Apoptosis induced by nucleosides in the human hepatoma HepG2

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AIM: To investigate the apoptotic effects of nucleosides on the human hepatoma HepG2.

METHODS: The nucleosides included inosine (I), cytidine (C), uridine (U), thymidine (T), adenosine (A), and guanosine (G). Cells were incubated by the mediums with or without nucleosides at 37 °C in a 50 mL/L CO₂ humidified atmosphere.

RESULTS: It was found that the cell viabilities were significantly decreased, when cells were treated with 30 mmol/L I, 30 mmol/L C, 30 mmol/L U, 30 mmol/L T, 0.5 mmol/L A, and 0.5 mmol/L G after 12 h incubation (P<0.05). About the apoptotic phenomenon, the cell percentages of sub-G₀ cells were significantly increased in the mediums containing nucleosides such as C, U, T, A, and G (P<0.05). Furthermore, the caspase-3 activity was increased, when the cells were incubated with T (P<0.05). The protein expressions of p53 and p21 showed no difference in each group. To investigate the mechanism of apoptosis induced by nucleosides, it was found that the contents of soluble Fas ligand contents were increased in HepG2 cells following I, U, T, and A treatment (P<0.05). But, TNF-α and cytochrome c were undetectable.

CONCLUSION: Thymidine may induce the apoptosis in HepG2, but the effective dosages and reactive time must be investigated in the future study. However, the apoptosis-inducing abilities of other nucleosides were still unclear in this study.

INTRODUCTION

According to the statistics tabulated by the Department of Health, Taiwan in 2003, cancer is the first leading cause of death in Taiwan. Among the various cancers, the morbidity of hepatoma is the highest in Taiwanese. The great medical expenses covering not only drugs but also in providing nutritional supplements for treating or preventing the liver diseases and hepatoma are the heavy burdens of Taiwanese. Food-derived inducers of apoptosis may be significant as exogenous anti-tumor substances in the control of malignant cell proliferation despite little understanding about their molecular and cellular basis of action. Many healthy foods that are rich in nucleosides have anti-carcinogenic effects, such as the hot water extract of chlorella[7-9]. Current evidence suggests that nucleosides induce cell death by apoptosis[10]. Nucleosides that act as signaling molecules are well documented and are mostly released from cells, when cells are under stress, such as anoxia or injury[7-9]. In addition, it has been presumed that a vast majority of tumor promoters are potent inhibitors of apoptosis, and cancer cells did not produce apoptosis-inducing nucleosides[10]. However, little is known about the signaling or the biochemical mechanisms of nucleosides-mediated cell death.

Thus, the purpose of this study was to investigate the apoptosis-inducing ability and the molecular mechanisms of nucleosides in human hepatoma HepG2.

MATERIALS AND METHODS

Cell culture and treatment

The human hepatoblastoma cell line, HepG2 (HB-8065), was obtained from the American Type Culture Collection. Cells were cultured in MEM supplemented with 5% FBS. Cells were grown in a humidified incubator under 50 mL/L CO₂ at 37 ℃ and medium was changed every 2 d. When the cell status was stable after several passages, the experiments started. Cells were seeded in 100-mm dishes at a density of 1×10⁶ cells/well. They were incubated with defined concentrations of nucleosides for 12 h. The cells and media were collected and analyzed after incubation. The nucleosides included inosine (I), cytidine (C), uridine (U), thymidine (T), adenosine (A), and guanosine(G).

Cell viability assay

The cell viability was determined using CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison,
Flow cytometric analysis
Cells were harvested by 0.25% trypsin release, washed twice with PBS, permeated with 75% ethanol, treated with 3 µL RNase A and finally stained with 1 mL propidium iodide (40 µg/mL final concentration). Distribution of cell-cycle phases with different DNA contents was determined using FACScan flow cytometer (Becton Dickenson, San Jose, CA, USA). Cells that were less intensely stained than G1 cells (sub-G1 cells) in flow cytometric histograms were considered as apoptotic cells.

Caspase-3 activity and soluble Fas ligand content
After treatment for 12 h, cells were collected and lysed with 50 µL cell lysis buffer. Then, the cell lysate was centrifuged and the supernatants were retained to determine the caspase-3 activity with Caspase-3/CPP32 Colorimetric Kit (Biosource International, Camarillo, CA, USA). Furthermore, the soluble Fas ligand contents (sFas-L) in the medium were detected with Human sFas Ligand ELISA Kit (Biosource International, Camarillo, CA, USA).

Protein extraction and Western blot assay
Cell proteins were extracted from cells in a buffer containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 4% protease inhibitors, and 1% phosphatase inhibitors. The cell lysate was rotated at 4 °C for 30 min, centrifuged at 10 000 r/min for 10 min and the precipitates were discarded. Protein contents were determined by the DC Bio-Rad protein assay kit (Biosource International). Cell proteins were added to an equal volume of 2× sample buffer containing 0.125 mol/L Trizma base, 4% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol for 10 min and the precipitates were discarded. Protein contents were determined by the DC Bio-Rad protein assay kit using bovine serum albumin as a standard. The cell lysates were separated by 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell BioScience, Keene, NH, USA), which was then incubated with primary antibody, such as p53 mouse monoclonal IgG (a) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and F-5 mouse monoclonal IgG(b) (Santa Cruz Biotechnology). HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was used as a secondary antibody. The immunoreactive bands were washed four times in PBS-T (PBS in 0.2% Tween 20), dried and exposed to Kodak film. The relative intensity of the bands was analyzed by densitometry.

Statistical analysis
All data are expressed as the mean±SD. One-way analysis of variance and Fisher’s least significant difference test were used to compare the differences of means using the SAS software (v.6.12, SAS Institute, Cary, NC, USA). Statistical significance was assigned at the 0.05 level.

RESULTS
Cell viability assay
The cell viabilities were significantly decreased when cells were treated with defined concentrations of nucleosides after 12-h incubation (Table 1). The most effective dosages were 30 mmol/L for I, C, U, and T, 0.5 mmol/L for A and G. Thus, these effective concentrations were used in later experiments.

Flow cytometric analysis
Figure 1 is the example of flow cytometer graph from all results in each group. The quantity of various phase percentages is shown in Table 2. About the apoptotic phenomenon, the cell percentages of sub-G1 phase were significantly increased in the media containing nucleosides such as C, U, T, A, and G (Table 2). In particular, thymidine induced the highest cell percentages of sub-G1 phase among the nucleosides. The cell-cycle progression of I, A, and G groups was similar to that of control group. Moreover, C and U treatments caused an increase in the cell numbers in the G0/G1 phase of the cell cycle. Certainly, the cell numbers of S phase in C and U groups were significantly decreased.

Caspase-3 activity and soluble Fas ligand content
Caspase-3 activity was increased when the cells were incubated with T (Table 2). To investigate the mechanism of apoptosis induced by nucleosides, it was found that the contents of sFas-L contents were increased in HepG2 cells following I, U, T, and A treatment (Table 2). But, TNF-α and cytochrome c were undetectable (data not shown).

Western blot assay
HepG2 is the wild type hepatoma with p53 gene. The expressions of p53 and p21 showed no difference in each group (Figure 1). The densitometric result was also unchanged (Figure 2).

Table 1 Effects of nucleosides on HepG2 cell viability by MTS assay

| Treatment | Inosine (%) | Cytidine (%) | Uridine (%) | Thymidine (%) | Adenosine(%) | Guanosine(%) |
|-----------|-------------|--------------|-------------|---------------|--------------|--------------|
| 0 mmol/L  | 103.9±3.5 a | 101.8±1.6 a  | 103.5±3.5 a | 102.2±1.9 a   | 101.3±1.2 a  | 103.1±2.7 a  |
| 7.5 mmol/L| 94.0±1.2 a  | 92.0±3.8 b   | 100.6±6.6 a | 101.5±5.1 a   | 99.0±0.3 a   | 101.8±2.7 a  |
| 15 mmol/L | 69.5±1.8 a  | 75.6±1.7 a   | 99.1±2.8 a  | 99.0±0.3 a    | 101.3±1.2 a  | 100.6±2.7 a  |
| 30 mmol/L | 67.4±0.7 a  | 61.4±5.9 a   | 86.1±1.3 a  | 84.5±3.2 a    | 97.0±2.0 a   | 98.5±2.7 a   |
| 0.1 mmol/L|             |              |             |               |             |              |
| 0.3 mmol/L|             |              |             |               |             |              |
| 0.5 mmol/L|             |              |             |               |             |              |

aCells were incubated with different treatments for 12 h. Cell viability was measured with MTS assay kits. Values are expressed as the mean±SD of six independent experiments. *P<0.05 vs 0 mmol/L.
However, it has been reported that 1 mmol/L I, C, U, and T did not increase the caspase-3 activity and sFas-L contents, whereas the contents of adenosine and guanosine were 0.5 mmol/L. Values are expressed as the mean±SD of four independent experiments. And, the example from all results in each group. M1, M2, M3, and M4 represented Sub G1, G0/G1, S, and G2/M phases, respectively (A-G).

Figure 1 Effects of nucleosides on the cell cycle progression of HepG2 cells. HepG2 cells untreated (control) or treated with nucleosides were stained with propidium iodide and analyzed using a flow cytometer. The contents of inosine, cytidine, uridine, and thymidine in mediums were 30 mmol/L, respectively. $\beta$-actin protein was also detected, respectively. This experiment was repeated thrice and obtained similar results were obtained. Figure 1 was the example from all results in each group. M1, M2, M3, and M4 represented Sub G1, G0/G1, S, and G2/M phases, respectively (A-G).

DISCUSSION

The cytotoxicity of nucleosides at defined concentrations in human hepatoma HepG2 cells was proved in this study. However, it has been reported that 1 mmol/L I, C, U, and T did not inhibit the proliferation of HL-60 and Caco-2 cells[12]. In addition, the inhibition effects were observed after 1 mmol/L A and G treatment[13]. Thus, it was speculated that the different cell type of tumor might lead to completely opposite influences.

The cell percentages of sub-G1 phase were increased after C, U, T, A, and G treatment. However, the variation of sub-G1 phase in C, U, A, and G groups was weaker than other apoptosis-related researches[13,14]. More apoptotic indicators or treatment dosages of nucleosides must be investigated further.

Thymidine increased the caspase-3 activity in HepG2 cells. Moreover, the sFas-L was also increased when cells were cultured with thymidine. However, these effects were not extremely obvious. These results indicated that thymidine might have the potential to induce the apoptosis, but the effective dosage and reactive time must be investigated further.

The cell percentages of sub-G1 phase and caspase-3 activities were still unchanged despite sFas contents were higher after inosine and uridine treatments. Similarly, cytidine did not increase the caspase-3 activity and sFas-L contents.

Table 2 Effects of nucleosides on the cell cycle progression, caspase-3 activity and sFas-L content in HepG2 cells

| Treatment | Control | Inosine | Cytidine | Uridine | Thymidine | Adenosine | Guanosine |
|-----------|---------|---------|----------|---------|-----------|-----------|-----------|
| SubG1 (%) | 2.6±0.8 | 3.2±0.3 | 6.9±0.2 | 6.5±1.5 | 26.8±2.3 | 9.6±1.5 | 5.7±1.2 |
| G0/G1 (%) | 67.7±1.1 | 69.2±0.1 | 74.1±1.3 | 84.8±2.6 | 54.4±1.8 | 64.8±1.4 | 68.0±1.9 |
| S (%)     | 15.7±0.4 | 18.8±0.3 | 8.5±0.2 | 3.1±0.3 | 12.0±0.2 | 10.6±0.4 | 17.3±0.7 |
| G2/M (%)  | 14.0±1.9 | 8.8±0.1 | 10.5±1.0 | 5.7±0.8 | 6.8±0.8 | 15.0±0.9 | 9.0±1.3 |
| Caspase-3 (%) | 100±4 | 101±5 | 97±6 | 98±5 | 148±2 | 104±4 | 100±3 |
| sFas-L (pg/mL) | 0.11±0.01 | 0.14±0.01 | 0.08±0.03 | 0.15±0.03 | 0.17±0.02 | 0.15±0.07 | 0.10±0.03 |

1Cells were treated with or without nucleosides for 12 h. The contents of inosine, cytidine, uridine, and thymidine in the mediums were 30 mmol/L, respectively, and, the contents of adenosine and guanosine were 0.5 mmol/L. Values are expressed as the mean±SD of four independent experiments. *P<0.05 vs others.

2Caspase-3 activity was shown as percentage to compare with control group.
but raised the cell percentages of sub-G 
phase. Insufficient data indicated the apoptosis-inducing action of inosine, uridine, and cytidine. There are two possibilities, which can explain these results. One is that the apoptosis induced by inosine, uridine, and cytidine might occur later than 12-h incubation. The other is that the cell death induced by inosine, uridine, and cytidine, which showed in MTS assays, was necrosis not apoptosis. Besides, the cell cycle progressions of uridine and cytidine were different from those of other nucleosides. It was observed that uridine and cytidine induced Go/G1 phase arrest in HepG2 cells. This effect may be associated with the growth inhibitory action of uridine and cytidine.

Chow et al. indicated that adenosine induced apoptosis of HL-60 cells by activating the G protein and increasing the cytosolic Ca++ concentration when adenosine combined with its receptors, P1[15,16]. Moreover, guanosine appears to improve the release of adenosine from cell, and induce cell death via the action of adenosine[17]. But, in this study, the apoptotic-related signals, such as sFas-L content and caspase-3 activity, were not altered in cells after adenosine treatments. Thus, the treatment concentration of uridine and cytidine might occur later than 12-h incubation to explain these results. One is that the apoptosis induced by thymidine might have the potential to induce the apoptosis; however, the apoptosis-induced abilities of other nucleosides were still unclear in this study. It is necessary to recheck the dosage and time of treatment and measure more apoptotic indicators, such as DNA fragmentation and morphological analysis.

REFERENCES

1 Tanaka K, Konishi F, Himeno K, Taniguchi K, Nomoto K. Augmentation of antitumor resistance by a strain of unicellular green algae, Chlorella vulgaris. Cancer Immunol Immunother 1984; 17: 90-94
2 Tanaka K, Tomita Y, Tsuruta M, Konishi F, Okuda M, Himeno K, Nomoto K. Oral administration of Chlorella vulgaris augments concomitant antitumor immunity. Immunopharmacol Immunotoxicol 1990; 12: 277-291
3 Noda K, Ohno N, Tanaka K, Kamiya N, Okuda M, Yadomae T, Nomoto K, Shoyama Y. A water-soluble antitumor glycoprotein from chlorella vulgaris. Planta Med 1996; 62: 425-426
4 Van Buren CT, Rudolph F. Dietary nucleotides: a conditional requirement. Nutrition 1997; 13: 470-472
5 Chow SC, Kass GE, Orrenius S. Purines and their roles in apoptosis. Neuropharmacology 1997; 36: 1149-1156
6 Ockner RK. Apoptosis and liver diseases: recent concepts of mechanism and significance. J Gastroenterol Hepatol 2001; 16: 248-260
7 Collins MG, Hourani SM. Adenosine receptor subtypes. Trends Pharmacol Sci 1993; 14: 360-366
8 Fishman P, Bar-Yehuda S, Ohana G, Pathak S, Wasserman L, Barer F, Multani AS. Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor. Eur J Cancer 2000; 36: 1452-1458
9 Wu JM, Lin JS, Xie N, Liang KH. Inhibition of hepatitis B virus by a novel L-nucleoside, beta-L-D4A and related analogues. World J Gastroenterol 2003; 9: 1840-1843
10 Wright SC, Zhong J, Larrick JW. Inhibition of apoptosis as a mechanism of tumor promotion. FASEB J 1994; 8: 654-660
11 Kwon KB, Kim EK, Lim JG, Joong ES, Shin BC, Jeon YS, Kim KS, Seo EA, Ryu DG. Molecular mechanisms of apoptosis induced by Scorpio water extract in human hepatoma HepG2 cells. World J Gastroenterol 2005; 11: 943-947
12 Meisel H, Günther S, Martin D, Schlimme E. Apoptosis induced by modified ribonucleosides in human cell culture systems. FEBS Lett 1998; 433: 265-268
13 Gali-Muhtasib HU, Abou Kheir WG, Kheir LA, Darwiche N, Crooks PA. Molecular pathway for thymoquinone-induced cell-cycle arrest and apoptosis in neoplastic keratinocytes. Anticancer Drugs 2004; 15: 389-399
14 Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997; 275: 1132-1136
15 Chow SC, Kass GEN, Orrenius S. Purines and their roles in apoptosis. Neuropharmacology 1997; 36: 1149-1156
16 Schrier SM, Tilburg EWP, Meulen HVD, Ijzerman AP, Mulder GJ, Nagelkerke. Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells studies on involvement of adenosine receptors and adenosine uptake. Biochem Pharmacol 2001; 61: 417-425
17 Soussi T. The p53 tumour suppressor gene: a model for molecular epidemiology of human cancer. Mol Med Today 1996; 2: 32-37
18 Miyashita T, Krajewska S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 1994; 9: 1799-1805
19 Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D. Immediate early up-regulation of bax expression by p53 but not TGF β 1a in a paradigm for distinct apoptotic pathways. Oncogene 1994; 9: 1791-1798
20 Kagawa S, Fujiwara T, Hizuta A, Yasuda T, Zhang WW, Roth JA, Tanaka N. p53 expression overcomes p21WAF1/ CIP1-mediated G1 arrest and induces apoptosis in human cancer cells. Oncogene 1997; 15: 1903-1909
21 Vogelstein B, Kinzler KW. p53 function and dysfunction. Cell 1992; 70: 523-526
22 Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21 waf/cipl. Gene Dev 1994; 8: 2817-2830

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