行政院國家科學委員會專題研究計畫成果報告

計畫題目:缺血再灌流誘發腎小管細胞凋亡之基因表現與預防機轉

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一、中文摘要

自由基的釋放可能是腎臟移植或缺血灌流所誘發細胞凋亡之主因。為驗證此一假說我們利用水溶性碳六十,一種自由基驅除之新藥，探究缺血灌流造成腎小管發生細胞凋亡的分子機轉與預防效應。我們以 TUNEL **免疫染色**, DNA **階梯生化證據及定量** PCR **與西方墨點法探討抗致死基因** bcl-xL **的表現。結果發現水溶性碳六十在缺血灌流前處理可加強** bcl-xL **之表現並而減低** TUNEL **正染色細胞**(apoptotic cell), **與** DNA **階梯之表現並進而減低腎缺血灌流所造成之傷害。**

關鍵詞： 細胞凋亡; **缺血灌流**; **自由基**; **水溶性碳六十**; **抗致死基因**

Abstract

Apoptosis is induced during the harvest and implantation of renal transplants or in ischemia/reperfusion (I/R) injury. The mechanism for inducing apoptosis might be related to the release of free radicals. Water-soluble hexa(sulfobutyl)fullerenes are tertiary structures consisted of sixty carbon atoms (C_{60}) and are shown to have strong free radical scavenge effects in vitro. The aims of this study were to elucidate the role of water-soluble hexa(sulfobutyl)fullerenes in the I/R induced renal tubular apoptosis of the rat kidneys. The bilateral renal artery occlusion was performed 45 min by hemoclip and then we removed the clip to reperfuse the damage kidneys for 1, 2, 4, 10, 15 and 24 hours. We used TUNEL, DNA laddering, quantitative PCR and western blot to evaluate the expression of apoptosis. Water-soluble C_{60} were administrated before or after I/R at a dose of 100 μg. We found that the severe apoptotic expression in the damaged kidneys was 45-min ischemia followed by 4-hour reperfusion and the expression of apoptosis could be attenuated by C_{60} pretreatment. Pretreated C_{60} up-regulated bcl-x_L expression, an anti-death gene, in the I/R kidney. It is suggested that water-soluble hexa(sulfobutyl)fullerenes can attenuate the I/R induced apoptosis in the rat kidney and the protective mechanism may be ascribed to the overexpression of bcl-x_L.

Keywords: Apoptosis; Ischemia/reperfusion; Free radicals; Water-soluble hexa(sulfobutyl)fullerenes; Anti-death gene

二、緣由與目的

Apoptosis is an inevitable event during the harvest and implantation of organs due to ischemia/reperfusion (I/R) injury [1]. The mechanism responsible for post-reperfusion apoptosis is attributed to the release of reactive oxygen species (ROS) or the increasing activity of endonuclease by elevation of calcium entry [2,3].

To preserve the renal function after renal surgery or transplantation, there are many

strategies, which have been performed. The use of chemopreventive agents with antioxidant activity becomes a recognized approach against the oxidative stress caused by the ROS in animals and humans [4]. Recently, novel highly water-soluble hexa(sulfobutyl)fullerenes were synthesized via electron reductions of C_{60} [5] . It showed a tertiary structure containing sixty carbon atoms with covalent bonds and 18 to 20 hydroxyl moieties in each molecule. In vitro studies have shown water-soluble fullerenols to be a potent antioxidant and free radical scavenger in biological systems [6]. Free radicals are involved in the I/R injuries during the harvest and implantation of renal grafts, and this mechanism is related to delayed functioning of the renal grafts or even acute tubular necrosis after transplantation [2,3,7]. In the in vivo study, we have found that this water-soluble C_{60} when added as an ingredient of the perfused solution, can improve the function of the canine renal grafts [7]. However, further investigations regarding the optimal concentration of water-soluble C_{60} in the perfusate and the possible protective mechanism to reduce tissue damages are necessary and are being undertaken to reveal the roles of in organ preservation before its clinical application.

Thus, in this study we hypothesized that I/R induced renal apotptosis could be minimized by the hexa(sulfobutyl)fullerenes.

三、方法

Animal and treatment. Male Wistar rat weighing 200~300 g were anesthetized with sodium pentobarbital (50 mg/kg, ip). The rats were randomly divided into three groups: I) nontreated I/R group (n=6), II) pretreated hexa(sulfobutyl)fullerenes (100 μg, intravenously) group (n=5), III) postreated hexa(sulfobutyl)fullerenes (100 μg, intravenously) I/R group (n=5). After a laparotomy, the left renal artery was clamped for 45 min, and then reperfused for 0, 1, 2, 4, 6, 10, 15 and 24 hours individually as described previously [8]. After I/R treated, kidney tissues were snap-frozen in liquid nitrogen and stored at -70℃ until they were used for the preparation of extracts.

Extraction of rat kidney DNA. DNA extraction and electrophoresis were performed as described previously by Facchinetti et al. [9].

TUNEL reaction. After removal, kidney sections were fixed in 10 % neutral buffered formalin and embedded in paraffin and stained for TUNEL reaction. The 6-μm thick sections of the kidney were prepared, deparaffinized, and stained by the terminal deoxynucleotidyl transferase-mediated nick-end labeling method (TUNEL) according to the method of Gavrieli et al. [10].

Quantitative and real time PCR. Total RNA preparations from rat kidney were performed by using trizol reagent (GIBCO/BRL). Quantitative PCR to measure the *bcl-*x_L transcript of treated and normal kidney tissue was used with two primers and one olignucleotide probe using an Applied Biosystems PRISM 7700 Sequence

Detector (Perkin-Elmer). The primers and probes sequences are：
bcl-x_L forward primer : 5-GCCACAGCAGCAGTTTGGAT-3
bcl-x_L reverse primer : 5-AAACTCATCGCCAGCCTCTCT-3
bcl-x_L probe : 5-CTCCCCATGGCAGCAGTGAAGC-3
For internal control of the RNA, expression of β-actin was examined by RT-PCR as described above.

Table 1. Effects of Hexa(sulfobutyl)fullerenes Parameters of ABs, DNA ladder and Bcl-xL Gene Expression in the Three Groups of Rats

Group	I	II	III
No. of ABs	23±4	7±2*	19±6
DNA ladder	++	+*	++
mRNA of Bcl-x_L	+	++*	+
Protien of Bcl-x_L	+	++*	+

Data are expressed as the mean of all animals at the 4-hour reperfusion after 45-min ischemia. No of apoptotic bodies (ABs) was calculated for each sample by counting the number of positive stained cells per 100 high-power fields. – = < 100 units; + =100 to 200 units; ++ = >200 units, with units calculated from the signal intensity from a densitometer. * $P<0.05$ when compared to group I.

Western blot. Protein samples were isolated from the kidneys and the expression of Bcl-x_L was determined by western blot. Polyclonal antibody, Bcl-x_L (Oncogene), generated by immunizing rabbits with a peptide corresponding to residue 201~216 of the Bcl-x_L protein. Bcl-x_L protein is similar in size and predicted structure to bcl-2 protein, and prevents apoptotic cell death. SDS-PAGE was performed on 12.5% separation gels in the absence of urea and stained with coomassie brilliant blue. Markers (Oncogene, low molecular weight calioretion kit) for estimation of molecular weight were obstained from: ovalbumin (43KD), carbonic anhydrase (29KD), β-lactoglobulin (18.4KD) and lysozyme (14.3KD). Proteins on the SDS-PAGE gels, each lane was loading 30 μg total protein, were transferred to nitrocellulose filters. The immunoreactive bands were detected by incubation with the antibody, followed by secondary antibody-alkaline phosphatase and finally with 4-chloro-1-naphthol.

四、結果與討論

With the evidence of TUNEL reaction and DNA laddering in the kidney tissue (Table 1), we found that the timing for most significant apoptosis formation was 45-min ischemia followed by 4-hour reperfusion. Pretreatment of hexa(sulfobutyl)fullerenes into the kidneys diminished the apotposis formation and

DNA laddering, whereas the diminished response was not found in the post-treated group. With the *bcl-x_L* analysis by QT-PCR, mRNA of *bcl-x_L* was up-regulated in the watre-soluble C_{60} pretreated kidney. Western blot of Bcl-x_L also consistently overexpressed in the hexa(sulfobutyl)fullerenes pretreated kidneys.

Reperfusion injury after ischemia has been linked to the generation of ROS and these ROS have been suggested to play a critical role in reperfusion-induced tissue damage [2,3,7]. Recent data implicated *bcl-x_L*, a member of the *bcl-2* family of apoptosis regulatory genes, as a potent death repressor and support the hypothesis that *bcl-x_L* regulates survival decisions within susceptible cells by functioning downstream of oxidant production [11]. In this study, administration of novel hexa(sulfobutyl)fullerenes into the kidney prior to I/R overexpressed Bcl-x_L and blocked apoptosis formation triggered by I/R injury. The beneficial effect to overexpress Bcl-x_L within the kidney and to protect the tubular cells without any apparent detrimental effects introduces the possibility of future therapeutic opportunity.

In conclusion, hexa(sulfobutyl)fullerenes pretreatment up-regulates some anti-death gene expression in the rat kidneys and attenuates renal I/R inducing apoptosis formation.

五、成果自評

本計畫之成果已發表在 Fullerene Science & Technology 1999, 7: 529-540. **本研究使用之技術及數據與實驗前之推論極為相近。水溶性碳六十已申請專利而以水溶性碳六十研發應用於器官保存液與減低器官缺血灌流傷害是極具醫學應用價值。**

六、參考文獻

1. Sasaki, H., and Matsuno, N., Transplant. P., 1996, 28, 1908.
2. Ueda, N., Walker P.D., Hsu, S.M., Shah, S.V., Proc. Natl. Acad. Sci. USA, 1995, 92, 7202.
3. Zager, R.A., Fuerstenberg, S.M., Baehr, P.H., Myerson, D., and Torok-Storb, B., J. Am. Soc. Nephrol., 1994, 4, 1588.
4. Imlay, J.A., and Linn, S., Science, 1988, 240, 1302.
5. Chiang, L.Y., Upasani, R.B., and Swirezewski, J.W., J. Am. Chem. Soc., 1992, 114, 10154.
6. Chiang, L.Y., Lu, F.J., and Lin, J.T., J. Chem. Soc. Chem. Commun. 1995, 1283.
7. Chueh, S.C., Lai, M.K., Chen, S.C., and Chiang, L.Y., Transplant. Proc. 1997, 29, 1313.
8. Chien, C.T., and Chen, C.F., Ren. Fail., 1994, 16, 287..
9. Facchinetti, A., Tessarollo, M., Mazzocchi, R., Hingston, D.C., and Blasi, C., J. Immunol. Methods, 1991, 136, 125.
10. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A., .J. Cell Biol., 1992, 119, 493.

11. Fang, W., Rivard, J.J., Ganser J.A., LeBien T.W., Nath, K.A., Mueller, D.L., and Behrens, T.W., J. Immunol., 1995, 155, 66.