Int J Legal Med (2005) 119: 16–21
DOI 10.1007/s00414-004-0453-4

ORIGINAL ARTICLE

Masataka Takamiya · Kiyoshi Saigusa · Reiko Kumagai · Nori Nakayashiki · Yasuhiro Aoki

Studies on mRNA expression of tissue-type plasminogen activator in bruises for wound age estimation

Received: 8 December 2003 / Accepted: 14 April 2004 / Published online: 17 June 2004

Abstract We investigated mRNA expression of tissue-type plasminogen activator (tPA) and inflammatory cell dynamics for wound age estimation of bruises in mice. Neutrophils were detected from 1 h post-injury. Up to 8 h, they accumulated in subcutaneous tissue and the lower part of the dermis, and thereafter they extended to all the layers. Macrophages became detectable 3 h post-injury, and moderate infiltration of lymphocytes was seen from 144 h. In addition, epidermal thickening was also seen from 72 h. tPA mRNA expression peaked at 1 h, and increased slightly at 72 h post-injury. tPA mRNA was detected in epidermal cells, fibroblasts, and endothelial cells before and after injury, from 3 h in neutrophils and from 72 h in macrophages, respectively. This study presents the time-dependent expression of tPA mRNA in bruises in relation to temporal histologic characteristics during wound healing, which was considered to be useful for wound age estimation. Furthermore, it is suggested that tPA plays an important role in the first step of tissue remodeling.

Keywords Tissue-type plasminogen activator · Bruise · Wound aging · Quantitative PCR · In situ hybridization

Introduction

Wound age estimation is one of the most critical issues for forensic pathologists (Jansen 1984). Immunohistochemical studies on macrophage phenotypes (Betz et al. 1995), fibronectin and tenascin (Ortiz-Rey et al. 2003), and cytochemical analysis of mast cells (Bonelli et al. 2003) have been performed. The accurate interpretation of bruises is essential at autopsy, and it is also considered that aging of bruises is an important component of many child abuse assessments. However, it has been mentioned that observing color change, which is a common method for bruise wound aging, included some difficulties. Location and skin complexion affect the time of appearance and color of bruises (Vanezis 2001), and assessment of color is rather subjective. Thus, the color shift of bruises varied between the textbooks and reports (Vanezis 2001). In addition, even inflammatory cell dynamics during the wound healing of bruises have not been resolved sufficiently in spite of their importance for the daily work of forensic pathologists.

Tissue-type plasminogen activator (tPA) is one of the plasminogen activators in the plasminogen activator/plasmin system which is organized as an enzymatic cascade, and the end product of the reaction is plasmin generated from the plasminogen by plasminogen activators (Dano et al. 1985). Plasmin is also thought to be of crucial importance whenever extracellular matrices are remodeled (Dano et al. 1985). Therefore, it is considered that tPA would play an important role during wound healing of bruises.

In this study, we investigated inflammatory cell dynamics, and analyzed tPA mRNA expression during wound healing of bruises to evaluate their utility for wound age determination.

Materials and methods

Animal experimental protocol

Six-week-old DDY male mice weighing 30–35 g were anesthetized by ether inhalation, and the dorsal region was shaved. Using 2 iron plates with holes (diameter 3 mm), the dorsal skin of each mouse was compressed to the width of 1 mm for 30 s (Fig. 1a, b). The mice were kept in clean cages and allowed food and water *ad libitum*, and killed at 1, 3, 8, 24, 72, 144, or 240 h post-injury (each n=5). A

M. Takamiya (✉) · K. Saigusa · R. Kumagai · N. Nakayashiki · Y. Aoki
Department of Legal Medicine, Iwate Medical University School of Medicine,
19–1 Uchimaru,
020–8505 Morioka, Japan
e-mail: mtakamiy@iwate-med.ac.jp
Tel.: +81-19-6515111 (ext. 3383)
Fax: +81-19-6222826

5×5 mm area surrounding the wound was excised. As a control, specimens from 5 mice without injury were examined in parallel.

Tissue sections and immunohistochemistry

Specimens were fixed in 4% buffered formalin, and immersed in 30% sucrose. After embedding in OCT compound (Bright Instrument Company, Huntingdon, England), serial 6 μm frozen sections were prepared and stained with hematoxylin-eosin. In order to classify the inflammatory cell dynamics, rat anti-neutrophils (Cedarlane Laboratories, Hornby, Ontario, Canada) for neutrophils, rat anti-CD11b (Harlan Sera-Lab, Loughborough, UK) for macrophages, and rat anti-CD3 (YLEM S.R.L, Roma, Italy) for lymphocytes were used as the primary antibodies. The sections were incubated for 2 h in a humid chamber at room temperature, and thereafter with anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Subsequently, peroxidase-labeled streptoavidin (Nichirei Tokyo, Japan) was added and color development was performed with 3,3'-diamino-benzidine. The slides were counterstained with hematoxylin.

Quantitative PCR

Extraction of total RNA and reverse transcription

Total RNA was extracted from the tissues by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). cDNA was synthesized using the Takara AMV-RT PCR kit (Takara Biomedicals, Otsu, Japan), following the manufacturer's instructions.

Positive control preparation

The following tPA primers and probe were used:

- Forward primer: 5'-AAT TAT TGT CGG AAT CCA GAT GGT-3'
- Reverse primer: 5'-CAC GTC AGC TTT CGG TCC TT-3'
- Probe: 5'-ATG CCA GAC CTT GGT GCC ATG TGA-3'.

As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed, and GAPDH primers and a probe from TaqMan Rodent GAPDH Control Reagents (Applied Biosystems, Foster City, CA) were used. PCR amplification was performed in a 50 μl reaction mixture which contained 2 μl cDNA, 2.5 U of Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer, and PCR buffer. After initial denaturation at 95°C for 2 min, amplification consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1.5 min was performed for 40 cycles. The amplified products were identified using electrophoresis on a 2% agarose gel and staining with ethidium bromide. Subsequently, the DNA was purified using GenElute Agarose Spin columns (Sigma-Aldrich, St. Louis, MO), ligated into the plasmids (3018 bp) and subcloned with DH 5α *E. coli* using pGEM-T Easy Vector with DH 5α (Promega, Madison, WI). Plasmid DNA was isolated using the PI-200 DNA automatic isolation system (Kurabou, Osaka, Japan), and DNA in the plasmid was confirmed by cycle sequencing.

Real-time quantitative PCR

Real-time quantitative PCR was performed using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA). Aliquots of 50 μl reaction mixture contained 0.2 μM of each primer and the probe, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Thermal cycler conditions were in accordance with the manufacturer's instruction (Applied Biosystems, Foster City, CA). The expression of tPA mRNA in

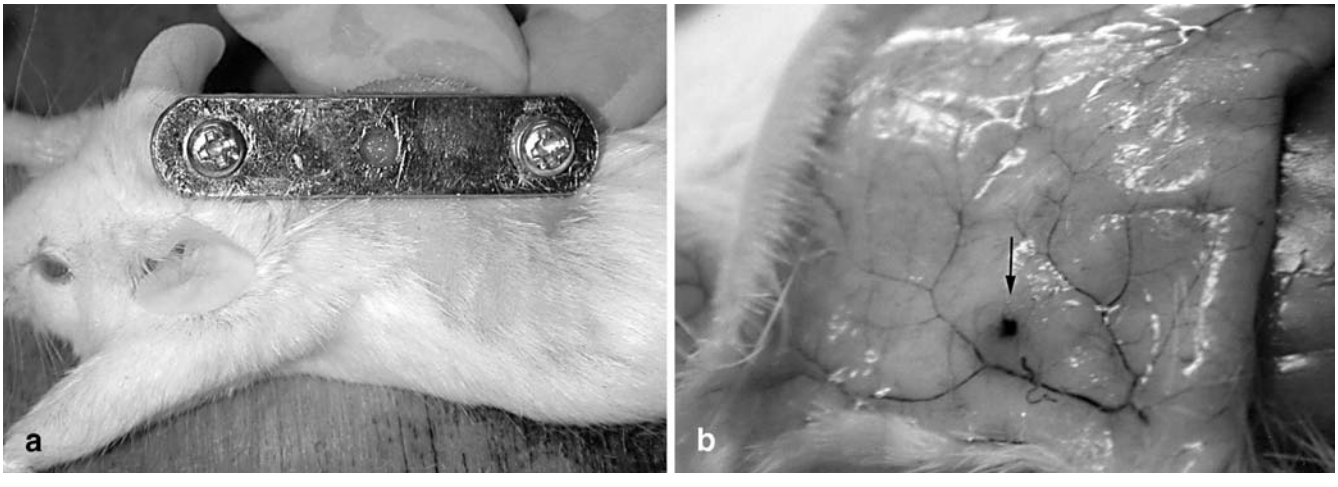

Fig. 1a, b Experimental bruise model. Using 2 iron plates with holes, the dorsal skin of each mouse was compressed **a** and a bruise (*arrow*) was seen near the vessel **b**

controls and each injured tissue was compared using the Dunnet test and *P*-values of 0.05 or less were considered statistically significant.

In situ hybridization (ISH)

After the plasmids were linearized by *STY I* or *SPE I* digestion, the digoxigenin-labeled anti-sense and sense RNAs were prepared using SP6 and T7 RNA polymerases, respectively, following the manufacturer's instructions (DIG RNA Labeling kit, Roche Molecular Biochemicals, Mannheim, Germany). Sections were treated with 0.2 N HCl (20 min) and digested with proteinase K (1 μg/ml) at 37°C for 15 min. After post-fixation with 4% paraformaldehyde for 5 min, they were kept in 50% deionized formamide in 4×SSC. The hybridization was carried out at 47°C for 16 h with a hybridization mixture which contained 1 μg/ml digoxigenin-labeled RNA probe. After washing 5 times with 50% deionized formamide in 2×SSC at 37°C for 1 h each, 40 μg/ml of the anti-digoxigenin-fluorescein Fab fragment (Roche Molecular Biochemicals, Mannheim, Germany) was added and allowed to stand for 20 min. Signals of anti-sense probes were judged to be significant only when signals of sense probes were absent.

The research described in this report was conducted in accordance with the guidelines for animal experimentation of Iwate Medical University.

Results

Histological analysis during wound healing of bruises

Neutrophils were detected from 1 h post-injury (Fig. 2a). Up to 8 h, they accumulated in subcutaneous tissue and the lower part of the dermis, thereafter they extended to all layers. Strong infiltration was seen at 72 h. Macrophages became detectable from 3 h post-injury (Fig. 2b) in subcutaneous tissue and the lower part of the dermis until 24 h, then infiltrated each part of the skin. A moderate infiltration of lymphocytes was seen in all layers at 144

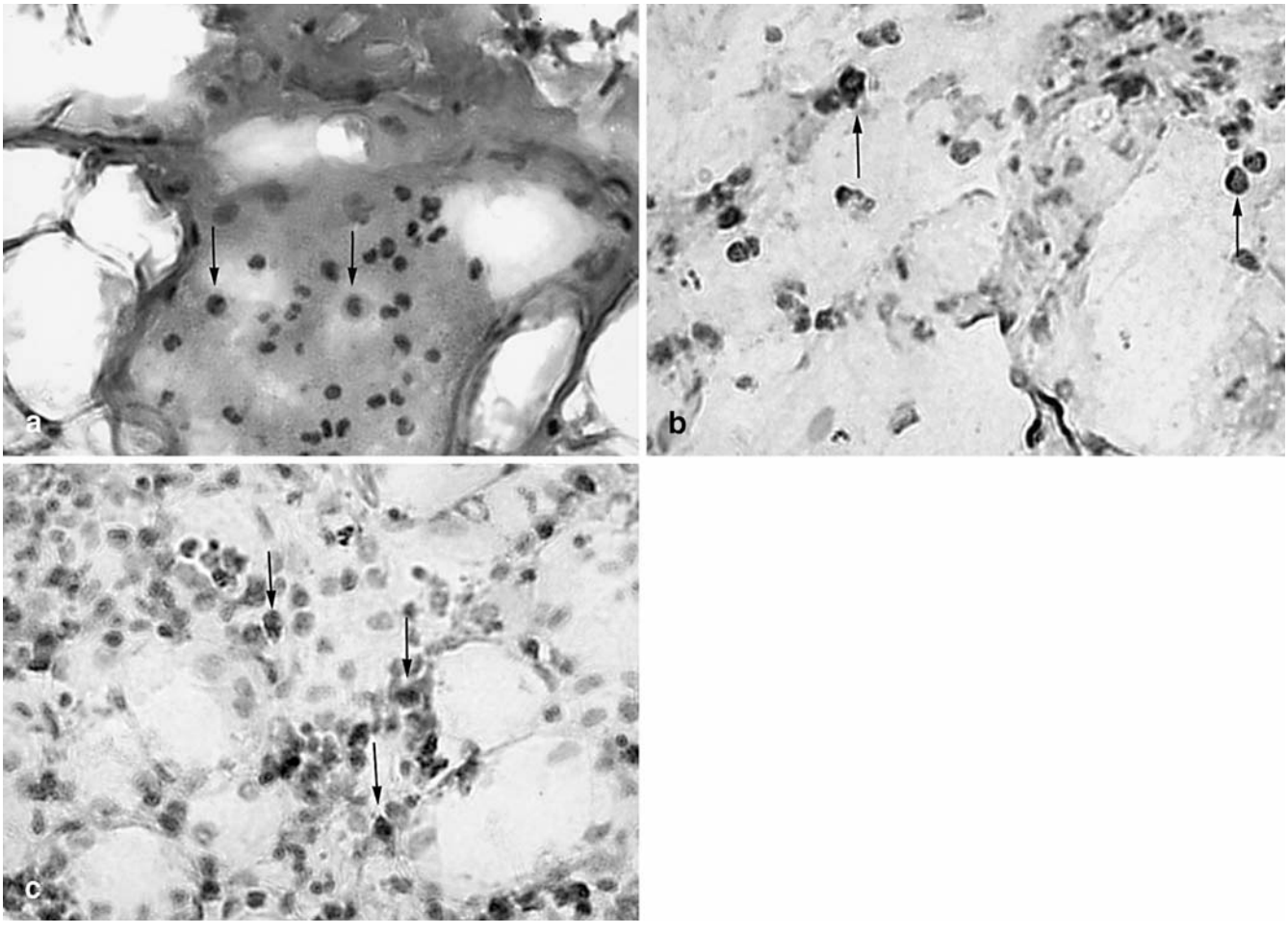

Fig. 2a–c Immunohistochemical analyses of inflammatory cell dynamics in the wound healing of bruise. Neutrophils accumulated in the bleeding of adipose tissue 1 h post-injury **a** (*arrow*), and **b** slight infiltration of macrophages (*arrow*) became detectable in the subcutaneous tissue 3 h post-injury. In addition, moderate infiltration of lymphocytes **c** (*arrow*) was seen at 144 h post-injury (magnification ×500)

Table 1 Histological analysis of the inflammatory cell infiltration in bruises

Time after injury (hours)	*Cell types*		
	Neutrophils	*Macrophages*	*Lymphocytes*
Intact tissue	Not found	Not found	Not found
1	Slight infiltration in ST and lower third of D	Not found	Not found
3	Slight infiltration in ST and lower half of D	Slight infiltration in ST and lower half of D	Not found
8	Moderate infiltration in ST and lower half of D	Moderate infiltration in ST and lower half of D	Not found
24	Moderate infiltration in all layers	Moderate infiltration in ST and lower half of D	Not found
72	Strong infiltration in all layers	Moderate infiltration in all layers	Slight infiltration in all layers
144	Slight infiltration in all layers	Moderate infiltration in all layers	Moderate infiltration in all layers
240	Slight infiltration in all layers	Slight infiltration in all layers	Moderate infiltration in all layers

D: Dermis.
ST: Subcutaneous tissue.

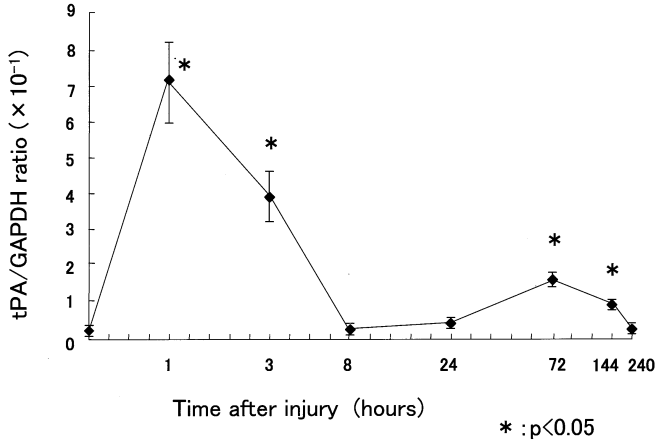

Fig. 3 Time course of tPA mRNA expression in the wound healing of bruise. The results represent the mean±SEM of 5 mice per group

and 240 h (Fig. 2c, Table 1). In addition, epidermal thickening was also detected from 72 h post-injury.

tPA mRNA expressions in bruises

tPA mRNA expression peaked at 1 h, increased again in the late phase of the wound healing (Fig. 3), and was detected in the epidermal cells, fibroblasts, and endothelial cells before and after injury (Fig. 4a,b,c), and in the neutrophils and macrophages from 3 h and 72 h, respectively (Fig. 4d,e, Table 2). No signals were seen in the lymphocytes throughout wound healing.

Discussion

Although histological changes during wound healing have been reported (Berg and Ebel 1969), we have demonstrated the appearance of the inflammatory cells in detail and systematically. These cells first became detectable in the subcutaneous tissue and deep part of the dermis, and then extended to all layers, indicating that the inflammatory reaction of the bruise started in the vicinity of the hemorrhage. Blood would be one of the strong triggers of the inflammatory reaction, and it was reported that injection of autogenous blood increased wound tensile strength (Myers and Rightor 1978).

Recently, it was revealed that tPA activity in dermal incised wounds peaked at 8 h (Arumugam et al. 1999). Therefore, tPA would play an important role in the early phase of dermal wound healing. tPA mRNA mobilization observed in the present study was consistent with this finding in respect of acute expression. tPA mRNA was detected in the epidermal cells, fibroblasts, and endothelial cells pre-injury and post-injury. In these cells, tPA production should be significantly enhanced by injury, although it seemed to be active throughout the life of the cells. Factor Xa (Ichinose et al. 1984) and thrombin (Grulich-Henn and Muller-Berghaus 1989) in the coagulation cascade would be important physiological stimulators of tPA production. Therefore, it was considered that vascular endothelial damage in bruises, a trigger of coagulation cascade, would induce tPA production.

Table 2 The number of specimens with tPA mRNA expression in bruises

Expressing cell	*Time after injury (hours)*							
	0	1	3	8	24	72	144	240
Epidermal cells	5	5	5	5	5	5	5	5
Fibroblasts	5	5	5	5	5	5	5	5
Endothelial cells	5	5	5	5	5	5	5	5
Neutrophils	0	0	4	4	5	5	5	0
Macrophages	0	0	0	0	0	5	5	0
Lymphocytes	0	0	0	0	0	0	0	0

The number of specimens examined was 5 of each.

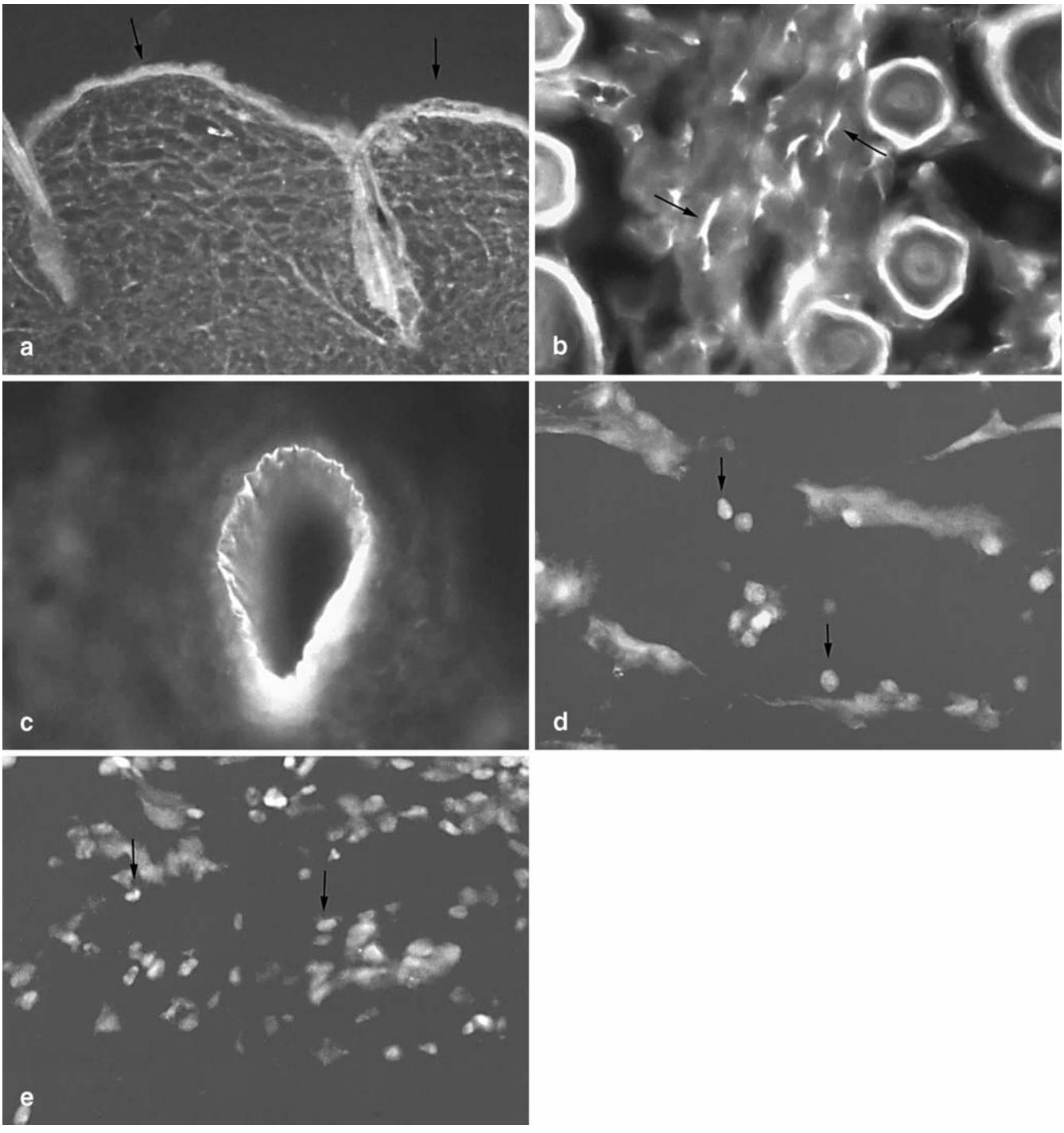

Fig. 4 tPA mRNA expression during the wound healing of bruise. tPA mRNA were in epidermal cells **a**(*arrow*), fibroblasts in dermis **b** (*arrow*), and endothelial cells **c** 1 h post-injury. In the subcutaneous tissue, neutrophils expressing tPA mRNA **d**(*arrow*) were seen sparsely 3 h post-injury, and tPA mRNA in accumulating macrophages **e**(*arrow*) were seen 72 h post-injury (magnification: a ×100, b–e ×500)

Polymorphonuclear leukocytes and monocytes are known to express PA (Hart et al. 1989; Heiple and Ossowski 1986). It was reported that degradation products of fibrin and the extracellular matrix triggered the recruitment of neutrophils (Stecher and Sorkin 1972) and monocytes (Postlethwaite and Kang 1976). In this study, tPA mRNA was detected in neutrophils and macrophages in injured tissue, suggesting that inflammatory cells also contribute to the local regulation of the PA system in wound healing. However, in contrast to a significant level of inflammatory cell infiltration, production of tPA mRNA in these cells would be relatively inactive, because tPA mRNA expressions in the later phase were low. A slight increase of tPA mRNA expression at 72–144 h during

wound healing might be related to epidermal proliferation rather than inflammatory cell infiltration (Grondahl-Hansen et al. 1988). The absence of tPA expression in macrophages between 3 and 24 h would be one of the important temporal characteristics. A possible explanation of this phenomenon is that prostaglandin produced in macrophages would suppress PA (Dore-Duffy et al. 1988; Vassalli et al. 1976). Although it was not evaluated in our study, prostaglandin in the bruised area would rapidly increase, as in burned tissue, as reported by Anggard and Johnson (1972).

In the present study, a reproducible, time-dependent expression of tPA mRNA, which should precede that of protein, was observed in experimental injuries. tPA mRNA expression, therefore, would be a potential parameter for wound age estimation in the acute phase. Consistent macroscopic and microscopic dating of bruises have been found to be difficult because of the variety of morphological features during wound healing (Vanezis 2001). However, examination of the correlation of microscopic changes, such as inflammatory cell dynamics and epidermal thickening, with mRNA expression of tPA and other cytokines, would be informative for bruise healing and dating in the acute and chronic phase.

Acknowledgements This study was supported by grants-in-aid for General Scientific Research (Y.A and M.T; 14570395) from the Ministry of Education, Science, Sports and Culture of Japan.

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