bFGF and TGFβ expression in rat kidneys after ischemic/ reperfusional gut injury and its relationship with tissue repair

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Subject headings ischemia-reperfusion injury, intestinal; basic fibroblast growth factor; transforming growth factor β; gene expression

INTRODUCTION
Intestinal ischemia/reperfusion (I/R) occur commonly in critically ill patients. It is well recognized that gut I/R may cause tissue damage and dysfunction of intestine, and induce remote organ injury including kidney, lung, and liver[1]. It may also lead to complications after severe burn or injury. Previous studies have focused on cellular elements, cytokines and inflammatory mediators. Relatively little attention has been paid endogenous protective mechanisms, i.e. the growth factors.

Both bFGF and TGFβ are important growth factors involved in tissue repair, these involve dermal and epidermal wound healing via promoting the initiation and regeneration of capillary vessels, effective chemotactants and increasing deposition of extracellular matrix[2]. Recent studies demonstrated that given exogenous growth factors could accelerate internal organ repair after gut I/R injury[3,4], however, little is known about its molecular mechanism.

The present study was carried out to evaluate endogenous bFGF and TGFβ expression of renal origin in a gut I/R rat model, and to explore the role of bFGF and TGFβ’s release in active repair.

MATERIALS AND METHODS
Animal model Male, pathogen-free Wistar rats were purchased from the Animal Center, Academy of Military Medical Sciences, weighing 200 g to 250 g. Animals were allowed water only for 24 hours before use, and anesthetized with pentobarbital sodium (30 mg/kg). Following midline laparotomy, intestinal ischemia was achieved by placing a microvascular clip across the proximal superior mesenteric artery (SMA) for 45 min, animals were then allowed reperfusion for different periods after removal of the clip and randomly divided into ischemia group (IR0; n = 12), reperfusion for 6 hours (IR1; n = 12) and 24 hours (IR2; n = 12) groups. Time-matched, sham-operated animals underwe nt laparotomy and dissection of the proximal SMA without occlusion served as controls (control; n = 12). Experimental animals were sacrificed and kidney tissues were fixed in 4% paraformaldehyde in PBS and 10% formalin respectively for analysis.

In situ hybridization
Tissues were dehydrated in an ascending series of ethanol and were embedded in paraplast. Five micro-thick sections were cut and prehybridized sequentially in PBS, 0.2N HCl for 20 min, Proteinase K1 mg/L (Sigma) for 20 min in 42°C, 2% glycine 15 min, post-fixation was performed with 4% paraformaldehyde in PBS for 20 min. Slides were dehydrated and prehybridized for 2 hours without probe and hybridized for 20 hours with probe at 42°C bFGF and TGFβ cDNA probes were obtained by polymerase chain reaction (PCR) and confirmed by sequencing analysis. The probes were labeled with DIG Labeling and Detection Kit (Boehringer Mannheim Co., Germany) with random primers. After hybridization, slides were washed with following solutions, 2× SSC, 0.1× SSC, maleic acid 0.1 mol/L, NaCl 0.15 mol/L, and 10% blocking solution. Sections were stained with NBT/BCIP. The positive staining was examined and photographed by a microscope equipped with camera.

Immunohistochemistry
Immunostaining was performed using polyclonal anti bFGF or TGFβ antibody by an indirect Streptavidin / Peroxidase (SP) technique. Antibodies and immunohistochemical SP kit were products of Santa Cruz Co. and Zymed Co.. Experiments were performed following the manufacturer. Briefly, sections we re incubated with polyclonal anti-rat bFGF or TGFβ antibody for 12 hours at 4°C. Slides were washed with phosphate buffered saline (PBS) solution. Biotinylated
secondary antibody IgG was added for 30 min, followed by horse radish peroxidase labeled streptomyein-avidin complex for 30 min. Slides were stained with hematoxylin/eosin and counterstained with Harris hematoxylin/eosin and examined under a light microscope equipped with a camera.

**Statistical analysis**
The percentage of positive staining cells of immunohistochemistry and in situ hybridization were expressed as mean±SD. Statistical analyses were performed using paired Student’s t test. \( P<0.05 \) was considered significant.

**RESULTS**

**Pathological alterations of kidney tissue**
Multiple cross-sections of hematoxylin and eosin stained sections of kidneys were examined for pathological changes. Compared with control group, IR0 had slight alteration, while IR1 showed significant injury. In IR1 sections, clearly tubulointerstitium fibrosis and inflammatory cells infiltrating could be seen, accompanied with vessel wall thickening, tubule narrowing and glomeruli basement membrane destruction, etc. After reperfusion for 24 hours, IR2 recovered gradually.

**Immunohistochemical localization of bFGF and TGFβ**
Both in situ hybridization and immunohistochemistry found expression of bFGF and TGFα in kidney tissues. bFGF was localized in cortex and glomeruli, particularly in mesangial cells, glomeruli epithelial cells and podocytes. TGFβ, however, was found predominantly in mesenchymal cells and glomeruli epithelial cells. Both bFGF and TGFβ positive staining of ISH were localized in the cytoplasm. While positive staining of immunohistochemistry was in cytoplasm or/and membrane.

**Detection of bFGF and TGFβ expression**
Immunohistochemistry and in situ hybridization showed both bFGF and TGFβ were expressed in controlled rat kidneys, and their expression level was enhanced slightly after 45 min ischemia. While significant elevation of bFGF and TGFβ expression was observed after reperfusion, peaking at 6 hours (IR1) and declined at 24 hours (IR2). Both bFGF and TGFβ expression changes have significant difference after reperfusion compared with control \( (P<0.05) \), and positive correlation was found between bFGF and TGFβ expression \( (r = 0.98, r = 0.97) \). Results of their expression of different groups are shown in Table 1.

| Group | n | mRNA | Protein |
|-------|---|------|---------|
| Control | 12 | 0.19±0.03 | 0.12±0.05 |
| IR0   | 12 | 0.24±0.07 | 0.21±0.06 |
| IR1   | 12 | 0.49±0.06 | 0.71±0.08 |
| IR2   | 12 | 0.35±0.05 | 0.50±0.05 |

\( ^{a}P<0.05, ^{b}P<0.01 \) (compared with controls).

**DISCUSSION**
Gut I/R has been implicated in the pathogenesis of intestine and other remote organs, and may potentiate the development of systemic inflammatory response syndrome (SIRS) and even multiple organ dysfunction syndromes (MODS). Much has been learned about the mechanisms that contribute to this injury process, which shows that this process is associated with activation of systemic inflammatory mediators including complement, cytokines, neutrophiles, oxygen free radicals, gut derived bacteria and endotoxin. Great attention has been paid to the potential therapeutic effect of antibodies to these elements. Relatively little is known about the endogenous protective mechanism responding to the gut I/R injury. bFGF and TGFα are important growth factors involved in wound healing and tissue repair and may be important mediators for renal repair after intestinal I/R injury.

bFGF is a potent mitogen, angiogenic agent, and chemoattractant. It is expressed in many kinds of cells, including macrophages, smooth muscle cells, vascular endothelial cells and fibroblasts, bFGF accelerate wound healing through promoting the initiation and regeneration of capillary vessels, accelerating the growth of epithelial cells and fibroblasts, and formation of granulation tissue. It does not have a signal sequence and thus is released independently of endoplasmic reticulum-Golgi pathway. bFGF is stored in inactive form in cell cytoplasm and is activated by cell injury. TGFβ is a growth factor having various functions, its biological activity depends on its concentration and cell types. TGFβ is a potent chemoattractant for inflammatory cells and fibroblasts. It can promote connective tissue formation and accelerate collagen synthesis, induce the release of many other growth factors, such as bFGF, vascular endothelial growth factor (VEGF) in wound healing and tissue repair.

In our present study, bFGF and TGFα mRNA and protein expressed after I/R injury in rat kidneys, and had close spatial and temporal association with the development of renal injury. Their expression levels were induced after I/R injury, peaking at 6 hours and decreased after 24 hours.
hours, correlated with the degree of tissue injury and compatible with other experiments of internal organ injury models. Iwata A et al[7] found in a model of transient focal ischemia, bFGF mRNA was markedly induced in the peri-infarcted white matter after reperfusion, persisted for 2 days and disappeared by 5 days. In rat livers after gut I/R injury[8], endogenous bFGF and TGFβ mRNA expression increased after reperfusion, and persisted for 24 hours. These results showed endogenous bFGF and TGFβ mRNA in internal organs were induced after injury, peaking at the point of severe damage. At this time point, rat kidneys showed markedly tubulointerstitial fibrosis and glomeruli basement membrane destruction. Whereas developmental assembly of endothelial, mesangial and epithelial cells into glomerular requires a coordinated, temporally defined series of steps occurred in an ordered sequence, growth factors and their receptors are important mediators of many of these events. Our results showed that bFGF and TGFβ might participate in this process. Experimental studies had also found that bFGF is a most potent angiogenic factor and TGFβ played an important role in angiogenesis. The significantly elevated expression of bFGF and TGFβ after renal damage suggests bFGF may be involved in renal blood vessel development. Furthermore, inflammation reaction after injury accumulation of inflammatory cells, such as monocytes, neutrophiles and macrophages etc results in release of large amount of TGFβ, and high local concentration of TGFβ induces the high expression of bFGF mediating the tissue repair. Our study shows that I/R injury up-regulate endogenous bFGF and TGFβ expression, and suggests this may serve as a compensatory protective response to remote organ injury.

Recent studies have demonstrated the feasibility of using exogenous growth factors in vivo, of which VEGF and bFGF can improve internal organ injury. Though the precise underlying mechanisms remain to be determined, the use of growth factors may represent a new therapeutic strategy for patients with gut I/R injury.

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Edited by Wu XN