β2-microglobulin induces epithelial-mesenchymal transition in human renal proximal tubule epithelial cells in vitro

Aiqing Zhang¹, Bin Wang², Min Yang³, Huimin Shi¹ and Weihua Gan¹*

Abstract

Background: The objective of this study was to investigate the influence of β2-microglobulin (β2-M) on the epithelial-mesenchymal transition (EMT) in renal tubular epithelial cells.

Methods: A human kidney proximal tubular cell line (HK-2) was used as the proximal tubular cell model. HK-2 cells were exposed to different concentrations of β2-M (5, 10, 25, and 50 μM) for up to 24, 48 and 72 h. The effects of β2-M on cell morphology were observed by phase contrast microscopy, and the possible associated mechanisms were assessed by immunofluorescence staining, western blot, RNA interference, immunoprecipitation, and induced coupled plasma mass spectroscopy.

Results: β2-M induced marked morphological alterations in the HK-2 cells, accompanied by the increased expression of extracellular matrix components and α-smooth muscle actin (α-SMA), vimentin and fibronectin and the reduced expression of E-cadherin. Our results also revealed that β2-M could induce the EMT in the HK-2 cells without significant affecting cell viability. Excess β2-M in the HK-2 cells led to a decrease in iron and an increase in hypoxia inducible factor-1α (HIF-1α), which induced EMT in the HK-2 cells. Additionally, disrupting the function of the β2-M/hemochromatosis (HFE) complex by HFE knockdown was sufficient to reverse β2-M-mediated EMT in the HK-2 cells.

Conclusion: These findings demonstrate that the activity of β2-M is mediated by the β2-M/HFE complex, which regulates intracellular iron homeostasis and HIF-1α and ultimately induces EMT in HK2 cells.

Keywords: β2-microglobulin, Epithelial-mesenchymal transition, Hemochromatosis

Background

Epithelial-mesenchymal transition (EMT) is a process by which fully differentiated epithelial cells undergo transition to a fibroblast phenotype. Emerging evidence has suggested that EMT is an important pathway leading to the generation of matrix-producing fibroblasts and myofibroblasts during the development of kidney fibrosis [1-5].

Protein overload models as well as animal models of proteinuria induced by renal injury have suggested that excessive protein reabsorption induces EMT in tubular cells [6,7]. Qiong Wen et al. [8] have proven that urinary proteins from patients with nephrotic syndrome alter signaling proteins regulating EMT. In vitro experiments have found that a high concentration of albumin up-regulates profibrogenic gene (transforming growth factor-β1, TGF-β1) expression in proximal tubular cells [9].

β2-microglobulin (β2-M) is an 11-kDa nonglycosylated protein with no transmembrane domain that usually associates with cells by interacting with the extracellular regions of heavy chains. It is typically filtered through the renal glomeruli, and 99% of it is absorbed and catalyzed by the renal tubular cells, while the non-absorbed portion is excreted in the urine. β2-M is also a major protein component of proteinuria. The appearance of β2-M in the urine depends on its plasma level, which must exceed its renal reabsorptive threshold of 5 mg/l, and/or proximal tubular damage [10]. Many studies have demonstrated that the serum or urine β2-M concentration is increased in a variety of diseases, including inflammatory or infectious diseases [11,12], prostate cancer, lung cancer, and...
particularly in lymphocytic malignancies, such as non-Hodgkin’s lymphoma and multiple myeloma [13-17]. However, the effect of excessive β2-M on proximal tubular cells is relatively unknown.

Therefore, the main aim of this study was to test the hypothesis that urinary β2-M can induce EMT human renal proximal tube epithelial cells and to elucidate the molecular mechanism associated with this process.

Methods

Cell cultures and treatments

HK-2 human renal proximal tubular epithelial cells (PTECs) were purchased from the American Type Culture Collection (Rockville, MD) and were maintained as described previously [8]. A β2-M stock solution (1 mM) was prepared by dissolving 118 mg of lyophilized powder into 10 ml of serum-free base media. Human serum albumin (HSA), which is the most abundant protein in nephrotic urine, was used as a protein control. We used TGF-β1, which is a classic profibrogenic factor, as a positive control. The HK-2 cells were exposed to different concentrations of β2-M, HAS, or TGF-β1 for various periods of time as scheduled. For neutralizing antibody assessments, cells were pre-treated for 1 h prior to the addition of β2-M and subsequently co-treated with a monoclonal anti-TGF-β1 antibody (TGF-β1 mAb; R&D Systems, Minneapolis, MN) at a concentration of 30 μg/ml.

Cell viability and preparation of cell lysates

After exposure to the β2-M (5, 10, 25, and 50 μM) or control medium for 24–72 h, cell viability was measured by a Cell Titer 96 Aqueous One solution Cell Proliferation Assay (Promega, Madison, WI). To evaluate the transition of PTECs to fibroblasts, we used HK-2 cells cultured in supplement-free fresh media in the presence or absence of various treatments. After the media were removed, cellular proteins were extracted by lysing the cells with Mammalian Cell Lysis Reagents (Sigma). The protein concentrations in the cellular lysates were determined by the Bradford method using DC protein assay reagents (Bio-Rad, Hercules, CA).

Western blot analysis

Western blot analysis was performed essentially according to an established procedure [18]. The primary antibodies used were as follows: anti-β-actin (Sigma, St. Louis, MO), anti-α-SMA (Sigma, St. Louis, MO), anti-E-cadherin (BD Transduction Laboratories, Lexington, KY), anti-fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-HFE (Santa Cruz Biotechnology, Santa Cruz, CA), anti-vimentin (Boster, Wuhan, China), and anti-HIF-1α (Millipore, USA). Quantifications were performed by measuring band intensities using Image J analysis software.

Transient transfection of cells with siRNA

For the knockdown experiments, HK2 cells were transiently transfected with siRNA specifically targeting HFE or a negative control siRNA using Lipofectamine PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were transfected with 20 nM HFE siRNA or control siRNA for 24 h before treatment. Cellular protein was extracted and subjected to western blot analysis for HFE.

Immunoprecipitation

Immunoprecipitation was performed using an Immunoprecipitation Starter Pack (GE Healthcare).

Iron measurements and iron chelator treatments on HK-2 cells

Iron concentrations were determined using induced coupled plasma mass spectroscopy (ICP-MS). A total of 10³ cells were pelleted and digested using 3% nitric acid. Samples were diluted and analyzed with a Perkin Elmer ICP-MS. The data are expressed as picomoles of metal. HK-2 cells were treated with 200 mmol/L of desferal (DES) for 48 h. Then, the DES was removed and replaced with normal media. A day later, the cells were photographed, and cell lysates were prepared for immunoblot analysis.

Statistical analysis

All data are expressed as the mean ± S.D. Multiple groups of values were compared using one-way analysis of variance (ANOVA) and Fisher’s LSD test. Data were analyzed using SPSS 13.0 for Windows. A P < 0.05 was considered significant.

Results

Effect of β2-M on PTEC cellular viability

Considering the absorbance at 490 nm in the control group as 1, the exposure of HK-2 cells to different concentrations of β2-M (5, 10, and 25 μM) produced no significant effects on cell viability at 24, 48 or 72 h; however, a significant decrease in cell viability (>20%) was observed at the higher doses of ≥50 μM β2-M after 48 h (Figure 1).

Effect of β2-M on PTEC morphology

The control cells showed a typical epithelial cuboidal shape with a cobblestone morphology (Figure 2A); however, they exhibited profound morphologic changes after exposure to β2-M (25 μM) for 72 h, becoming elongated in shape, disassociating from neighboring cells and losing their cobblestone monolayer morphology (Figure 2B). Because TGF-β1 is a well-characterized inducer of EMT in renal tubular epithelial cells, it was used as a positive control (Figure 2C). HSA, which was used as a protein control, had no significant effect on the morphological
changes of the HK2 cells at 50 μM (Figure 2D). More interestingly, a neutralizing TGF-β1 antibody could not block the β2-M-induced morphological changes of the HK2 cells (Figure 2E), indicating that β2-M EMT occurred in a TGF-β1-independent manner.

**β2-M induced EMT in HK-2 cells**

We further assessed the expression of E-cadherin, which is a marker for the epithelial phenotype, and α-SMA, vimentin, and fibronectin, which are markers for the myofibroblast phenotype. We observed that in HK-2 cells exposed to 25 μM of β2-M, the expression of E-cadherin decreased after 72 h of treatment, while that of α-SMA, vimentin, and fibronectin increased, as indicated by western blot analysis (Figure 3A and B). These results suggest that these cells lost their epithelial characteristics and acquired mesenchymal cell properties.

**β2-M interacted with hemochromatosis (HFE) protein and inhibition of HFE reversed EMT**

HFE has been previously shown to interact with β2-M. We therefore assessed the presence of β2-M and HFE complexes in HK-2 cells. The physical interaction between β2-M and HFE as a complex was demonstrated by coimmunoprecipitation (co-IP) followed by western blot analyses (Figure 4A). To determine the possible functional roles of the β2-M/HFE complex in β2-M-induced EMT in HK-2 cells, we transfected these cells with either siRNA-HFE or irrelevant siRNA-con. After 24 h, HFE expression was assessed by western blot. Our results indicated that the level of this protein was markedly lower in the HK-2 cells transfected with siRNA-HFE compared with the control cells and those transfected with siRNA-con (Figure 4B). Interestingly, a decrease in the HFE protein level blocked the increased
expression of vimentin and α-SMA and decreased expression of E-cadherin induced by β2-M (Figure 4B and C). Additionally, we observed that the down-regulation of the HFE protein altered the morphology of the HK-2 cells to a cobblestone-like appearance (Figure 4D). Thus, disrupting the function of the β2-M/HFE complex by HFE knockdown is sufficient to reverse β2-M-mediated EMT in HK-2 cells.

**β2-M attenuated intracellular iron but increased HIF-1α expression in HK-2 cells**

Because the β2-M protein is known to directly regulate iron levels in cells as demonstrated by the prevention of iron uptake by the β2-M/HFE complex and the iron overload observed in β2-M and HFE knockout mice (20), we assessed whether cellular iron levels were lower in β2-M-stimulated HK-2 cells compared to control cells.
using inductively coupled plasma mass spectroscopy (ICP-MS). Intracellular iron was significantly lower in the β2-M-stimulated HK-2 cells compared to the control cells (Figure 5A). Additionally, iron-responsive HIF-1α, which is a factor that has been widely proven to participate in the EMT process, increased after stimulation with β2-M for 72 h; however, HFE knockdown reversed the increase in HIF-1α induced by β2-M (Figure 5B and C).

**Iron modulated EMT in HK-2 cells**

To further determine whether iron is involved in the regulation of EMT, we used an iron chelator (DES) to induce EMT-like changes. DES increased the expression of iron responsive HIF-1α and induced mesenchymal characteristics (Figure 6A through C). These results collectively demonstrate that β2-M overload in HK-2 cells leads to decreased iron and increased HIF-1α, which induces EMT in HK-2 cells.

**Discussion**

The role of β2-M has long been documented in many kidney diseases. In this study, we documented for the first time that (i) β2-M can promote apoptosis and EMT in tubular epithelial cells *in vitro*; (ii) induced the stable expression of EMT biomarkers, including the decreased expression of E-cadherin and increased expression of α-SMA and fibronectin in HK-2 cells; and (iii) formed a complex with its receptor, HFE, and this complex regulated intracellular iron and activated HIF-1α in HK-2 cells (Figure 7). To our knowledge, this is the first report to demonstrate the manner by which β2-M functionally promotes EMT in tubular epithelial cells.

β2-M is a nonglycosylated protein with a molecular mass of 11,800 Da that is synthesized by all nucleated cells and forms a small invariable light chain subunit of major histocompatibility complex (MHC) class I antigen through noncovalent linkages on cell surfaces. The β2-M protein is present at low levels in serum, urine and other body fluids under normal physiological conditions and is almost exclusively catabolized within the kidney. Many studies have demonstrated that the serum or urine β2-M concentration is increased in a variety of diseases, including inflammatory or infectious diseases [10,11] and cancers [12-16]. When the elevation in plasma β2-M exceeds its renal reabsorptive threshold of 5 mg/l or the proximal tubules are damaged, β2-M appears in the urine. In chronic kidney disease, as kidney function gradually declines, β2-M filtration is reduced and the serum level of β2-M decreases.
β2-M is increased, which leads to the exposure of relatively normal residual glomeruli to high concentrations of β2-M. The excess β2-M filtered from the original urine can cause damage to renal tubular cells [18].

However, the actual concentration of β2-M in the proximal tubular ultrafiltrate in patients with proteinuric renal diseases is unknown. In systemic lupus erythematosus and chronic kidney disease patients, the maximum serum β2-M protein concentration has been reported to reach ~25 mg/dl [19,20]. Because the relatively small molecular weight of β2-M permits its filtration through the glomerular membrane, we chose to use the dose of

![Figure 6](image-url)  
Figure 6 Effect of iron on EMT in HK-2 cells. (A) HK-2 cells were stimulated with DES for 48 h, and photographs were taken using a Nikon TMS microscope at 400x magnification. (B) HK-2 cells were cultured in the presence or absence of DES for 48 h. Cell lysates were immunoblotted with antibodies against HIF-1α, E-cadherin, vimentin, and α-SMA. (C) Graphical representation of relative HIF-1α, E-cadherin, vimentin, and α-SMA protein abundances after normalization with β-actin as indicated. The data are presented as the mean ± SE of three experiments. **P < 0.05 versus controls.

![Figure 7](image-url)  
Figure 7 Working model of β2-M-mediated EMT. The β2-M/HFE complex maintains low iron levels and activates EMT via iron-responsive pathways, such as HIF-1α signaling.
5–50 μM in our *in vitro* study. As shown in Figure 1, a significant decrease in cell viability (>20%) was observed at higher β2-M doses of ≥50 μM after 48 h. In addition, the induction of fibronectin, vimentin, and α-SMA expression by β2-M *in vitro* was dose dependent, and the maximal induction was observed following treatment with 25 μM of β2-M (data not shown). We therefore selected 25 μM of β2-M as the treatment in most of our cell experiments.

Although a conclusive demonstration of EMT *in vivo* in patients with kidney diseases remains a challenge, it has emerged as an important pathway leading to kidney fibrosis in diseased kidneys in recent years. The process of EMT is characterized by a loss of epithelial proteins, such as E-cadherin and cytokeratin, and the acquisition of new mesenchymal markers, including vimentin, α-SMA, fibroblast-specific protein-1 (FSP1), interstitial matrix components type I collagen, and fibronectin [21,22]. These alterations in protein expression are usually accompanied by a change in morphology to a fibroblastoid appearance and an enhanced migratory capacity. It has been proven that there is a correlation between the severity of tubular injury and urine protein quality in various renal diseases [23]. Additionally, Sajni Josson et al. [24] have found that β2-M, which is an important component of urine protein, induces epithelial to mesenchymal transition and confers cancer lethality and bone metastasis in human cancer cells. However, the molecular biological mechanism of β2-M in the damage of renal tubular epithelial cells is far from clear. In our study, β2-M induced tubular cells to undergo significant morphologic changes to a fibroblastoid appearance and promoted the expression of EMT-related proteins in these cells. These results indicate that this protein may also be an important factor in the peritubular microenvironment that induces or promotes EMT. Although we did not find any new molecular mechanism by which β2-M induces EMT, our results support the function of the β2-M/HFE complex as a mediator participating in β2-M-induced EMT in both renal proximal tubule epithelial cells and cancer cells. Further, this mechanism may play an important role in tumor-associated nephropathy because the concentrations of serum and urine β2-M have been shown to be increased in many types of cancer [13–17].

HFE is an MHC class 1b protein that is likely to assume a signaling role in association with β2-M [25]. β2-M/HFE has been found to regulate negatively intracellular iron, activate HIF-1α and promote EMT in cancer cells. Our study demonstrated that HFE is a β2-M receptor as shown by the following results: (1) HFE was found to physically interact with β2-M, as demonstrated by the immunoprecipitation results for the HK-2 cells (Figure 4A); and (2) the knockdown of HFE resulted in a reversal of EMT in the HK-2 cells with supportive morphologic, biochemical, and behavioral characteristics (Figure 4B–D). Thus, β2-M/HFE interactions are important for β2-M-mediated EMT. In addition, β2-M protected the influx and accumulation of intracellular iron. Higher β2-M/HFE levels led to the downregulation of intracellular iron in the HK-2 cells, and low levels of this complex in these cells enhanced intracellular iron levels (Figure 5A). Lower levels of intracellular iron activated HIF-1α and its target genes in the HK-2 cells (Figures 5B–C and 6), driving EMT. The downstream functional significance of the β2-M/HFE complex is depicted in Figure 7.

**Conclusion**

In summary, we demonstrated the importance of β2-M in EMT in tubular epithelial cells. The activity of β2-M is mediated by the β2-M/HFE complex, which regulates intracellular iron homeostasis and HIF-1α and ultimately induces EMT. The inhibition of this complex could affect the process of EMT.

**Abbreviations**

β2-M: β2-microglobulin; EMT: Epithelial-mesenchymal transition; PTEC: Proximal tubular epithelial cells; α-SMA: α-smooth muscle actin; HFE: Hemochromatosis; HIF-1α: Hypoxia-inducible factor-1α.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

WHG and AQZ participated in the design of the study, drafted the manuscript and contributed to its revision. BW and HMS performed the cell culturing and molecular studies, helped to draft the manuscript and participated in its revision. MY participated in the discussions about the manuscript. All authors read and approved the final manuscript.

**Acknowledgments**

This work was sponsored by the Distinguished Researcher Program of Jiangsu Province of China, the Science and Technology Projects of Nanjing (No. 20121089), the Medical Science and Technology Development Key State Projects of Nanjing (No. ZKX11177), and Nature Science Research of Jiangsu Province University (No. 12KJB320002)."
4. Fan JM, Ng YY, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, et al. Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation in vitro. Kidney Int. 1999;56(4):1455–67.

5. Frazier KS, Paredes A, Dubé P, Styer E. Connective tissue growth factor expression in the rat remnant kidney model and association with tubular epithelial cells undergoing transdifferentiation. Vet Pathol. 2000;37(4):328–35.

6. Abbate M, Zoja C, Rottoli D, Coma D, Tomasoni S, Remuzzi G. Proximal tubular cells promote fibrogenesis by TGF-beta1-mediated induction of peritubular myofibroblasts. Kidney Int. 2002;61(6):2066–77.

7. Harris RC, Neilson EG. Toward a unified theory of renal progression. Annu Rev Med. 2006;57:365–80.

8. Wen Q, Huang Z, Zhou SF, Li XY, Luo N, Yu XQ. Urinary proteins from patients with nephrotic syndrome alters the signalling proteins regulating epithelial-mesenchymal transition. Nephrology (Carlton). 2010;15(1):63–74.

9. Yard BA, Chorianopoulos E, Herr D, van der Woude FJ. Regulation of endothelin-1 and transforming growth factor-beta1 production in cultured proximal tubular cells by albumin and heparan sulphate glycosaminoglycans. Nephrol Dial Transplant. 2001;16(6):1769–75.

10. Kalandada V, Vandercaem B, Bernard A, Lauweys R, van Ypersele DSC. Low molecular weight proteinuria in human immunodeficiency virus-infected patients. Am J Kidney Dis. 1996;27(6):803–8.

11. Akay H, Akay A, Koklu S. Serum and tissue beta-2 microglobulin levels in patients with Helicobacter pylori infection. Dig Dis Sci. 2008;53(2):358–62.

12. Braaten DC, McClellan JS, Messaoudi I, Tibbetts SA, McClellan KB, Nikolić-Zugich J, et al. Effective control of chronic gamma-herpesvirus infection by unconventional MHC Class Ia-independent CD8 T cells. PLoS Pathog. 2006;2(5), e37.

13. Faderl S, Keating MJ, Do KA, Liang SY, Kantarjian HM, O’Brien S, et al. Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL). Leukemia. 2002;16(6):1045–52.

14. Bataille R, Durie BG, Grenier J. Serum beta2 microglobulin and survival duration in multiple myeloma: a simple reliable marker for staging. Br J Haematol. 1983;55(3):439–47.

15. Abdul M, Hoosein N. Changes in beta-2 microglobulin expression in prostate cancer. Urol Oncol. 2000;5(4):168–72.

16. Molica S, Levato D, Cascavilla N, Levato L, Musto P. Clinico-prognostic implications of simultaneous increased serum levels of soluble CD23 and beta2-microglobulin in B-cell chronic lymphocytic leukemia. Eur J Haematol. 1999;62(2):117–22.

17. Nissen MH, Bjerrum OJ, Plesner T, Wilken M, Roth M. Modification of beta-2-microglobulin in sera from patients with small cell lung cancer: evidence for involvement of a serine protease. Clin Exp Immunol. 1987;67(2):425–32.

18. Wang B, Zhang A, Zheng J, Gong J, Li S, Zeng Z, et al. Bufalin inhibits platelet-derived growth factor-BB-induced mesangial cell proliferation through mediating cell cycle progression. Biol Pharm Bull. 2011;34(7):967–73.

19. Donadio C. Serum and urinary markers of early impairment of GFR in chronic kidney disease patients: diagnostic accuracy of urinary beta-trace protein. Am J Physiol Renal Physiol. 2010;298(6):F1407–23.

20. Hermansen ML, Hummelshøj L, Lundsgaard D, Homum L, Keller P, Fleckner J, et al. Increased serum beta2-microglobulin is associated with both clinical and immunological markers of disease activity in systemic lupus erythematosus patients. Lupus. 2012;21(10):1098–1104.

21. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. J Am Soc Nephrol. 2004;15(1):1–12.

22. Strutz FM. EMT and proteinuria as progression factors. Kidney Int. 2009;75(5):475–81.

23. Bakouch O, Grubb A, Rippe B, Tencer J. Urinary excretion of protein HC in protonuric glomerular diseases correlates to urinary IgG but not to albuminuria. Kidney Int. 2001;60(5):1504–9.

24. Josson S, Nomura T, Lin JT, Huang WC, Wu D, Zhou HE, et al. Chung LW. beta2-microglobulin induces epithelial to mesenchymal transition and confers cancer lethality and bone metastasis in human cancer cells. Cancer Res. 2011;71(7):2600–10.

25. Lebron JA, Bennett MJ, Vaughan DE, Chinno AJ, Snow PM, Mintier GA, et al. Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. Cell. 1998;93(1):111–23.