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ORIGINAL ARTICLE

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Studies on mRNA expression of basic fibroblast growth factor in wound healing for wound age determination

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Abstract We investigated the mRNA expression of basic fibroblast growth factor (bFGF) for wound age determination during dermal, cerebral, hepatic and renal wound healing in mice. The bFGF mRNA expression in the injured skin peaked at 1 h and was detected in epidermal cells, fibroblasts, endothelial cells and neutrophils. In the injured cerebrum the expression increased from 1 h and peaked at 48 h. In the intact cerebrum, bFGF was detected exclusively in the endothelial cells, whereas it was also detected in astrocytes during wound healing. Time-dependent expression of bFGF mRNA in skin and cerebrum was considered to be useful for wound age determination. On the other hand it was suggested that bFGF mRNA in astrocytes could be a vital sign of the acute phase. In hepatic and renal injuries, however, bFGF mRNA expression increased slightly in endothelial cells at 24 h, in neutrophils of the liver and in the glomeruli of the kidneys.

Keywords bFGF · Injury · Wound aging · Quantitative PCR · In situ hybridization

Introduction

Wound age determination is one of the most critical issues for forensic pathologists. Esterase, ATPase, acid phosphatase and alkaline phosphatase have all been reported to increase during the early phases of cutaneous wound healing [14]. Recently, immunohistochemical studies on IL-8, MCP-1, MIP-1α [8] and melanocyte migration into human scar tissue [3] were performed, and the mRNA dynamics of inflammatory cytokines, such as IL-1α, IL-1β, IL-6, TNFα [15] and IL-10 [13, 16] were examined for dermal wound age determination.

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It was shown that basic fibroblast growth factor (bFGF) stimulated wound healing in impaired mice [20] and was a positive regulator in angiogenesis [10, 17]. Therefore, bFGF was considered to play an important role in wound healing. In our immunohistochemical analyses it was demonstrated that bFGF protein was induced in the early phase of dermal wound healing [18], which suggested that bFGF could be used as a marker for dermal wound age determination. In this study we analyzed the bFGF mRNA expression during wound healing of skin and some organs, namely cerebrum, liver and kidney, to evaluate the 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.

Dermal wounds. A 2 cm full thickness incision was made on the dorsal skin of each mouse using a scalpel and the incision was covered with sterile cotton gauze. 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 (n =5 each). After shaving the dorsal region a 5×5 mm area surrounding the wound was excised.

Cerebral, hepatic and renal wounds. To make cerebral injuries the scalp was incised and a small hole was made with a 21 gauge needle (diameter: 0.8 mm) in the right side of the skull 5 mm anterior to the bregma and 2 mm lateral to the midline. Another 21 gauge needle was then inserted through the hole to a depth of 3 mm from the skull and left in place for 2 min. Subsequently, the needle was pulled out slowly, and the skin was sutured. For hepatic injuries after sterile preparation the right subcostal region was incised with a 3 mm incision to the inner right lobe of the liver from the edge to the center. The abdominal wall was then sutured by two strata. To make renal injuries, the left lateral subcostal skin was cut after sterile preparation and after opening the fascia, a 3 mm vertical incision was made through the mid-portion of the kidney from the cortex to medulla. The flank was then sutured by two strata. The mice were kept in clean cages and allowed food and water and killed at 1, 3, 8, 24, 48, or 96 h post-injury (n =5 each) and a 3×3 mm area around the wound was excised.

As controls specimens from five mice without injury were examined in parallel.

Quantitative PCR

Extraction of total RNA and reverse transcription

Total RNA was extracted from the tissues by the acid guanidinium thiocyanate-phenol-chloroform method [1] and cDNA was synthesized using the Takara AMV-RT PCR kit (Takara Biomedicals, Otsu, Japan) following the manufacturer's instructions.

Positive control preparation

The following bFGF primers and probe were used:

– forward primer: 5'-TTC AAG GAC CCC AAG CGG-3'
– reverse primer: 5'-TAG TTT GAC GTG TGG GTC GCT C-3'
– probe: 5'-CGG CTT CTT CCT GCG CAT CCA-3'.

As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed and GAPDH primers and probe from TaqMan Rodent GAPDH control reagents were used (Applied Biosystems, Foster City, CA). 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 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 55°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 the QIA quick gel extraction kit (Qiagen), ligated into plasmids (3018 bp) and subcloned with DH 5α *E. Coli* using the 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 each primer and probe, TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) and thermal cycler conditions were according to the manufacturer's instructions. The expressions of bFGF mRNA in controls and each injured tissue were compared using the Dunnet test and P values of 0.05 or less were considered statistically significant.

In situ hybridization (ISH)

Preparation of the RNA probe and tissue sections

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 polymerase, respectively, following the manufacturer's instructions (DIG RNA labeling kit, Roche Molecular Biochemicals, Mannheim, Germany). Specimens were fixed in 4% buffered formalin and immersed in 30% sucrose. After embedding in OCT compound (Bright Instrument, Huntingdon, UK), serial 6 µm frozen sections were prepared and stained with hematoxylin-eosin.

Hybridization and immunodetection of ISH signals

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 conclusive only when signals of sense probes were absent.

Immunohistochemistry

In order to classify the glial cells, rabbit anti-GFAP (DAKO, Carpinteria, CA) for astroglial cells and rat anti-CD11b (Harlan Sera-Lab, Loughborough, UK) for microglial cells were used as the primary antibodies. The sections were reacted with these antibodies for 2 h in a humid chamber at room temperature and thereafter with anti-rabbit IgG (Nichirei, Tokyo, Japan) and anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, respectively. Subsequently peroxidase labeled streptoavidin (Nichirei Tokyo, Japan) was added and color development was performed with 3,3'-diaminobenzidine. The slides were counterstained with hematoxylin.

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

Results

The bFGF mRNA expression in the injured skin peaked at 1 h and the increase was sustained until 24 h post-injury (Fig. 1). The expression was detected in epidermal cells, fibroblasts, and endothelial cells in intact skin, and in neutrophils it became detectable at 24 and 72 h post-injury (Fig. 2a,b; Table 1). The bFGF mRNA expression of the injured cerebrum increased from 1 h, and peaked at 48 h (Fig. 3). mRNA was detected only in the endothelial cells in the intact cerebrum, whereas it was found within the cells surrounding the injury (Fig. 2c). In the serial sections, the region contained many cells stained by anti-GFAP, rather than by anti-CD11b (Fig. 2d; Table 2). In hepatic and renal injuries, bFGF mRNA expression increased slightly at 24 h without showing significant differences (Fig. 4). mRNA was detected in the endothelial cells of the liver (Fig. 2e) and in the endothelial cells and glomeruli of the kidneys (Fig. 2f). In addition, mRNA in the neutrophils was detected 24 and 48 h post-hepatic and post-renal injury (Table 2).

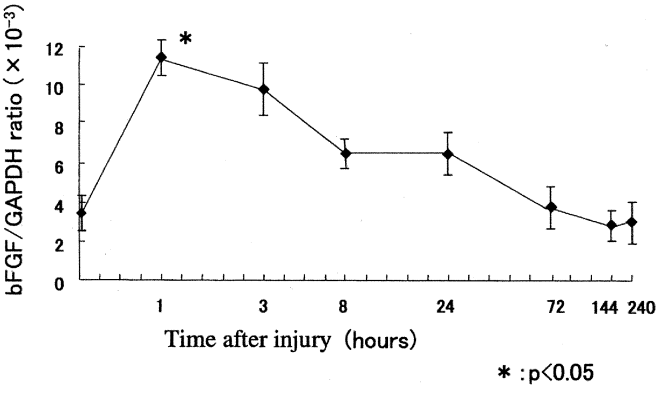

Fig. 1 Time course of bFGF mRNA expressions in dermal injury. The results represent the mean±SEM of 5 mice per group

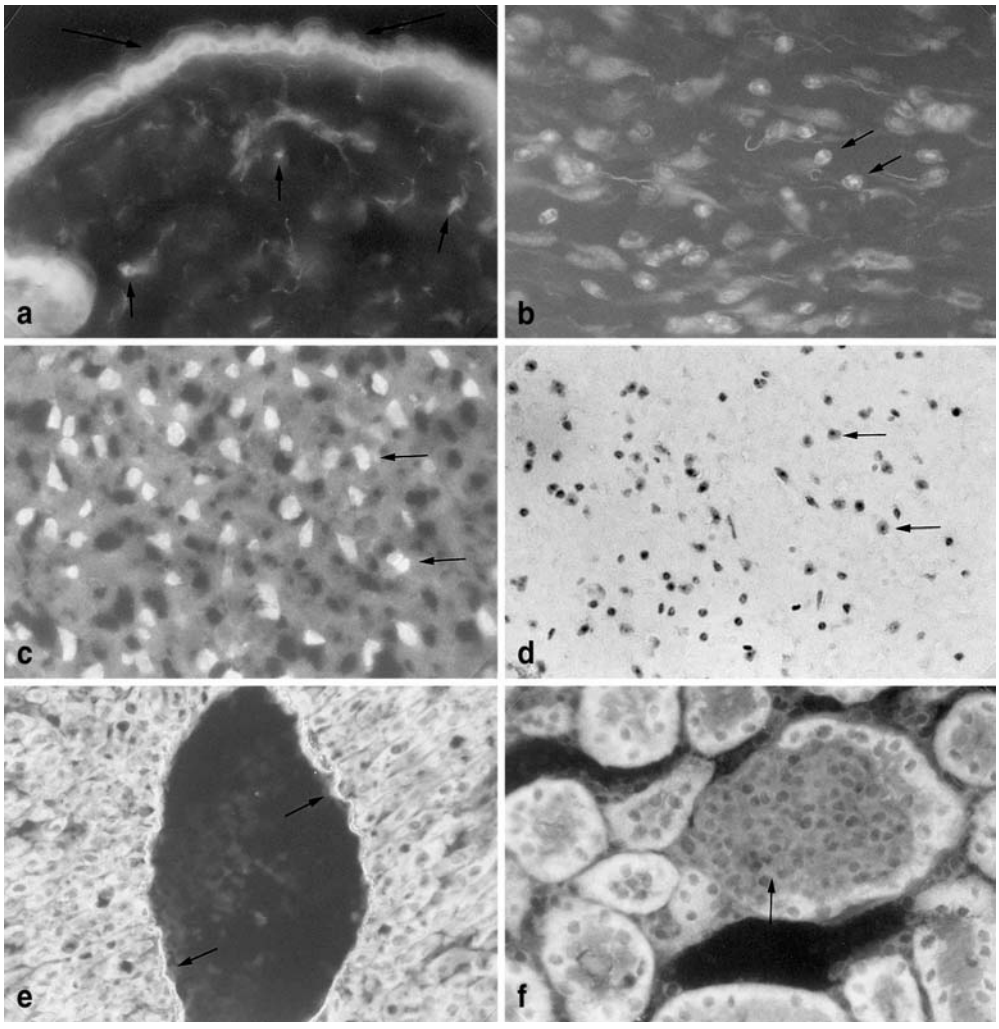

Fig. 2 Expressions of bFGF mRNA after dermal **a**, **b**, cerebral **c**, **d**, hepatic **e** and renal **f** injuries. bFGF mRNA was detected in epidermal cells (*long arrow*) and fibroblasts in dermis (*short arrow*) at 1 h **a** and in neutrophils in subcutaneous tissue at 24 h post-dermal injury **b**. They were also within the cells near the lesion at 48 h post-cerebral injury **c**. In the serial sections, the region contained many cells stained by anti-GFAP **d**. mRNA was detected in endothelial cells of the central vein at 24 h post-hepatic injury **e**, and weak signals were found in the glomeruli at 24 h post-renal injury **f** (magnification a ×100, b–f ×200)

Table 1 Specimens with bFGF mRNA expressions in dermal wound healing

Cells	Time after injury (h)							
	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	0	0	5	4	0	0

For each investigation five specimens were examined.

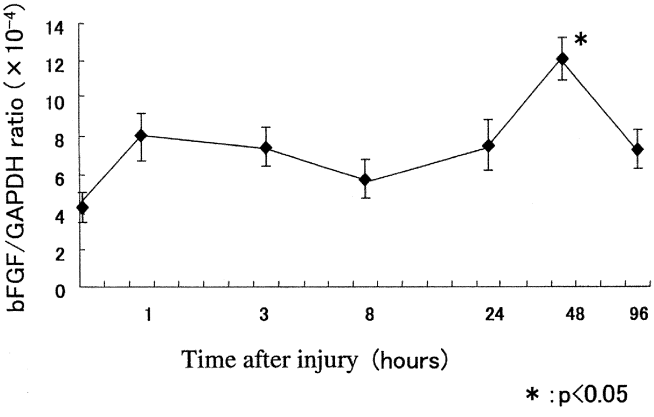

Fig. 3 Time course of bFGF mRNA expression in cerebral injury. The results represent the mean±SEM of 5 mice per group.

Discussion

It has been shown that bFGF increases rapidly during dermal wound healing of humans and animals. Analysing surgical drainage fluid, Nissen et al. [11, 12] reported that bFGF protein levels were highest on the first day after an operation. In our previous study bFGF protein in epidermal cells and fibroblasts became most significant 1 h post-injury [18], whereas in equine limb wounds the dermal bFGF protein peaked within 12 h [19]. Therefore it was presumed that bFGF plays an important role in the early phase of post-dermal injury, more specifically as a initiator of dermal wound healing.

The dermal bFGF mRNA expression observed in the present study was consistent with these findings in respect to the acute expression which peaked 1 h post-injury and

Table 2 Specimens with bFGF mRNA expressions in cerebral, hepatic, and renal wound healing

Tissue	Time after injury (h)						
	0	1	3	8	24	48	96
Cerebrum							
Astroglias	0	4	4	5	5	5	5
Endothelial cells	5	5	5	5	5	5	5
Liver							
Endothelial cells	5	5	5	5	5	5	5
Neutrophils	0	0	0	0	5	4	0
Kidney							
Endothelial cells	5	5	5	5	5	5	5
Glomeruli	5	5	5	5	5	5	5
Neutrophils	0	0	0	0	5	5	0

For each investigation five specimens were examined.

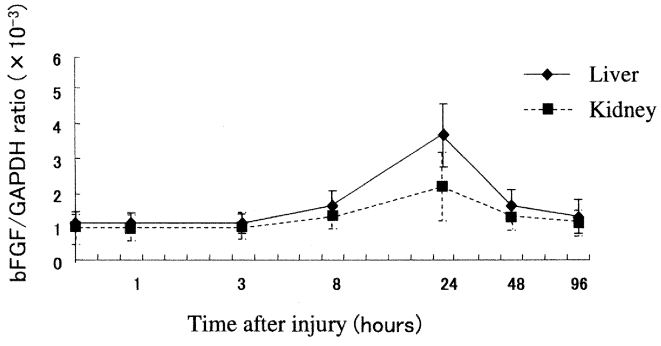

Fig. 4 Time course of bFGF mRNA expression in hepatic and renal injuries. The results represent the mean±SEM of 5 mice per group

this increase was maintained until 24 h post-injury. A time-dependent expression of dermal bFGF mRNA, which should precede that of the protein, could be a potent parameter for wound age determination. mRNA was detected in the epidermal cells, fibroblasts, and endothelial cells before and after injury, although the staining intensity was variable. In these cells, therefore, bFGF production seemed to be active throughout life. In our previous study, bFGF protein in epidermal cells and fibroblasts peaked in the early phase and increased again in the late phase during wound healing, showing therefore a biphasic course [18]. Since mRNA from endothelial cells and neutrophils was added to the analysis in this study, the time course was considered to have one peak. However, the dermal bFGF mRNA expression pattern was close to that of bFGF protein of epidermal cells and fibroblasts, which indicated that the value of mRNA expression in endothelial cells and neutrophils during wound healing was rather low.

Earlier research revealed that astrocytes were the main source of the bFGF supply in the brain [7]. In experiments using a rat brain contusion model, it was indicated that the expression of bFGF mRNA in the astrocytes peaked on the second day, then decreased gradually until 15 days. In the present study with more temporally detailed analyses, time-dependent expression of bFGF mRNA in the astrocytes was also observed in the acute phase of the ablated brain. The cells originate from monocytes, namely microglias in the brain and macrophages in visceral organs, both of which have no expression of bFGF. Besides angiogenesis, bFGF was considered to enhance survival of neurons [22]. On the other hand astrocytes, which could be proliferated by bFGF [5], supported neurite outgrowth by producing extracellular matrix proteins, such as tenascin, laminin, and fibronectin [2]. Recently, immunoreactivity of tenascin was detected in traumatized brain tissue [6]. Therefore, it was suggested that bFGF contributes not only to the initiation of wound healing but also to the protection of critical neurons from secondary damage.

It was demonstrated that hepatocytes could produce bFGF after hepatectomy [9] and immunoreactivity was localized in glomerular mesangial cells [4]. In this study, however, only slight increases were found in the bFGF mRNA expressions 24 h after renal and hepatic injuries, presumably because production of bFGF is not strongly stimulated by mechanical injury. Thus it was suggested that it was impossible to use bFGF for hepatic and renal injuries. The endothelial cells were deemed to be one of the major sources of bFGF mRNA production [21]. However, productivity would be relatively constant during wound healing. It seemed that the mRNA dynamics of skin and brain derived from ectoderm were quite different from those of liver and kidney, therefore, bFGF could be a useful wound age marker for organs of ectodermal origin.

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