

Extraction and purification of TGF β and its effect on the induction of apoptosis of hepatocytes

Xiao-Hui Si¹ and Lian-Jun Yang²

¹Research Institute of Stomatology, the Ninth People's Hospital, Shanghai Second Medical University, Shanghai 200011, China

²Department of Pathology, Fourth Military Medical University, Xi'an 710033, Shaanxi Province, China

Correspondence to Dr. Xiao-Hui Si, Research Institute of Stomatology, the Ninth People's Hospital, Shanghai Second Medical University, Shanghai 200011, China.

Tel: 0086-21-63135412

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Abstract

AIM To extract and purify the transforming growth factor β (TGF β), and to demonstrate its biological activity *in vivo* and induction of apoptosis of hepatocytes *in vitro*.

METHODS TGF β was isolated from fresh bovine platelets by acid/ethanol extraction method and purified with ion exchange and gel chromatography. The extracted TGF β as injected subcutaneously to mice, and its biological activity *in vivo* was observed 72 hrs post-injection by HE staining. The morphological changes were observed by HE staining and the occurrence of apoptosis was detected by TUNEL method after the human normal hepatic cell line QZG was treated with $8\mu\text{g}\cdot\text{L}^{-1}$ TGF β for 12 hrs *in vitro*.

RESULTS The molecular mass 25 ku TGF β protein was successfully extracted. It was able to induce localized granulation tissue formation *in vivo*. TGF β -treated hepatocytes showed obvious apoptotic morphological changes, including the pyknosis and dense-stained nuclei and cytoplasm, the fragmentary, annular or crescent nuclei, and the "bubbling" cytoplasm. Moreover, its apoptotic rate was significantly higher than that of the control group ($P < 0.05$).

CONCLUSION Biological active TGF β protein is extracted and purified successfully from bovine platelets, and it is able to induce the apoptosis of hepatocytes.

Subject headings transforming growth factor beta/ isolation & purification; transforming growth factor beta/ pharmacology; liver/cytology; apoptosis

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INTRODUCTION

Liver diseases are very common in China^[1-10], and apoptosis is the research focus in recent years^[11-20]. Transforming growth factor β (TGF β) is a kind of polypeptide growth factors that is extensively present in most tissues and cells. A variety of cell types, both nonneoplastic and neoplastic, synthesize TGF β and most of these cells have specific high-affinity receptors for TGF β ^[21-24]. It is a multifunctional

molecule which is known to play an important regulatory role in cell growth, migration and differentiation^[25], embryogenesis^[26,27], tumorigenesis^[28], wound healing^[29-31], bone formation^[32-35] and immunomodulation^[36], acting by both autocrine and paracrine mechanisms. It is also suggested that TGF β may be responsible for some pathological process, such as scarring and fibrosis, renal diseases and immunosuppressant^[37-39]. Furthermore, TGF β is able to induce many kinds of cells, including hepatic and hepatoma cells to undergo apoptosis^[40,41]. The molecular mechanisms underlying TGF β induction of the apoptosis of hepatocytes is still unclear. In this study, TGF β was extracted from the fresh bovine platelets by acid/ethanol procedure and purified by ion exchange and gel chromatography, then its biological activity was detected *in vivo* and its induction of apoptosis of cultured hepatocytes was observed *in vitro*, and to provide the basis for the study of the relationship between TGF β and the signal transduction of hepatocellular-apoptosis.

MATERIALS AND METHODS

Isolation of platelets

The fresh anticoagulant bovine blood was collected in bags containing 0.1 volume of $8.78\text{g}\cdot\text{L}^{-1}$ NaCl and $22.50\text{g}\cdot\text{L}^{-1}$ EDTA (pH 7.4). The blood was centrifuged at $2000\text{r}\cdot\text{min}^{-1}$ for 15 min and the supernatant was recentrifuged at $4000\text{r}\cdot\text{min}^{-1}$ for 15 min. Then the supernatant was discarded and the precipitated platelets was washed twice with PBS (pH 7.4) by centrifugation at $5000\text{r}\cdot\text{min}^{-1}$ for 30 min^[42,43]. All the centrifugations were carried out at 0°C .

Extraction procedure

TGF β was extracted by a modified acid/ethanol procedure of Roberts and others^[44,45]. The platelets were suspended in acid/ethanol extraction solution containing 375mL of $950\text{mL}\cdot\text{L}^{-1}$ ethanol and 7.5mL of concentrated HCl, plus 33mg phenylmethylsulfonyl fluoride(PMSF) and 1.9mg pepstatin A as protease inhibitors. The mixture was sonicated in ice-bath, extracted overnight at 4°C , and centrifuged at $15000\text{r}\cdot\text{min}^{-1}$ for 40 min at 0°C . The supernatant was adjusted to pH 3.0 with concentrated ammonium hydroxide. Then 2 volumes of cold anhydrous ethanol (-20°C) and 4 volumes of cold anhydrous ether (-20°C) were immediately added. After the mixture stood at -20°C for 48 hrs, the resulting precipitate was collected by centrifugation at $20000\text{r}\cdot\text{min}^{-1}$ for 30 min at 0°C and redissolved in $1\text{mol}\cdot\text{L}^{-1}$ acetic acid. After extensive dialysis at 4°C against $0.17\text{mol}\cdot\text{L}^{-1}$ acetic acid in a dialyzing tube (molecular mass cutoff, 10000ku), the sample was then subjected to the next purification.

Ion-exchange chromatography

The above crude sample was centrifuged at $20000\text{r}\cdot\text{min}^{-1}$ for 40 min at 0°C to remove the small precipitate and the supernatant was then applied to a CM-Sepharose column

(1.6cm×11cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 0.17mol·L⁻¹ acetic acid. The column was eluted successively with 0.17 mol·L⁻¹ acetic acid, 41.02g·L⁻¹ NaOAc, 82.04g·L⁻¹ NaOAc and 4g·L⁻¹ NaOH at a flow rate of 90mL·h⁻¹ at room temperature. The fraction that eluted by 4g·L⁻¹ NaOH was immediately neutralized by adding 100mL·L⁻¹ acetic acid. The eluted fractions were collected respectively and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE).

Gel chromatography

The above fractions containing 25ku component were collected, concentrated with glycol polyethylene and extensive dialyzed at 4°C against tridistilled water and then 1 mol·L⁻¹ acetic acid. After being centrifuged at 20000r·min⁻¹ for 10 min, the supernatant was applied to a Superdex 75 column (1.6cm×70cm, Phamacia Biotech, Uppsala, Sweden) equilibrated with 1mol·L⁻¹ acetic acid. The elution was carried out at a flow rate of 36mL·h⁻¹ with 1mol·L⁻¹ acetic acid containing 11.69g·L⁻¹ NaCl at room temperature. The eluted fractions were collected and analyzed by SDS-PAGE. The fraction containing the 25ku component was concentrated using glycol polyethylene. After extensive dialysis at 4°C against tridistilled water and 0.17mol·L⁻¹ acetic acid, the samples were stored at -20°C for the following assays. The protein content was determined by Coomassie brilliant blue G-250 method and bovine serum albumin (BSA) was used as control.

Detection of TGFβ activity in vivo^[46]

Nine male Balb/c mice (10 days old) were injected subcutaneously each day in the back with the purified TGFβ (0.5g·L⁻¹). After 72 hrs, the tissues at the injection sites were removed and fixed in 100mL·L⁻¹ formalin and paraffin sections were then subjected to the routine HE staining. The other 3 mice were injected with equivalent BSA as controls.

TGFβ induction of hepatocellular apoptosis

Human normal hepatic cell line QZG (purchased from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured routinely on cover slides. After having grown to logarithmic phase, 8μg·L⁻¹ of TGFβ was added and then for a further 12 hrs culture. BSA (8μg·L⁻¹) was added as control. The cells were subjected to HE staining. In addition, the cells were stained by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) method to detect apoptosis^[47]. A random field of cells was chosen with a magnification of ×400, and adjacent non overlapping fields were counted until the minimum 500 limits for each slide was obtained. TUNEL index was expressed as the number of positive cells/the total number of cells.

RESULTS

Purification of TGFβ

The crude TGFβ was purified by ion exchange chromatography on a CM-Sepharose column, as shown in Figure 1. SDS-PAGE analysis showed that peaks 2 and 3 contained the 25ku component. The peaks 2 and 3 fractions were collected and then purified by gel chromatography on a Superdex-75 column (Figure 2). SDS PAGE analysis showed that peak 2 predominantly contained 25ku component (Figure 3). The protein content was 1.1g·L⁻¹.

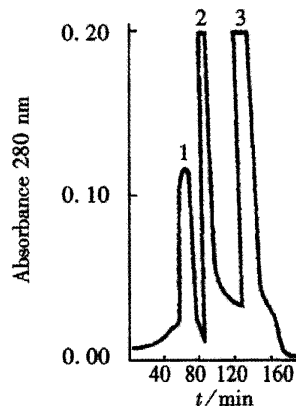


Figure 1 Ion-exchange chromatography of acid/ethanol extract on a CM-Sepharose column.

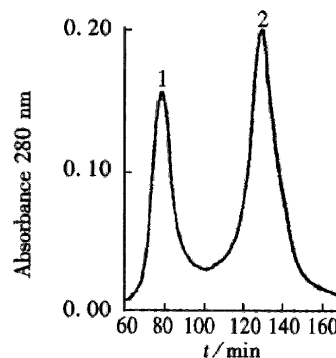


Figure 2 Gel chromatography of the CM-Sepharose peak 2 and peak 3 fractions on a Superdex-75 column.

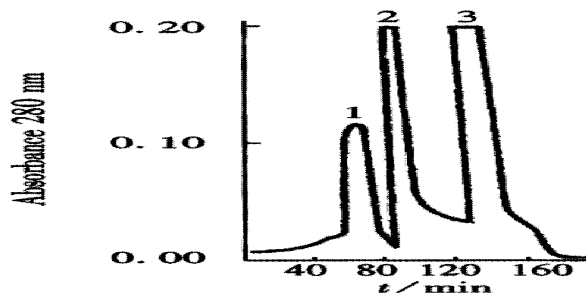


Figure 3 Molecular mass analysis of the extracted TGFβ (SDS PAGE). Right Lane: marker proteins.

TGFβ activity in vivo

Seventy-two hrs after subcutaneous injection of TGFβ, there was evident proliferation of fibroblasts and a few infiltration of inflammatory cells at the injection sites. The newly formed blood capillaries were also seen at the injection sites (Figure 4). The granulation tissue was confined at the injection sites. No obvious tissue change was observed in the control group.

Detection of apoptosis of hepatocytes

HE staining revealed that apoptotic hepatocytes exhibited the pyknosis and dense-stained nuclei and cytoplasm. Moreover, the nuclei demonstrated fragment, annular or crescent body, and the cytoplasm showed “bubbling” (Figure 5). TUNEL positive signals were olivine or yellow florescence in the nuclei. The TUNEL index of the TGFβ treated group was 0.31 and distinctly higher than that of control group (0.09), ($P < 0.05$)

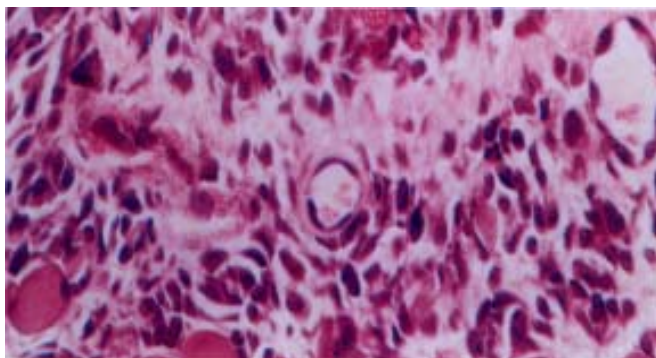


Figure 4 The granulation tissue formation after 72 hrs subcutaneous injection of TGF β . HE \times 400

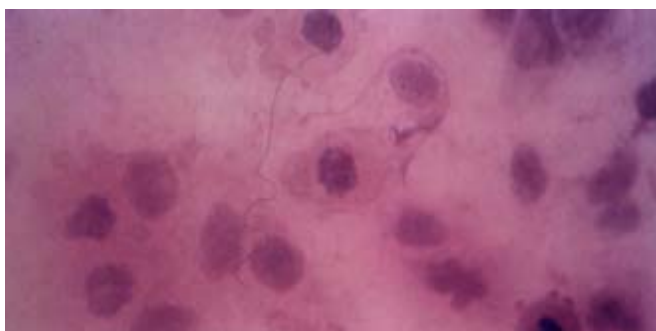


Figure 5 Hepatocytes QZG displayed apoptosis after being treated with 8 μ g/L TGF β for 12 hrs. HE \times 400

DISCUSSION

TGF β is a disulfide-linked homodimeric 25ku protein that consists of two identical 112 amino acid subunits, and only the dimer is biologically active. It is acid and heat stable and the presence of 18 half cystine residues in each dimeric molecular contributes to this stability^[48-50]. There are at least five described subtypes of TGF β , encoded by distinct but closely related genes. TGF β 1, 2 and 3 have been found in many species, including humans; TGF β 4 has been found in chickens and TGF β 5 in amphibians^[51]. Bone is the richest source of TGF β in the body, as it contains more than 200 μ g per kg of wet weight, whereas blood platelets represent the most concentrated source of TGF β (up to 20mg per kg of wet weight) and is released from β granules of platelets when blood clots^[52,53]. Therefore, TGF β is generally extracted from the fresh platelets. It is suggested that TGF β is highly stable under acidic condition and can be activated by heating in boiling water for 5 min, or treatment with 1N acetic acid or 6M urea. The acid/ ethanol procedure is a practical way and previously used to extract biologically active polypeptides such as insulin, insulin like growth factor and platelet-derived growth factor^[54,55]. Some other studies verify the effectiveness of this extraction procedure for isolation of TGF β from many tissues, including platelets, placenta and kidney^[53,56,57]. Isolation of TGF β from platelets includes five steps: collection and washing of platelets, acid/ethanol extraction, ethanol/ether precipitation, ion exchange chromatography and gel chromatography. In the present study, crude TGF β was isolated from the fresh bovine platelets and then purified by ion exchange and gel chromatography. The SDS-PAGE analysis showed that the molecular weight of its main component was 25ku, which

corresponded to that of standard TGF β protein.

Wound healing and tissue repair involve a complex series of biological events which include inflammation, cellular migration, fibroblasts proliferation, production of collagen and tissue remodeling. Growth factors have been reported to enhance the repair process in animal models by increasing the degree of cellularity, the rate of angiogenesis, and the amount of collagen accumulated^[58,59]. TGF β has been studied in association with wound healing and the ability of TGF β to initiate a cascade of events leading to enhanced wound healing has been clearly demonstrated by many reports. Pierce and co-workers^[60-65] have reported that TGF β significantly accelerate soft tissue repair by attracting fibroblasts into the wound and stimulating rapid synthesis, deposition and maturation of collagen *in vitro* and *in vivo*. Other major activities of TGF β are its abilities to promote the synthesis and deposition of various extracellular matrix (ECM) proteins and increase the expression of integrins and fibronectin, receptors that mediate cellular interactions with ECM proteins^[66,67]. Different assay systems have been developed to measure the TGF β activities, including cell proliferation and inhibition assays, radio receptor assays, immunoassays and matrix formation or cell surface antigens expression assays^[68,69]. In the present study, the analysis of activity *in vivo* demonstrated that there were fibroblast proliferation and blood capillaries formation after subcutaneous injection of the extracted TGF β . Moreover, the granulation tissue was only located in the injection sites and had no tendency to diffuse. That the action of the extracted TGF β *in vivo* led to granulation tissue formation suggested the successful extraction of TGF β and its participation in repair of tissue injury.

TGF β has been shown to either stimulate or inhibit proliferation in different cell types, and within same cell types, depending upon the stage of cell differentiation, *in vitro* condition and the presence of other growth factors. TGF β has a stimulatory effect on the proliferation of cells of mesenchymal origin, such as fibroblasts, osteoblasts and Schwann cells, yet is a growth inhibitor for cells of epithelial or neuroectodermal origin, including epithelial cells, osteoclasts, keratinocytes, T and B lymphocytes, endothelial cells and hepatocytes^[70-72].

Apoptosis is a genetically and highly conserved process. Regulation of the balance between cell proliferation and apoptosis is essential for development and maintenance of multicellular organisms^[73-77]. Previous studies suggest that TGF β is able to induce evidently apoptosis of hepatocytes and hepatoma cells *in vitro*. The animal experiments *in vivo* also manifest that the hepatocytes undergoing apoptosis have obviously elevated level of TGF β expression^[78,79]. Furthermore, hepatoma cells which have a high apoptotic incidence rate, simultaneously demonstrate a high level of TGF β expression^[80]. These studies suggest an involvement of TGF β in the initiation of apoptosis of hepatocytes. In our HE staining, normal hepatic cell line QZG showed remarkable morphological changes of apoptosis, including the pyknotic and hyperchromic cytoplasm and nuclei, and the fragmentary, crescent form or annular nuclei, and the "bubbling" cytoplasm, after being treated with exogenous TGF β . TUNEL staining also showed that the incidence rate of apoptosis was distinctly higher in the TGF β treated group than that of the control group. The present study further supported the apoptotic induction of hepatocytes by TGF β , and verified the good biological activity of the extracted TGF β as well. Better understanding of the relationship between TGF β and the signal transduction of hepatocellular apoptosis requires further investigations.

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