The Reduced Coenzyme Nicotnamide Adenine Dinucleotide (NADH) Prevents Hepatic Cells from Apoptosis by Mitochondria-dependant Signaling Pathway

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ABSTRACT Mitochondria are well known to have a critical function in energy metabolism and damage to mitochondria has been related to apoptosis. NADH acts as direct positive involvement in the production and regulation of important compounds in vivo. Chemotherapeutic drugs cause DNA damages and kill not only cancer cells but also normal cells chiefly by apoptosis. The role of NADH in preventing apoptosis was elucidated. When cells were treated with 10 μ Mol Cisplatin for 12h, the microvilli became shortened and decreased. Cell membrane appeared to be convoluted and blebbing. Cell nuclear events included nuclear condensation, chromatin cluming and pronounced pyknosis. In the group treated with NADH and then with Cisplatin(NADH/Cisplatin), no obvious apoptotic morphological changes of cells were found and the shape of mitochondria was normal. In the group treated with Cisplatin alone. the fluorescence intensity of Rhodamine 123 was 1952.43 ± 305.44, but in the group of NADH/Cisplatin it was 1145.09±83.92 ($P<0.05$). In the group of Cisplatin ROS(3184.37±247.63) was higher than that(1230.69±94.65) in the group of NADH/Cisplatin ($P<0.05$). Compared with the group of Cisplatin, Bcl-2 mRNA expression in the group of NADH/ Cisplatin increased obviously. 113kD PARP was cleaved into 89kD fragments, which served as an early specific marker of apoptosis. The cytosolic fractions of 15 kD cytochrome c were detected. It was responsible for the release of cytochrome c from mitochondria during apoptosis. In the group of NADH/Cisplatin. those phenomenon were not observed. Caspase-3 activity rised quickly when cells were incubated with 1 μ mol Cisplatin, especially after 24h. However, if cells were cultured with NADH/ Cisplatin. Caspase-3 activity was kept in a low level. The results showed NADH could prevent normal cells from apoptosis by mitochondria-dependant Signaling Pathway and reduce the side effects of chemotherapeutic drugs on normal cells.

KEY WORDS NADH, Apoptosis, Cisplatin, Caspases

Mitochondria are well known to have a critical function in energy metabolism. In many types of chemical-induced liver or renal damages, toxic effects are thought to happen on the mitochondria. The recognition of mitochondrial signaling provides better understanding of apoptosis [1]. Numerous chemotherapeutic drugs often have severe side effects that limit their efficacy. Agents that could protect healthy tissue from these side effects and thus permit higher dosage of chemotherapy would increase efficacy of treatment [2]. NADH plays a vital role in the generation of ATP (adenosine triphosphate). Clinical studies have proven that in vivo NADH increases the production of depleted brain chemicals called neurotransmitters, which are used to improve memory and thinking. Birkmayer [3,4,5] studied the effects of NADH and found that NADH not only alleviated the impairment in motor skills caused by Parkinson's. but also effectively treated the corresponding cognitive dysfunction. Zhang [6,7] found that NADH could repair DNA damage of PC12 cells induced by Doxorubin and Rotenone. NADH is also needed for the regeneration of glutathione after it has become oxidized. NADH plays an active role in the immune response system. NADH is the most potent biologically antioxidant in nature. The objective of this study was to elucidate the molecular mechanisms of NADH in preventing human normal cells from apoptosis.

MATERIALS AND METHODS

Induction of apoptosis Human hepatic cell line L02 was provided by Institute of Cell Biology. Chinese Academy of Medical Science. Cells was cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, 4mM glutamine. 100 u/ml penicillin, 1 μ g/ml glucose, 0.25 u/ml insulin under 37 ℃, 5% CO_2, 95% air and 95% humidity. Exponentially growing cells on log phase were treated as follows: (1) in the group of Cisplatin. cells were incubated in medium containing 0.1 μ M, 1 μ M, 10 μ M, 100 μ M Cisplatin for 12h to 48h; (2) in

the group of control, cells were incubated in RPMI-1640 medium for 48h; (3) in the group treated with NADH and Cisplatin.

Electron microscopy Samples were prepared by the methods of agar envelope. Cell pellets were fixed with 2.5% glutaraldehyde, and then 1% osmic acid. Samples were observed after conventional rinse, dehydration, osmosis, enveloped by epoxy resin, ultrathin section by LAB-2088V ultramicrotome and doubling staining of uranium acetate and plumbum citrate. Determination of Mitochondrial membrane potential ($\Delta\psi m$) and reduced oxygen species (ROS) production.To measure $\Delta\psi m$ and ROS generation, treated or untreated cells were incubated with Rhodamine 123 (5 μg/ml) for $\Delta\psi m$ and 2′,7′ dichlorofluorescein (5 μg/ml) for ROS generation for 15 min at 37℃ in the dark followed by analysis on a confocal microscopy.

Western blot analysis of PARP cleavage and cytochrome c Crude cell extracts were obtained by suspending about 10^6 in 150 μl extraction buffer. Sonicate and incubate for 15min at 65℃ before loading. The lysates were separated through SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Cleavage of PARP was determined using PARP polyclonal antibody. Cytochrome c was detected by H-104 polyclonal antibody. Blots were stained with the chromogenic detection of alkaline phosphatase-labeled antibodies on western blots.

Semi-quantitative RT-PCR analysis of Bcl-2 transcript Total cellular RNA was isolated by Tripure isolation reagent kit. cDNA reaction for use in the different gene specific amplification was prepared as follows: 1×RT buffer, 1mM dNTP, 1mM OligdT, 25 u RNasin, 200 u Moloney Murineleukemia Virus Reverse Transcriptase and 500ng of total cellular RNA was incubated at 37℃ for 60min. The cDNA reaction mixture of 10× buffer, 1mM dNTP, 25pM primers, 2 u Taq was used for amplification of specific DNA sequences. A total of 35 cycles at 93℃ for 45s, 55 ℃ for 45s, 72℃ for 50s, a final extension at 72℃ for 3min in a thermocycler (Cetus Perkin Elmer, CA). PCR product was electrophoresed in 2% agarose gels containing ethidium bromide.

Caspase activity assay 2×10^6 cells were Counted and centrifuged at 200g for 10min.Cells were resuspended in chilled cell lysis buffer. Cell lysates were centrifuged to precipitate cellular debris. Supernatants were transferred to new microcentrifuge tubes.2× reaction buffer(containing DTT)was added to each reaction. Then 50 μM conjugated substrate DEVD-pNA (for caspase-3 detection) or 200 μM conjugated substrate IETD-pNA (for caspase-8 detection) was added to each tube and incubated at 37℃ for 2 hr in water bath. Samples were read in a spectrophotometer at 405 nm.

RESULTS

Protection against apoptosis by NADH When cells were treated with 10 μM Cisplatin for 12h, the microvilli became shortened and decreased. Cell membrane appeared to be convoluted and blebbing. Cell nuclear events included nuclear condensation, chromatin cluming and pronounced pyknosis. The apoptotic cells shrank markedly, whose cytoplasm concentrated and vacuolized, and the mitochondria were swelled and decreased. Normal L02 cells were found to have different length microvilli, cell nuclei appeared to be globular, mitochondia and nucleoli were visible and clear. In the group of NADH/Cisplatin, no obvious apoptotic morphological changes of cells were found and the shape of mitochondria was normal. It suggested that low dosage of Cisplatin could induce apoptosis of L02 cells and NADH could prevent cells from apoptosis.

Mitochondrial membrane potential $\Delta\psi$ reduced oxygen species ROS and Bcl-2 transcript in the anti-apoptosis process In the group of Cisplatin, the fluorescence intensity of Rhodamine 123 was 1952.43±305.44, but in the group treated with NADH then Cisplatin it was 1145.09±83.92 ($P<0.05$). It meant a fall of $\Delta\psi m$ occurred during apoptosis. In the group of Cisplatin, ROS was 3184.37±247.63, higher than 1230.69±94.65 in the group of NADH/ Cisplatin ($P<0.05$). It showed the involvement of mitochondrial ROS signaling in cell apoptosis pathways. Bcl-2 is an integral Intracellular membrane protein that inhibits apoptosis induced by multiple insults in a variety of cell types. Both biochemical and genetic evidence indicates the Bcl-2 family can regulate cell death induced by Caspases molecular. In the group of Cisplatin, the expression of Bcl-2 mRNA was decreasing sharply after exposing cells to Cisplatin for 12h to 48h. The level of mRNA expression increased obviously in the group of NADH/Cisplatin compared with the group of Cisplatin.

The function of PARP cleavage, cytochrome c and

Caspases We found that the 113kD PARP was cleaved during apoptosis into 89kD fragments, which served as an early specific marker of apoptosis. In the group of NADH/Cisplatin, those phenomenon were not observed. Our finding showed that Caspase-3 activity rised quickly when cells were incubated with 1 μ m Cisplatin especially after 24h. However, if cells were cultured with NADH then with Cisplatin, Caspase-3 activity was kept in a low level. The alteration pattern of Caspase-8 appeared to be similar. Differently, activation of downstream Caspase-3 occurred after a lag phage of 12h compared with Caspase-8 activation, which was incompatible with the viewpoint that they should be activated directly by apical Caspases. The cytosolic fractions of 15 kD cytochrome c were detected, which was responsible for the release of cytochrome c from mitochondria during apoptosis.

DISCUSSION

Apoptosis is essential in many physiological processes. In oncology, extensive interest in apoptosis comes from the mode of cell death that is triggered by a variety of antitumor drugs. Chemotherapy has provided clinical remissions and treatment for many patients with malignancies. However, normal tissues need to be protected from the immediate and delayed effects of cytotoxic agents. A better understanding of the mechanisms that regulate cell death pathways might make it possible to improve the current therapeutic strategies. NADH is an essential component of enzymes for many metabolic reactions and energy production in cells. Our previous research confirmed that NADH had no significant effect on abnormal proliferation of cancer cells in vitro, suggesting that supplementary therapy of NADH for patients with cancer may not promote the growth of tumor. The results of electron microscopy and cell viability assessment proved that NADH could inhibit and rescue liver cells from the Cisplatin-induced apoptosis.

Mitochondria are presented as the important executioner of apoptosis. This crucial position of mitochondria in apoptosis control is proven by the results obtained from the changes of Mitochondrial membrane potential ($\Delta\psi m$) and reduced oxygen species (ROS) production [8]. The level of intracellular ROS is activated to commit cells to apoptosis. However, NADH could inhibit this kind of ROS signal. Bcl-2 is a membrane-associated protein that is largely found in the mitochondria of intact cells. More attention has been focused on its function as an opponent of apoptosis. In the group of Cisplatin, the expression of Bcl-2 gene was decreasing sharply after exposing cells to Cisplatin for 12h to 48h. The level of gene expression increased obviously in the group of NADH/Cisplatin compared with the group of Cisplatin. Reports that overexpression of Bcl-2 prevented the release of Cyto c into the cytosol provided an important insight into the possible anti-apoptotic mechanisms. Cyto c is on the outside of the inner mitochondrial membrane while Bcl-2 is associated with outer membrane, extending to the inner surface [9]. Anti-apoptotic members probably function as mitochondia membrane stabilizing or vesicle fusion moleculars. A cascade of Caspases, a family of cysteine proteases that cleave selected substrates at aspartic acid residues. Caspases activation is indispensable for apoptosis to occur. Caspases that are activated as a consequence of cell membrane signaling events can be classified as initiating or upstream Caspases, such as Caspases-8, and effector or downstream Caspases, such as Caspases-3[10]. Our results indicated that Caspase-3 activity rised quickly when cells were incubated with 1 μ m cisplatin especially after 24h. But when cells were cultured with NADH and then with Cisplatin, Caspase-3 activity kept in a low level. The alteration pattern of Caspase-8 appeared to be similar. Caspase-8[11] is a member of caspases family of cysteine proteases. Caspase-8 is activated by the signaling pathways for Fas and TNF, and is the most upstream caspase in the Fas apoptotic pathway. These data suggest the existence of mitochondria signaling pathway in which NADH acts as an anti-apoptosis agent.

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New Positioning System of 3-D Conformal Radiotherapy

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ABSTRACT Objective Through the new system (ExacTrac), we can determine isocenter more easily and improve the accuracy of repositioning. So it provides permanent position fiducial coordination and ensures repeated localization of treatment target for 3-dimentional Conformal Radiotherapy(3D-CRT). **Method** The ExacTrac system has been designed by BrainLab, Inc. to immobilize, reposition, and plan patients for 3D-CRT. It includes a series of reflective CT-markers, two IR cameras and localization software. The reflective CT-markers are placed on the relatively immobile parts of patient's body for automated repositioning. When CT scanning, patients with the markers are scanned. These markers are included in the initial planning CT scans and are easily visualized in the CT slices which intersect the individual marker .The markers are reproducible reference points .The BrainLab localization software automatically identifies these markers and records their positions relative to the CT coordinate system. Therefore, the relationship between CT coordinate system and the radiotherapy coordinate system is determined by the markers. The key point in the radiotherapy treatment coordinate system is the isocenter which is the point of intersection of the axis of rotation of the linear accelerator with the central axis of the radiation beam. The software can put the isocenter in the geometrical center of the target which is drawn by the physicists. Additionally, it can calculate the relative 3-D coordinate of the isocenter. With two infrared cameras, the system determines the coordinate of markers through stereo-photogrammetry. The system locates automatically the target relative to the markers at the isocenter of the linear accelerator. **Conclusion** By clinical test, the ExacTrac system repositions a target on a patient within 2 mm of it's initial position .If stationary markers are used, the system consistently repositions a target within 1mm of isocenter. The advantages of the system are atraumatic, accurate, convenient and automatic. It provides a new position technology for 3D-CRT.

KEY WORDS conformal radiotherapy, isocenter, reposition, marker