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Synergism between heparin and adriamycin on cell proliferation and apoptosis in human nasopharyngeal carcinoma CNE2 cells[1]

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KEY WORDS heparin; flow cytometry; electrophoresis; apoptosis; doxorubicin

ABSTRACT

AIM: To measure the effect of addition of heparin to adriamycin (ADM) on cell proliferation and apoptosis in CNE2 cells and investigate the possible molecular mechanisms of heparin and ADM interactions. **METHODS**: Cell viability and cell cycle were determined by MTT assay and flow cytometry. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and agarose gel electrophoresis. The expression of Bax, Bcl-2, p53, and p21 was examined by Western blot. **RESULTS**: ADM (5 mg/L) alone inhibited the growth of CNE2 cells, which was magnified when heparin was added. ADM elicited typical apoptotic morphologic changes. Compared with ADM or heparin alone, ADM plus heparin obviously enhanced the number of TUNEL-positive cells from 12.6 % ± 1.1 % to 65.7 % ± 1.3 %, and the DNA ladder was more clearly observed. After exposure to different concentrations of heparin (with or without ADM) for 24 h, CNE2 cells were accumulated in G_0/G_1 phase. There was a decrease in the number of cells in S phase by the combined heparin and ADM treatment compared to heparin or ADM alone. The ratio of Bax/Bcl-2 was elevated, and p53 and p21 mRNA were over-expression. **CONCLUSION**: Heparin and ADM appear to interact in a synergistic manner, which may be related to the over-expression of p53 and p21 mRNA and the elevated ratio of Bax/Bcl-2 mRNA.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant disease of the head/neck region and endemic in Southeast Asia and Southern China, especially in Guangdong province, mainly affecting young patients in their 40's. The molecular events leading to the development of the tumor are still not well understood. Similar to other human cancers, development of NPC is likely to involve multiple genetic events including activation of oncogenes and inactivation of tumor suppressor genes. Recent studies showed that the over-expression of p53, p16, Rb2, and Bcl-2 proteins had a high percentage in NPC cases[1,2]. However, we still do not know which is the key gene(s) responsible for this geographically/ethnically specific malignancy.

The aminoglycoside antibiotic adriamycin (ADM) is widely used in antineoplastic chemotherapy. ADM is frequently used for the treatment of cancers via intravesical or intra-arterial delivery as a monotherapy, or in combination with other chemotherapeutic agents intravenously[3]. It causes DNA damage and induces apoptosis in some cancers. Heparin is a polysulfated glycosaminoglycan with a high negative charge. The antiproliferative effect of heparin has been studied in hepatoma cells and smooth muscle cells (SMC)[4]. Heparin binds tightly to specific SMC surface receptors, accumulates within the cell, and blocks a critical point in the G_0/G_1 to S transition of the cells. Some reports suggested that heparin might facilitate apoptosis in human peripheral blood neutrophils[5], lymphoblasts, and mononuclear cells[6]. Others, however, suggested that heparin inhibited apoptosis in cultured mesangial cells and explanted glomeruli[7]. In previous studies, we have found that heparin induced apoptosis in CNE2 cells, which was associated with the inhibition of PKC (data not shown). The aim of the present work was to measure the effect of addition of heparin to ADM on cell proliferation and apoptosis in CNE2 cells, and further investigate its possible molecular mechanisms.

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MATERIALS AND METHODS

Drugs and reagents Heparin and adriamycin, RPMI-1640 medium, RNase A, agarose gel, sodium dodecyl sulfate (SDS), propidium iodide (PI), and proteinase K were purchased from Sigma Chemical Co (St Louis, MO, USA). Anti-Bcl-2, Bax, p53, p21, and β-actin antibody were purchased from Santa Cruz. CNE2 cell line was provided by Cancer Institute, Sun-Yet Sen University of Medical Sciences (China).

Cell culture The CNE2 cell line was cultured in RPMI-1640 medium, containing 10 % fresh bovine serum, benzylpenicillin (100 kU/L), and kanamycin (0.1 g/L) at 37 ℃ in 5 % CO_2 – 95 % air atmosphere.

MTT assay Cell growth was measured by a modified MTT assay. Cells about 1×10^5 per well were plated in 96-well microtiter plates and incubated overnight. Cells were then treated with various concentrations of heparin and/or ADM for 48 h. Then stock MTT 10 μL (0.5 g/L) was added to each well, and the cells were further incubated at 37 ℃ for 4 h. The supernatant was removed, and 100 μL of HCl 0.04 mol/L in isopropanol was added to each well to solubilize the formazan production. The absorbance at wavelength of 570 nm was measured by a microELISA reader (Sigma), the negative control well contained medium only. The ratios of the absorbance of treated cells relative to those of the control wells were calculated and expressed as percentage of growth inhibition.

TUNEL assay TUNEL assay was performed using the apoptosis detection system. Cells were fixed by 4 % paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 ℃. The samples were washed three times with PBS and were permeabilized by 0.2 % Triton X-100 in PBS for 15 min on ice. After washing twice, cells were equilibrated at room temperature for 15 to 30 min in equilibration buffer (potassium cacodylate 200 mmol/L, dithiothreitol 0.2 mmol/L, bovine serum albumin 0.25 g/L, and cobalt chloride 2.5 mmol/L in Tris·HCl 25 mmol/L, pH 6.6) and then incubated in the presence of pluorescein-12-dUTP 5 μmol/L, dATP 10 μmol/L, edetic acid 100 μmol/L, and terminal deoxynucleotidyl transferase at 37 ℃ for 1.5 h in dark. The tailing reaction was terminated by 2 × standard saline citrate (SSC). The samples were washed three times with PBS and were analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

Ladder detection assay After induction of apoptosis, cells (5×10^6 per sample, both attached and detached cells) were lyzed with 150 μL hypotonic lysis buffer(edetic acid 10 mmol/L, 0.5 % Triton X-100 Tris·HCl, pH 7.4) for 15 min on ice and were precipitated with 2.5 % polyethylene glycol and NaCl 1 mol/L for 15 min at 4 %. After centrifugation at 16 000 × *g* for 10 min at room temperature, the supernatant was incubated in the presence of proteinase K (0.3 g/L) at 37 ℃ for 1 h and precipetated with isopropanol at −20 ℃. After centrifugation, each pellet was dissolved in 10 μL of Tris-edetic acid (pH 7.6) and electrophoresed on a 1.5 % agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Cell cycle phase distribution The proportion of cells in G_0/G_1, S, and G_2/M phases of cell cycle was determined by flow cytometric analysis of DNA content. Cells were treated with ADM and/or heparin for 24 h. DNA content was then determined after labeling cells with propidium iodide. Cell suspension was prepared as before, fixed in ice-cold 70 % ethanol in PBS, and stored at −20 ℃. Prior to analysis, the cells were washed and resuspended in PBS and incubated with RNase I 1 g/L and propidium iodide 20 mg/L at 37 ℃ for 30 min. The analysis of samples was performed by a flow cytometer.

Western blot analysis The cells were lysed in lysis buffer [hepes 25 mmol/L, 1.5 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, NaCl 0.5 mol/L, edetic acid 5 mmol/L, NaF 50 mmol/L, sodium vanadate 0.1 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L, and leupeptin 0.1 g/L (pH 7.8)] at 4 ℃ with sonication. The lysates were centrifuged at 15 000 × *g* for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (Tris·HCl 42 mmol/L, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol, and 0.002 % bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamidel gel. Proteins were transferred to nitrocellulose and incubated sequentially with anti-Bcl-2, Bax, p53, p21 antibody, and then with peroxidase-conjugated secondary antibodies in the second reaction. Detection was performed with enhanced chemiluminescence reagent.

Statistical analysis The data shown were mean values of at least three independent experiments and expressed as $\bar{x} \pm s$. Student's *t*-test was used to

compare data. $P < 0.05$ was considered statistically significant.

RESULTS

MTT assay Compared with control, the growth rate was significantly inhibited after treatment with heparin (20 kU/L and 40 kU/L). ADM 5 mg/L also markedly inhibited the growth of CNE2 cells. The combination of heparin 20 kU/L and ADM 5 mg/L showed greater inhibition than ADM or heparin 40 kU/L alone (Tab 1).

Tab 1. Effect of heparin and ADM on cell proliferation in CNE2 cells. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* heparin + ADM.

Treatment	$A_{570\ nm}$	Inhibitory rate/%
Control	0.96 ± 0.03	–
ADM 5 mg/L	0.43 ± 0.04^{cf}	59.4 ± 0.7
Heparin 20 kU/L	0.891 ± 0.023^{bf}	6.0 ± 0.3
Heparin 40 kU/L	0.663 ± 0.021^{cf}	32.4 ± 2.2
Heparin 20 kU/L + ADM 5 mg/L	0.15 ± 0.03^{c}	84 ± 3

TUNEL assay TUNEL analysis was performed after CNE2 cells treated with various concentrations of heparin and/or ADM; ADM 5 mg/L or heparin alone weakly induced apoptosis, but ADM 5 mg/L plus heparin (20 kU/L) dramatically increased the number of TUNEL-positive cells, the percentage of apoptosis was enhanced from 12.6 % ± 1.1 % to 65.7 % ± 1.3 % (Fig 1).

Ladder detection assay Agarose gel electrophoresis exhibited DNA ladder formation in CNE2 cells after exposure to different concentrations of heparin (with or without ADM) for 48 h. Compared with ADM or heparin alone, the DNA ladder was more clearly observed by the treatment with heparin (20 kU/L) plus ADM (5 mg/L) (Fig 2).

Cell cycle phase distribution After exposure to different concentrations of heparin (with or without ADM) for 24 h, CNE2 cells were accumulated in G_0/G_1 phase. There was a significant decrease in the number of cells in S phase by the combined heparin and ADM treatment compared to heparin or ADM alone. No significant changes were found in the number of cells in G_2/M phase following drug challenge (Tab 2).

Effect of heparin on protein p53, p21, Bax, and Bcl-2 expression Western blot analysis showed

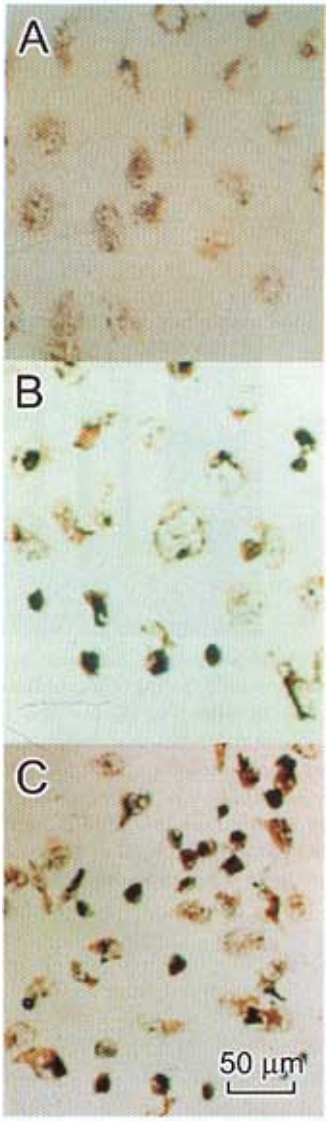

Fig 1. TUNEL assay demonstrating markedly morphological changes in CNE2 cells after treatment with heparin (with or without ADM). A) Control; B) ADM 5 mg/L; C) Heparin 20 kU/L + ADM 5 mg/L. × 200.

that ADM or heparin alone induced expression of Bcl-2, Bax, p53, and p21 in CNE2 cells, ADM plus heparin augmented the expression of these genes too. However, the Bax/Bcl-2 ratio was apparently enhanced by the treatment with heparin plus ADM (Fig 3, Tab 3).

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Tab 2. Effect of heparin and ADM on cell cycle in CNE2 cells. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^eP < 0.05$, $^fP < 0.01$ *vs* heparin + ADM.

Treatment	Concentration	G_0/G_1 (%)	S/%	G_2 M(%)
Control	–	61.7 ± 1.5	22.7 ± 1.5	15.6 ± 2.0
ADM	5 mg/L	71 ± 3^{ce}	12.3 ± 1.5^{cf}	16.4 ± 2.1
Heparin	20 kU/L	66 ± 4^{bf}	17.6 ± 2.1^{bf}	16.7 ± 1.6
qHeparin	40 kU/L	72 ± 3^{ce}	12.3 ± 1.5^{cf}	16.0 ± 1.0
Heparin + ADM	20 kU/L + 5 mg/L	76.7 ± 2.1^c	7.3 ± 0.6^c	16.0 ± 1.7

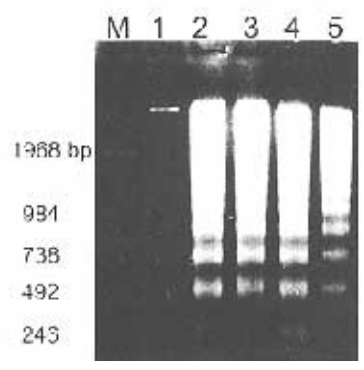

Fig 2. DNA ladder pattern formation in CNE2 cells after treatment with different concentrations of heparin (with or without ADM) for 48 h. The formation of oligonucleosomal fragments was determined by 1.5 % agarose gel electrophoresis. Lane M: DNA markers; Lane 1: control; Lane 2: heparin 20 kU/L; Lane 3: heparin 40 kU/L; Lane 4: ADM 5 mg/L; Lane 5: heparin 20 kU/L + ADM 5 mg/L. The result presented is typical of three separate experiments.

DISCUSSION

In previous work, we have found that heparin may facilitate apoptosis in human nasopharyngeal carcinoma CNE2 cells (data not shown). The present study demonstrated that heparin or ADM inhibited the growth of CNE2 cells and induced cell death by apoptosis. The combination of heparin and ADM resulted in a significant effect compared to ADM or heparin alone. It suggests that apoptosis contributes to the growth inhibitory effect of ADM and heparin and the two agents appear to be interacting in a synergistic manner in CNE2 cells. This finding of apoptosis in CNE2 cells is in agreement with our previous studies demonstrating the ability of heparin to induce apoptosis in CNE2 cells.

The ability of elevated levels of Bcl-2 protein to block apoptosis induced by most cytotoxic agents including adriamycin defines a new multidrug resistance mechanism[8-11]. Bax, a homologue of Bcl-2, which forms heterodimers with Bcl-2, blocks the action of Bcl-2 and thus acts as a promoter of apoptosis. When Bax predominates, programmed cell death is accelerated and the death repressor activity of Bcl-2 is countered. Therefore, it appears to be the relative ratios of Bax and Bcl-2 that determine the fate of a cell, rather than the absolute concentrations of either. Our present study showed that ADM or heparin alone could induce the expression of Bcl-2 and Bax, and ADM plus heparin augmented the expression of two genes. Furthermore, significantly increased ratio of Bax/Bcl-2 was observed by combined ADM and heparin. This may imply that the synergistic interaction between heparin and ADM on apoptosis in CNE2 cells is regulated via preferential overexpression of Bax over Bcl-2.

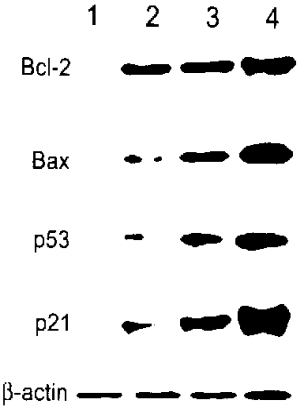

Fig 3. Bcl-2, Bax, p53, and p21 protein levels in CNE2 cells treated with heparin for 8 h. The whole cellular protein was electrophoresed in SDS-PAGE gel. Western blot was performed using antibodies against Bcl-2, Bax, p53, and p21. Beta actin was used as a lane-loading control. 1) control; 2) heparin 20 kU/L; 3) ADM 5 mg/L; 4) heparin 20 kU/L + ADM 5 mg/L. The result presented is typical of three separate experiments.

P53 is one of the most frequently studied tumor-suppressing genes. Any reagent or genotoxic stress that

Tab 3. Relative changes in Bcl-2, Bax, p53, and p21 protein expression in CNE2 cells treated with heparin 20 kU/L and/or ADM 5 mg/L. $n=3$. $x \pm s$. $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* heparin + ADM.

	Control	Heparin	ADM	Heparin + ADM
Bcl-2	0.04 ± 0.021	2.08 ± 0.06^{cf}	2.17 ± 0.14^{cf}	2.9 ± 0.1^{c}
Bax	0.03 ± 0.06	1.96 ± 0.22^{cf}	2.36 ± 0.05^{cf}	4.56 ± 0.07^{c}
p53	0.025 ± 0.013	1.31 ± 0.04^{cf}	1.97 ± 0.05^{cf}	2.67 ± 0.09^{c}
p21	0.072 ± 0.012	1.55 ± 0.07^{cf}	3.28 ± 0.07^{cf}	5.45 ± 0.07^{c}

produces DNA damage can cause a specific cellular response whereby the p53 protein accumulates to high level[12]. This increase in expression of p53 seems to be responsible for the arrest of cells in the G_1 phase of the cell cycle, presumably to enable cells to repair their damaged DNA prior to the onset of DNA synthesis and mitosis[13]. Our results revealed a reduction of cells in the S/G_2 phase 24 h after heparin or ADM plus heparin treatment, suggesting that entry of cells into the S phase is hindered, thus provoking arrest in G_1 of the cell cycle.

One of the genes for which transcription is induced by p53 is the cyclin-dependent kinase (CDK)-inhibitor p21. G_1 arrest is triggered by p53-mediated transactivation of p21 gene. P21 inhibits the activity of several CDK-cyclin complexes[14,15]. P21 has been implicated in the induction of p53-dependent or independent apoptosis in various cell types in response to a wide variety of stimuli[16]. P21 may exert an indirect influence to either induce or protect from apoptosis. In this study, the results demonstrate a significant increase of p21 mRNA after ADM and/or heparin treatment for 8 h. Our data suggest that enhanced expression of p53 in response to heparin plus ADM treatment may be followed by an increase of p21, resulting in decreased cell proliferation rates. This decreased proliferation rate was due to G_1 arrest, as demonstrated by cell cycle analysis and occurrence of apoptosis.

In conclusion, our results demonstrate that ADM and/or heparin can inhibit CNE2 cells proliferation and induce apoptosis. Heparin and ADM appear to interact in a synergistic manner, which is associated with increased expression of Bax, p53, and p21. The observation of the synergistic interaction between heparin and ADM in CNE2 cells *in vitro* illustrates the potential application of this strategy in the management of human patients with NPC. Further studies will investigate this strategy in *in vivo* models with NPC xenografts in immune-deficient mice, which will allow a more precise determination of the potential of this strategy to patients with NPC.

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肝素和阿霉素对人鼻咽癌 CNE2 细胞增殖与凋亡的协同作用[1]

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关键词 肝素；流式细胞术；电泳；细胞凋亡；阿霉素

目的：观察肝素联合阿霉素对人鼻咽癌细胞的 = 增殖与凋亡的影响及其可能的分子机制. 方法：用 MTT 法、流式细胞术观察肝素联合阿霉素对人鼻咽癌细胞增殖及细胞周期的影响；而用 TUNEL、琼脂糖凝胶电泳、Western blot 等方法观察肝素与阿霉素联合应用对人鼻咽癌细胞凋亡的作用. **结果**：肝素与阿霉素联合应用后对鼻咽癌细胞的生长抑制及其凋亡具有显著增强作用，同阿霉素单独应用于诱导细胞凋亡相比，低剂量的肝素与阿霉素联合应用后发现：TUNEL 阳性细胞从 12.6 % ± 1.1 % 增加到 65.7 % ± 1.3 %；G_0/G_1 期细胞阻止，S 期细胞明显减少，DNA“梯形”变化更加明显；Bax/Bcl-2 的比率及 p53 和 p21 基因的表达明显升高. **结论**：肝素与阿霉素对人鼻咽癌细胞在抗增殖及促凋亡方面有协同作用，这可能与 Bax/Bcl-2 比率的升高及 p53 和 p21 的表达上调有关.

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