X-ray induced L02 cells damage rescued by new anti-oxidant NADH

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INTRODUCTION

Recent radiobiological studies have demonstrated that ionizing radiation can induce cell death. Exposure of cell to ionizing radiation over a wide dose range results in activation of cellular response pathways, including p53-dependent and p53-independent ways\(^1,2\). At the same time, apoptosis resulted from a coordinate sequence of biochemical events eventually leads to cell death. Among these, the generation of ROS with perturbation of prooxidant/antioxidant ratio, alterations in mitochondria structure and membrane potential have been investigated\(^3,4\). The stabilized electrochemical gradient relies on a functional ion exchange via the electrogenic transporter Na+/K+-ATPase. Na+/K+-ATPase is an energy hungry process which consumes a major of cellular ATP production. Therefore, there may be a decrease of ATP level when apoptosis starts within a few minutes after ionizing irradiation. NADH, an important coenzyme, participates in three carboxyl cycles and ultimately produces ATP molecules. We added NADH to L02 cells undergoing X-ray irradiation in vitro, and observed change of survivals and apoptosis as well as radiation associated proteins which take part in signal transduction of apoptosis.

MATERIALS AND METHODS

**Reagents**

NADH (purity: 97 %) was gifted by Professor Birkmay from Chemical Department of Graz University. Monoclonal mouse anti-p53, bcl-2, bax antibodies and rat anti-Fas, FasL antibodies were from Beijing Zhongshan Biotechnology Company. PI/Annexin V kits were purchased from Immunotech (France). High FITC-labeled goat anti-mouse antibodies were from Zhongshan Company. H2DCF probe was purchased from America Molecular Probe Company.

**Cell lines and culture**

Normal human liver cell line L02 was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 supplemented with streptomycin (50 U/ml), glutamate (2 mM) and 10 % fetal bovine serum.

**Induction of apoptosis**

The L02 cells were seeded in 6-well tissue cultured plates, the supernatant was discarded and 0.01 M PBS (pH 7.4) was added. The L02 cells were X-ray-irradiated with 2.5, 5.0 and 7.5 Gy. The cells were then cultured in a complete medium in the presence or absence of NADH at a concentration of 400 ug/ml, respectively. Non-irradiated culture served as control.

**MTT cell viability assay**

The cells were seeded into 96-well dishes (5×10\(^3\) cells/well), incubated for 24 h to allow attachment, treated with X-ray at 5.0 Gy, and continued to culture for 12, 24, 36, 48 h in the presence or absence of NADH. Absorbance was read at 570 nm by using DG3022 ELISA according to the routine MTT methods. The cellular viability was calculated as the amount of MTT uptake.

**Assessment of apoptotic cells**

The L02 cells were seeded at 5×10\(^4\)-5×10\(^5\)/ml in 6 well tissue cultured plates and cultured for 48 hours in RPMI-1640 medium containing 10 % FBS. Apoptosis was induced by X-ray irradiation, the L02 cells were continued to culture for 24 h. A total of 5×10\(^4\)-5×10\(^5\)/ml cells were collected by centrifugation at 200 g (≤5 min) and washed twice with ice-cold PBS (pH 7.4). Percent age of apoptosis was detected by flow cytometry according to PI/Annexin V kits.
Scanning electron microscope
24 hours following exposure to 2.5 Gy X-ray radiation, the L02 cells were fixed for 1 hour at room temperature using 4 % glutaraldehyde in PBS and proceeded for scanning electron microscopy as routine methods.

Protein expression of bcl-2, bax, fas, FasL and p53
The L02 cells were collected and then washed twice with ice-cold PBS, followed by fixation in 0.5 % paraformaldehyde at 4 °C for 30 min. The fixated cells were treated with PBS containing 0.1 % Triton-100 and washed twice. The treated cells of every tube were divided into five tubes and washed. The supernatant was aspirated. The antibodies against bcl-2, bax, p53, fas, FasL were added into each tube, mixed and incubated for 1 h at 37 °C. Then, the cells were washed twice with PBS and resuspended with 500 µl PBS. 10 000 events were analyzed and the positive rate of protein expression was detected by FCM.

Determination of intracellular ROS concentration
The cell suspension was dispensed into special culture plates at a density of 2×10⁴ cells per ml and incubated at 37 °C, 5 % CO₂ for 48 h. The supernatant was removed and replaced with Hank’s solution, then exposed to 2.5 Gy X-ray radiation. The supernatant was discarded at once, and replaced with RPMI-1640 medium with or without NADH at a concentration of 400 µg/ml for 4 h, respectively. Non-irradiated culture served as control, followed by washing three times with Hank’s solution. Measurement of intracellular ROS concentration was described in literature. Briefly, the cells were loaded with 0.5 ml H₂DCF in DMSO solution at 5 µg/ml and incubated at 37 °C for 30 min. After washed three times with PBS, 0.5 ml PBS was loaded and the change of intracellular ROS was detected by scanning fluorescence intensity under confocal microscope.

RESULTS
X-ray treatment inhibited growth of L02 cells
The L02 cells were treated with different doses of X-ray irradiation. Cell survival was determined after 12, 24, 36, 46 h. Inhibition of growth in X-ray treated cells occurred in a dose-dependent manner (Figure 1). Survival of L02 cells decreased as the dose of X-ray increased. It was most obvious at 24 h post-irradiation.

Figure 1 X-ray induced inhibition of growth of L02 cells.

NADH antagonized apoptosis of X-ray treated L02 cells
The L02 cells were treated with 2.5, 5.0, 7.5 Gy X-ray irradiation, then post-incubated in fresh complete RPMI 1640 medium containing NADH or NADH free drug for 24 h. Percent age of apoptosis was determined by FCM using PI/Annexin V stain method. The results showed that NADH diminished apoptosis of L02 cells exposed to X-rays. The percent age of apoptosis was (7.08±2.34) %, (28.16±2.46) %, (47.30±3.43) % in the absence of NADH. However, it was (6.04±0.86) %, (8.25±1.64) %, (15.30±1.98) % in the presence of NADH. The difference was significant between L02 cells treated with 5.0 Gy, 7.5 Gy X rays and cultured for 24 h in the presence of NADH and L02 cells cultured in the absence of NADH (P<0.05). These findings suggested that NADH was involved in cytoprotection by blocking the induction of apoptosis.

NADH rescued L02 cells damage from X-ray radiation
X-ray radiation could induce L02 cells damage. As shown in Figure 2, Part(b) and part(c) represented different morphologic changes of 2.5 Gy X-ray radiated L02 cells in the absence or presence of NADH. Part(a) represented the shamly irradiated L02 cells, which had normal liver cell surface structure with normal protuberance and volume. But, Part(b) had decreased protuberance and atrophy. However, degree of damage in L02 cells of part(c) group was becoming less than that of part(b). These suggested that NADH could rescue L02 cells damage from X-ray irradiation.

Expression of p53, bax, bcl-2, Fas and Fas-L in L02 cells
The results of FCM analysis for p53, bax, bcl-2, Fas and Fas-L protein expression in X-ray irradiated and mockly irradiated
L02 cells are summarized in Table 1. Significantly high levels of p53, bax, Fas and Fas-L protein expression were detected in cells irradiated and cultured in the absence of NADH than in those cells cultured in the presence of NADH and mockly irradiated, but expression of bcl-2 protein tended to be low in L02 cells. Our results showed that NADH up-regulated expression of bcl-2 protein and down-regulated expression of p53, bax, bcl-2, Fas and FasL protein in L02 cells undergoing X-ray irradiation. It might be one of the mechanisms that NADH rescues L02 cells injury from ionizing irradiation.

Table 1  Effect of NADH on regulation of apoptosis associated proteins in L02 cells treated with X-ray (n=3, x±s)

| Group          | p53        | Fas        | Fas-L      | bcl-2       | bax        |
|----------------|------------|------------|------------|-------------|------------|
| Mock IR        | 22.40±0.91 | 7.01±0.21  | 66.66±1.60 | 5.27±0.12   | 74.71±1.81 |
| IR             | 37.4±1.11  | 13.40±0.78 | 74.40±1.09 | 2.22±0.18   | 86.76±2.14 |
| IR+NADH        | 26.93±6.73 | 11.29±1.40 | 68.93±1.88 | 3.62±1.34   | 71.60±2.92 |

Based on t test, “Mock IR” represented L02 cells of non-irradiated group. “IR+NADH” represented L02 cells irradiated and continued to culture in the presence of NADH. *P<0.05 vs Mock IR group and IR+NADH group.

**DISCUSSION**

When a cell exposed to ionizing irradiation, at least two signal-generating targets are activated; one at the membrane and the other at the DNA. Signal may also originate in cytoplasm[6,7]. These signal targets ultimately result in cell death or non-death stress response. Apoptosis, also called programmed cell death (PCD), is a peculiar form of cell death characterized by several morphological and biochemical aspects which are different from necrosis, an other form of death. X-ray irradiation is one of the ionizing radiations, which can cause both membrane and DNA damage to cells and result in cell apoptosis. How ionizing radiation triggers apoptosis is not known. It was reported that apoptosis mediated by DNA damage occurred via p53-dependent and p53-independent pathways[1,2]. However, several pathways of apoptosis have been reported. One of these is the Fas/FasL pathway, which involves binding of a death receptor to a death domain, initiating a cascade of proteases that leads to cell apoptosis[8]. In the present study, we tested whether apoptosis induced by X-ray irradiation...
occurred via DNA damage or Fas/FasL pathway. Our results demonstrated that X-ray irradiation led to cell apoptosis by increasing positive rate of L02 cells expressing p53 and bax proteins, and decreasing positive rate of bcl-2 protein. At the same time, Fas and FasL expression in L02 cells exposed to irradiation was up-regulated as compared with that in mockly irradiated-cells.

Cellular sensitivity to radiation reflects a culmination of distinct molecular pathway including DNA repair, cell cycle checkpoint fidelity, and particularly apoptosis. Several oncogenes and tumor suppressor genes play a pivotal role in modulating the response of cells to radiation. An important molecule, p53, initiates responses to DNA damage, and affects the sensitivity of cells to apoptosis. Functional inactivation of p53 is associated with resistance to radiotherapy. Overexpression of wt-p53 gene was found to be associated with increased cellular sensitivity to apoptosis induced by ionizing irradiation[9,10]. Bcl-2, an important regulator of apoptosis, was found to be associated with anti-apoptosis response. Over expression of bcl-2 by transferring bcl-2 gene into deficient cells has been associated with increased cellular resistance to induction of apoptosis by a variety of DNA-damaging agents including ionizing irradiation, drugs. However, bax, another member of bcl-2 family, as an inhibitor of apoptosis, can bind bcl-2 to form homo- and heterodimers. Rate of bcl-2 to bax may determine the extent to which apoptosis is induced or suppressed[11,12]. At the same time, high expression of wild-type p53 protein induced by ionizing irradiation appears to regulate expression of bcl-2, bax, p21 and p16 genes[13]. Our results showed that X-ray radiation induced DNA damage resulted in an increase of positivity of p53 and bax protein expression, and decrease of bcl-2 protein expression in L02 cells. But it is necessary to further confirm whether p53 may regulate expression of bcl-2 and bax genes.

Recently, it has been suggested that Fas/FasL system plays a key role in the regulation of apoptosis. Fas is located on the plasma membrane of hepatocytes abundantly, and FasL, a glycoprotein of 40 kd is located on the plasma membrane of the lymphocytes. The interaction of the Fas ligand and Fas receptor initiates a chemical process in the cells that leads to apoptosis. Fas, named CD95, expresses in alcoholic liver disease, viral hepatitis, hepatocarcinomas, and hepatocirrhosis[14-16]. Fas-FasL interaction may contribute to hepatocyte apoptosis in these patients. It was rarely reported that apoptosis induced by ionizing irradiation occurred via Fas-FasL interaction pathway, particularly apoptosis induced by Fas-FasL interaction itself in hepatocytes. It was recently reported that the Fas/Fas ligand system was involved in modulating keratocyte apoptosis induced by UV irradiation[17]. Newton reported that apoptosis induced by ionizing radiation required p53 and was regulated by the Bcl-2 protein family but did not require signals transduced by Fas and FADD/MORT1[18]. Our experiment indicated that X-ray irradiation enhanced the positive rate of L02 cells expressing Fas and FasL protein as compared with shamly irradiated cells. It is suggested that Fas-Fas interaction play an important role in liver cell apoptosis induced by X-ray irradiation. It is different from the model in which ionizing irradiation triggers apoptosis via p53-dependent activation of caspase-9.

Several biochemical alterations, including excessive generation of reactive oxygen species (ROS), calcium flux, Caspase activation, have been shown to be essential in cell apoptosis[19-21]. ROS plays a pivotal role in ionizing radiation-induced cell apoptosis[22-24]. ROS, as a signal molecule, could regulate gene expression and mitochondria membrane potential[25,26]. Free radicals are an integral part of metabolism and formed continuously in the body. Many sources of stress heat, irradiation, hyperoxia, inflammation and any increases in metabolism including exercise, injury, and even repair processes lead to increased production of free radicals and associated reactive oxygen or nitrogen species (ROS/RNS)[27-29]. Evidences have shown that free radicals have important functions in the signal network of cells, including induction of growth and apoptosis and as killing tools of immunocompetent cells[30,31]. Massive intervention into the redox state by pharmaceutical doses of exogenous antioxidants should be considered with caution due to the ambiguous role of free radicals in regulation of growth, apoptosis, and cytotoxicity of immunocompetent cells. Our results showed that X-ray irradiation could give rise to increases of ROS generation after 4 h irradiation, but NADH could reverse the effect of X-ray irradiation, lower the level of intracellular ROS. These results indicated that NADH rescued L02 cells damage from X-ray irradiation by regulation of ROS generation.

NADH, a kind of important coenzyme, takes part in triggering biological anti-oxidation and regulating the expression of membrane glycoprotein receptors[32]. But it was seldom reported that NADH played a role in antagonizing ionizing radiation induced apoptosis and regulating expression of membrane receptors. Recently, it was reported that the content of intracellular NADH changed after UV or ionizing irradiation[33,34]. Most of the results indicated that the content of intracellular NADH declined after ionizing irradiation or PDT treatment by confocal microscope scanning analysis. Pogue reported that the endogenous fluorescence signal attributable to reduced nicotinamide adenine dinucleotide (NADH) was measured in response to photodynamic therapy (PDT)-induced damage. Measurements on cells in vitro have showed that NADH fluorescence decreased relatively to that of controls after treatment with a toxic dose of PDT, as measured within 30 min after treatment. Similarly, assays of cell viability indicated that mitochondria function was reduced immediately after treatment in proportion to the dose delivered[35]. It was seldom reported that signal transduction molecules changed after ionizing irradiation by extraneously added NADH. Recent studies of repairing ionizing irradiation injury focused on antioxidant drugs. Bush reported that NAC could protect immune function and regulate expression of oncogenes in bone marrow cells exposed to ionizing irradiation[36]. Zhou found that bilobalide might block PC12 cells from reactive oxygen species-induced apoptosis in the early stage and then attenuate the elevation of c-Myc, p53, and Bax and activation of Caspase-3[37]. Our results indicated that expression of p53, bax, Fas and FasL proteins was up-regulated and expression of bcl-2 protein was down-regulated in L02 cells undergoing X-ray irradiation. However, when L02 cells undergoing X-ray irradiation continued to culture in the presence of NADH, expression of p53, bax, Fas and FasL proteins were down-regulated and expression of bcl-2 protein was up-regulated. At the same time, level of intracellular ROS declined, survival of cell increased. Our observations provide evidence that NADH is a new kind of radiation protector.

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