen which is a heterodimer containing MRP14 and MRP8 units. It is expressed in short term activated monocytes/macrophages and can be detected in cultured monocytes one day after stimulation, its maximum expression being at days 2 and 3. Granulocytes and some endothelial cells are also detected with this antibody (Zwadlo et al. 1986; Bhardwaj et al. 1992). MRP14 is expressed in short term activated monocytes/macrophages and it is the first detectable antigen to react with the antibodies investigated. In stimulated monocyte cultures it can be demonstrated after several hours and in tissue sections for the first days (days 1–8) after the lesion was initiated. The antibody also detects granulocytes, which serves as an internal control (Odnik et al. 1987; Hessian et al. 1993). MRP8 is expressed in more differentiated macrophages, tissue macrophages, and granulocytes for several days up to weeks after injury (Odnik et al. 1987; Zwadlo et al. 1988). The antibody 25F9 recognizes a 86 kDa surface-bound antigen expressed in tissue macrophages, macrophages of chronic inflammatory lesions and in some melanoma cells. In monocyte cultures it is expressed 3 days after activation and reaches its peak expression after day 7 (Zwadlo et al. 1985).

The entire monocyte/macrophage activity in lesions can be estimated with the pan-macrophage antibody Ki-M1P. This antibody detects a membrane-bound 68 kDa protein expressed in monocytes/macrophages at all stages of differentiation and activation. It does not bind to monocyte precursors, granulocytes or dendritic cells (Radzun et al. 1991).

Results

An acute injection lesion (several hours up to 1 or 2 days old) is characterized morphologically by hemorrhaging with extravasation of erythrocytes, migration of granulocytes and monocytes/macrophages from the capillaries, and erythrocyte adhesion or erythrophagia by macrophages. The following macrophage subtyping pattern was demonstrable: perivascular monocytes/macrophages and erythrophagic monocytes expressed antigens detectable with the antibodies MRP14 and 27E10 (Fig. 1). The antibody reaction with intravascular and perivascular granulocytes was used as an internal standard for the quality of the method. Antigens on granulocytes were detectable by the antibody MRP8, but only few monocytes/macrophages expressed MRP8-detectable antigens. The erythrophagic macrophages detectable at an early stage were MRP8-negative. Although the antibody Ki-M1P recognized antigens in all perivascular monocytes/macrophages, no reaction was seen with the antibody 25F9. In a control case with an acute cutaneous hemorrhage several hours before death, perivascular granulocytes were detected by the antibodies MRP14 and MRP8. Some scattered macrophages were recognized in subcutaneous tissue by the antibody Ki-M1P, the same pattern as in a negative control (without any bleeding).

Lesions over 1 day old are histologically characterized by dense perivascular granulocyte and monocyte/macrophage infiltration after acute erythrophagia and showed no significant differences in the expression of antigens detected by antibodies MRP14 and MRP8 on their macrophages. In the control cases, 27E10 was strongly expressed in a 1 to 2-day-old infliction. MRP 14 and MRP8 showed similar patterns in a 1 to 2 and a 10-day-old skin lesion, whereas 16 days after infliction the macrophages were stained more strongly with the antibody MRP8 than with the antibody MRP14.

Multinucleated giant cells are frequently found at the site of foreign body reactions, and hematoma-phagocytosing multinucleated macrophages are characteristic of residues from injection paravasates and subcutaneous hemorrhages weeks after the lesion. Antigens in these cells were recognized by the pan-macrophage antibody Ki-M1P and antibody 25F9 (Fig. 2 a, b). MRP8 showed a non-specific homogeneous colour reaction with necrotic material, but multinucleated macrophages or hemosiderophages were negative, as were reactions with antibodies 27E10 and MRP14. With increasing inactivity of foreign body granulomas and hematophagocytosis, macro-

phages lost their antigenicity to antibody 25F9. They were, however, always detectable by the pan-macrophage antibody Ki-M1P (Fig. 2c, d).

Discussion

Mononuclear cells of the monocyte/macrophage system are essential effector cells in inflammatory lesions. Previous studies revealed a certain degree of macrophage heterogeneity in these lesions, suggesting a differentiation process after mediator-associated activation (Sorg 1988). The detection of macrophage activation antigens has allowed a chronologically-based grading of inflammatory lesions which can be correlated with morphological landmarks. This detection method can be used to estimate the time lag between the initial lesion and the inflammatory reaction (Betz et al. 1995). The present study provides evidence for a sequential expression of macrophage activation antigens in the inflammatory reactions in the sites surrounding needle punctures. MRP14 is an antibody which recognizes a 13.2 kDa calcium binding protein found in neutrophils and monocytes (Odnik et al. 1987). It indicates acute inflammatory activity since MRP14 is expressed in the first days of stimulated monocyte culture cells and, for example, defines the earliest stage of demyelinating activity in multiple sclerosis lesions (Ozawa et al. 1994). The antigen detected by MRP14 is not expressed by resident monocytes/macrophages, as was recently described (Akiyama et al. 1994).

The antibody 27E10 recognizes a cell surface protein which is formed by noncovalent association of the two calcium-binding proteins MRP14 and MRP8 (Goebler et al. 1994). The antigen is expressed by granulocytes but is absent from resident mature tissue macrophages. It is expressed by monocytes/macrophages in acute inflammation of tissue and sometimes also by endothelial cells, while it is absent in chronic inflammatory lesions (Zwadlo et al. 1986). In our study the antibodies MRP14 and 27E10 recognized monocytes/macrophages as early-inflammation antigens, and erythrophagia by single cell macrophages.

The antibody MRP8 detects a 8 kDa calcium-binding protein present on granulocytes and monocytes/macrophages (Odnik et al. 1987). Its expression indicates the subacute nature of an inflammatory process. This antigen was expressed in inflammatory processes a few days old and after the stage of acute erythrophagia.

The 25F9 antibody was raised against cultured human blood monocytes/macrophages. The expression of this antigen increases with time after stimulation of monocyte cultures, which seems to reflect the process of maturation from monocytes to macrophages. It recognizes mature tissue macrophages and macrophages of chronic inflammatory reactions, but not blood monocytes or monocytes/macrophages of acute inflammatory reactions (Zwadlo et al. 1985). In our study the antibody detected multinucleated macrophages from foreign body reactions and hematophagia as well as hemosiderin and hematoidin-containing macrophages. The antigen expression correlated with the activity of macrophages. In inactive granulomas neither multinucleated nor hemosiderin-containing macrophages were recognized by the antibody 25F9. The panmacrophage antibody Ki-M1P recognized these macrophages as well as all other monocytes/macrophages in the different activation states and therefore can be used as a routine marker for macrophages.

On the basis of the results obtained, it seems reasonable to investigate the time-dependent expression of monocyte/macrophage subtyping antigens in lesions with questionable chronological infliction. These findings show that the macrophage subtyping pattern may give additional information to enable the determination of the age of inflammatory reactions especially at the site of injection marks.

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