Enhancement of Sodium Fluoride-induced Cell Death by Centrifugal Force

CHIEN-HUAN CHIEN[1], SHAHEAD ALI CHOWDHURY[2],
HIROSHI SAKAGAMI[3] and HARUHIDE KANEGAE[1]

[1]*Division of Orthodontics, Department of Human Development and Fostering,*
[2]*Meikai Pharmaco-Medical Laboratory (MPL) and* [3]*Division of Pharmacology,*
Department of Diagnostic and Therapeutic Sciences, Meikai University
School of Dentistry, Sakado, Saitama 350-0283, Japan

Abstract. *As an initial step in the study of the influence of orthodontic force on cellular function in vitro, the effects of centrifugal force on the cytotoxicity induced by various apoptosis inducers were investigated. When human oral squamous cell carcinoma (HSC-2) and human promyelocytic leukemia (HL-60) cell lines were treated with increasing magnitudes of centrifugal force (evaluated by g-value), the viability assessed by the MTT method and trypan blue dye exclusion began to decline. Centrifugal force enhanced the cytotoxicity of sodium fluoride (NaF), but not that of redox compounds (hydrogen peroxide, sodium ascorbate, gallic acid) or chemotherapeutic agents (daunorubicin, doxorubicin, idarubicin, mitoxantrone, peplomycin, 5-FU). The combination of NaF and centrifugal force enhanced caspase-3 activity. The present study suggests that centrifugal force is an additional factor that modifies the biological activity of NaF.*

When patients undergo orthodontic treatment, they are exposed to orthodontic force. The biological effects of orthodontic force have been investigated extensively in animal models (1-4). However, their effects at the cellular level have not been clarified. Recently, the effect of pressure on apoptosis induction was reported (5, 6). When human osteoblasts were stimulated by orthodontic force (hypergravity stimulation through centrifugation, at 200 xg, for 30 minutes), RUNX2 gene expression was transiently enhanced (7). Orthodontic force may affect RUNX2-

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: (+81)49-285-5511 ex 336, 429, Fax: (+81)49-285-5171, e-mail: sakagami@dent.meikai.ac.jp, bakemono@ed.ejnet.co.jp

Key Words: Centrifugal force, cell death, caspase, fluoride, orthodontic force.

directed bone development and homeostasis. However, the effect of orthodontic force on the biological activities of clinically-used medications has never been investigated, suggesting the necessity for model systems for these studies.

We previously reported that redox compounds [hydrogen peroxide (H_2O_2) (8), ascorbic acid (9), gallic acid (10)], chemotherapeutic agents [doxorubicin (11, 12), peplomycin (12), 5-Fluorouracil (5-FU) (12), daunorubicin, idarubicin, mitoxantrone (unpublished data)] and sodium fluoride (NaF) (13) induced apoptosis in human promyelocytic leukemia HL-60 cells. The growth and apoptosis induction by these compounds were affected by various factors, such as the temperature (14), pH (15), metal (16), nutrients (amino acid in culture medium and serum components) (17) and oxygen levels (18). In this study, whether centrifugal force (used as a model system for orthodontic force) affects cell death induction in the human promyelocytic leukemia HL-60 and human oral squamous cell carcinoma HSC-2 cell lines was investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI 1640 (Gibco BRL, Grand island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA); daunorubicin HCl, doxorubicin HCl, idarubicin HCl, mitoxantrone 2HCl, 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St Louis, MO, USA); H_2O_2, NaF, dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan); peplomycin sulfate (Nihonkayaku, Tokyo, Japan); sodium ascorbate, gallic acid (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); 5-FU (Kyowa, Tokyo, Japan).

Cell culture. The HSC-2 cells (kindly provided by Prof. Masao Nagumo, Showa University, Japan) were cultured in DMEM supplemented with 10% heat-inactivated FBS. The HL-60 cells (provided by Prof. Kazuyasu Nakaya, Showa University) were cultured in RPMI 1640 supplemented with 10% heat-inactivated
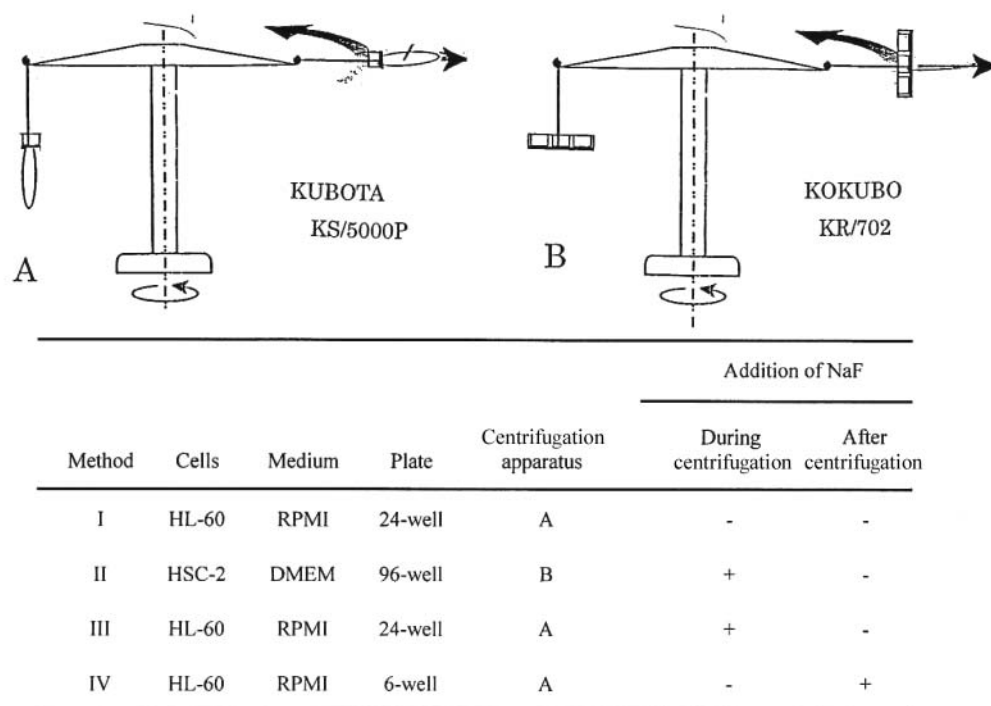
Figure 1. *Method of cell treatment with centrifugal force.*

FBS. The cell density of the HL-60 cells was kept under 1.6×10^6/mL, since higher cell density caused significant cell death.

Assay for cytotoxic activity. The HL-60 cells were resuspended in fresh medium at 1×10^6/mL in a 15-mL centrifugal tube. The cells were then treated with different centrifugal forces (200, 780, 1,700 xg, KUBOTA KS/5000P centrifugation apparatus) (Figure 1A). The cells were resuspended in fresh culture medium and then incubated for an additional 24 h to determine the viable cell number by trypan blue exclusion (Method I, Figure 1).

The HSC-2 cells were inoculated at 12×10^3 cells/well in a 96-microwell plate (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 h, the medium was removed by suction with an aspirator and was replaced with 0.1 mL of fresh medium. The cells were then treated for 5 to 60 min, with or without apoptosis-inducing agents under various magnitudes of centrifugal force (145 xg, KOKUBO KR/702 centrifugation apparatus) (Figure 1B). The medium was replaced with fresh drug-free culture medium and the cells were incubated for an additional 24 h without centrifugal force. The relative viable cell number was then determined by the MTT method (Method II, Figure 1). In brief, the cells were incubated for 4 h with 0.2 mg/mL MTT. The cells were lysed with 0.1 mL of DMSO and the absorbance at 540 nm (A_{540}) of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The A_{540} of control cells were usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Assay for DNA fragmentation. The cells were washed once with phosphate-buffered saline without Ca^{2+}/Mg^{2+}(PBS (–)) and lysed with 50 μL lysate buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate) and incubated for 2 h at 50°C with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K. The DNA was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with ethanol. Subsequently, the DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0). The DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and the DNA from apoptotic HL-60 cells induced by UV irradiation were run in parallel. After staining with ethidium bromide, the DNA was visualized by UV irradiation and photographed by a CCD camera (Bio Doc Inc, UVP).

Assay for caspase activation. The cells were washed twice with PBS(–) and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 min on ice and centrifugation for 5 min at 10,000 rpm, the supernatant was collected. The lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing the substrate for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)). After incubation for 2 h at 37°C, the absorbance at 405 nm of the liberated chromophore pNA was measured by a plate reader.

Results

Effect of centrifugal force on the viability of HL-60 cells. When the HL-60 cells were exposed to increasing magnitudes of centrifugal force (using the KUBOTA
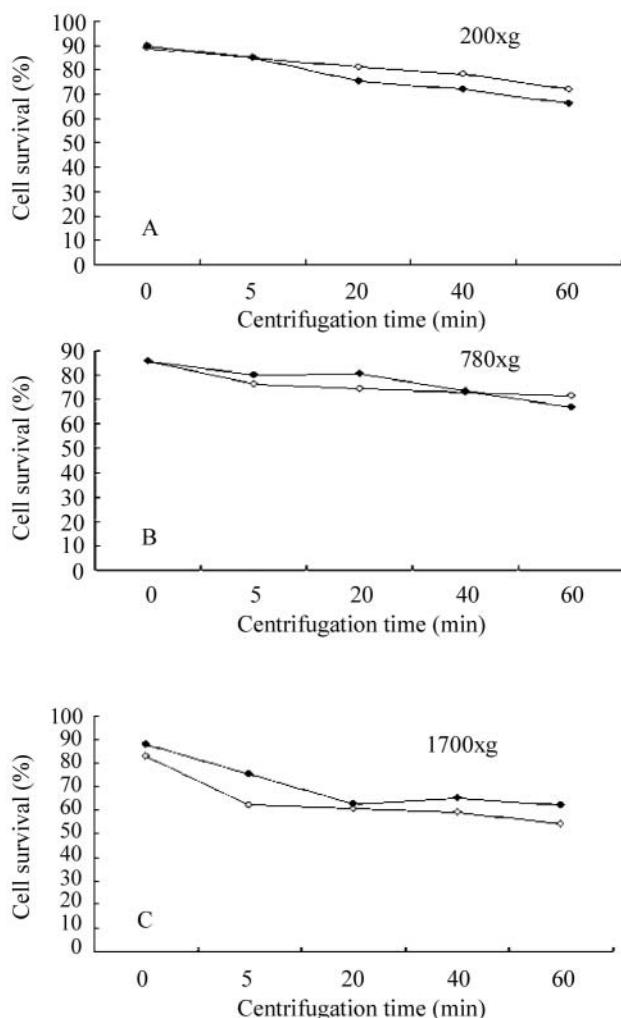
Figure 2. *Relationship between centrifugal force and viability. HL-60 cells ($1x10^6$/mL) were exposed for various times to 200 xg (A), 780 xg (B) or 1,700 xg (C) by the KUBOTA KS/5000P centrifugation apparatus and the viability of the cells was determined immediately (0 h) (○) and 24 (●) h after incubation in a 5% CO_2-incubator (Method I in Figure 1). Each value represents the mean from 4 independent experiments.*

Figure 3. *Effect of centrifugal force on the cytotoxicity induction by various apoptosis inducers. HSC-2 cells were seeded at $6x10^4$/well in 96-microwell plates. After 48 h, the medium was changed with fresh medium containing various concentrations of NaF (A), H_2O_2 (B), gallic acid (C) or sodium ascorbate (D) and was left to stand at room temperature (26˚C) without centrifugation (○) or immediately centrifuged for 30 min (●) at 145 xg with the KOKUBO KR/702 centrifugation apparatus. The medium was replaced with fresh drug-free medium and the cells were incubated for an additional 24 h to determine the viable cell number by the MTT method (Method II in Figure 1). The relative viable cell number was expressed as % of control. Each value represents the mean±S.D. from 4 independent experiments.*

KS/5000P centrifugation apparatus), the number of dead cells increased as a function of exposure time. At 200 xg, the viability of the cells declined after 20-min centrifugation (Figure 2A). At 780 xg, the viability declined after 5-min centrifugation (Figure 2B), while at 1,700 xg, the viability started to decline after 5-min, reaching a maximum cell death after 20 min (Figure 2C).

Effect of centrifugal force on the cytotoxicity induction by apoptosis inducers. When the HSC-2 cells were incubated with various concentrations of H_2O_2 (Figure 3A), sodium
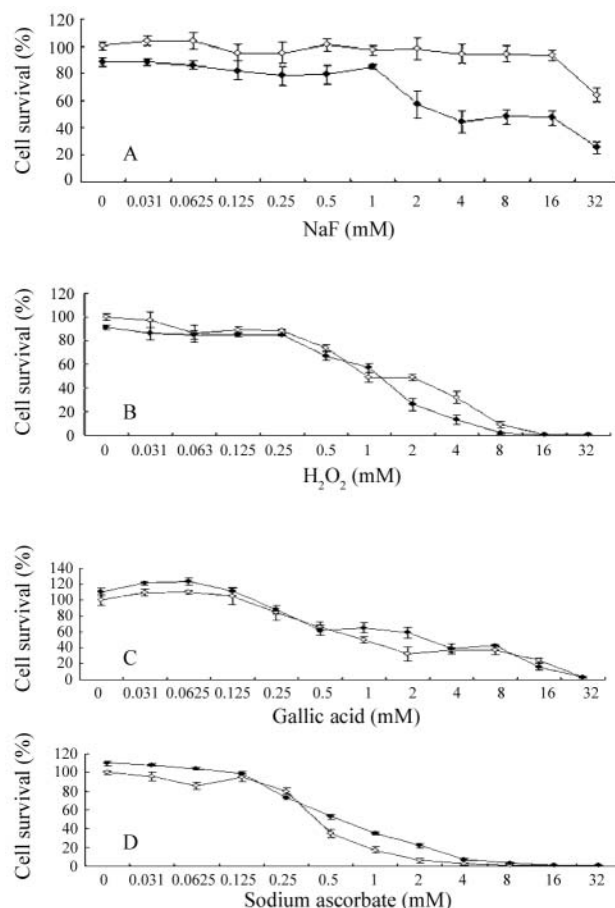
ascorbate (3B), gallic acid (3C) or NaF (3D), the viable cell number was dose-dependently reduced (CC_{50} of these compounds was 0.98, 0.42, 1.0 and >32 mM, respectively). Even when centrifugal force (145 xg, 30 minutes) was applied to these cells during drug treatment, the cytotoxic activities of H_2O_2, gallic acid and sodium ascorbate were only marginally changed (Figure 3B-D). However, the application of the same magnitude of centrifugal force dramatically increased the cytotoxic activity of NaF (CC_{50} was reduced to 3.1 mM) (Figure 3A). Similar experiments were repeated and it was found, reproducibly, that centrifugation significantly
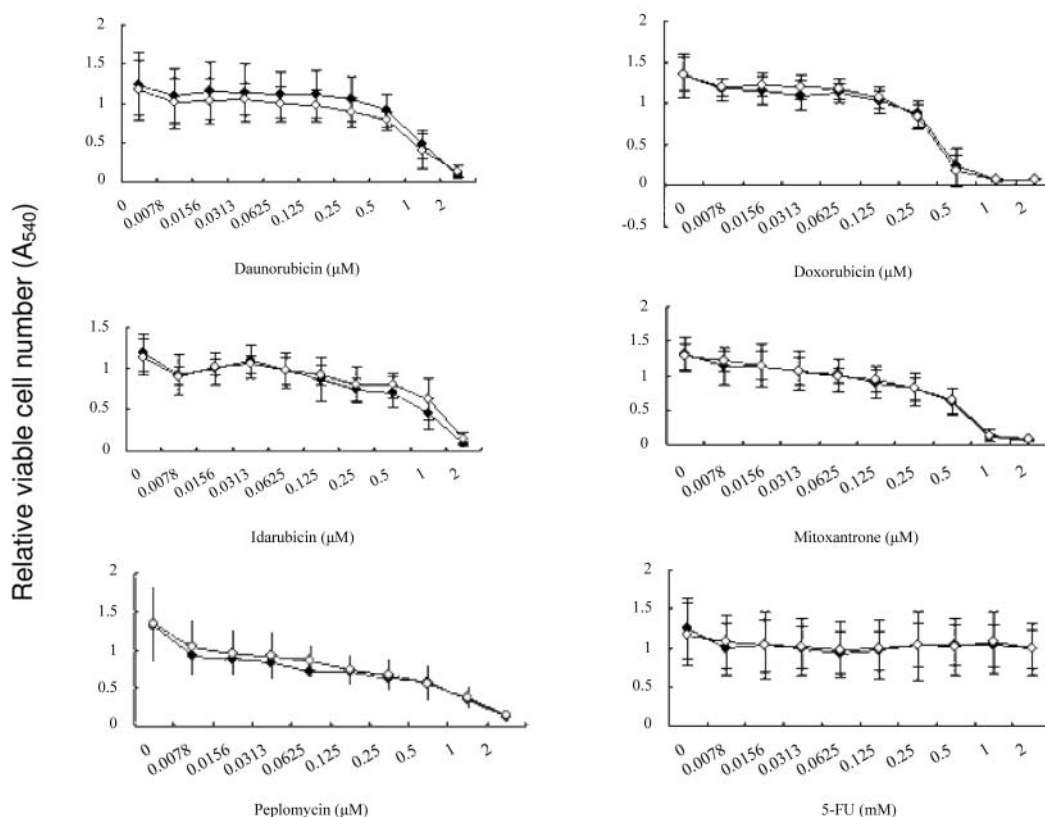
Figure 4. *Effect of centrifugal force on cytotoxicity induction by various chemotherapeutic agents. HSC-2 cells were seeded at 6x10^4/well in 96-microwell plates. After 48 h, the medium was changed with fresh medium containing various concentrations of daunorubicin (A), doxorubicin (B), idarubicin (C), mitoxantrone (D), peplomycin (E), or 5-FU (F) and were left to stand at room temperature (26°C) without centrifugation (○) or were immediately centrifuged for 30 min (●) at 145 xg using the KOKUBO KR/702 centrifugation apparatus. The medium was replaced with fresh drug-free medium, and the cells were incubated for an additional 24 h to determine the viable cell number by MTT method (Method II in Figure 1). The relative viable cell number was expressed as a % of the control. Each value represents the mean±S.D. from 4 independent experiments.*

enhanced the cytotoxic activity of NaF (2.3-fold) [>32 mM (without centrifugal force) *vs.* 13.7±10.1 mM (with centrifugal force)], but not those of H_2O_2 (0.9±0.6 *vs.* 1.5±0.6 mM), sodium ascorbate (0.7±0.3 *vs.* 0.5±0.2 mM) and gallic acid (1.4±0.5 *vs.* 1.7±1.0 mM) (Table I).

Centrifugal force (145 xg, 30 min) only marginally affected the cytotoxicity of the 5 chemotherapeutic agents (daunorubicin, doxorubicin, idarubicin, mitoxantrone, peplomycin) (Figure 4A-E, Table I), while the combination of centrifugal force and 5-FU did not induce cytotoxicity against HSC-2 cells (Figure 4F).

Enhancement of NaF-induced caspase activation by centrifugation. NaF was found to dose-dependently induce internucleosomal DNA fragmentation in the HL-60 cells (Figure 5), confirming our previous finding that NaF induced the activation of caspases-3, -8 and -9, the loss of mitochondrial membrane potential, the production of apoptotic bodies, the expression of the pro-apoptotic protein BAD and the accumulation of BAD protein in the

mitochondrial fraction near the nuclear periphery, as demonstrated by the co-localization of mitochondrial markers and BAD-GFP (13). The extent of DNA fragmentation, with or without centrifugal force, was comparable (Figure 5).

DNA fragmentation is a qualitative, but not quantitative, marker of apoptosis. Therefore, caspase activation was adopted as a quantitative marker of apoptosis for the evaluation of the combination effect of NaF and centrifugal force. Caspase-3 was activated approximately 10-fold by NaF, reaching a maximum level at 3 h and declining thereafter (Figure 6). The stimulation effect of NaF was significantly (*p*<0.01) augmented by centrifugation (780 xg, 20 min) prior to the NaF treatment. The caspase-3 activity was slightly increased by centrifugal force alone, indicating that NaF and centrifugal force may additively enhance cytotoxicity against HL-60 cells. However, the extent of caspase-3 activation by NaF and centrifugal force was much lower than that attained by actinomycin D, a positive control (Figure 6).
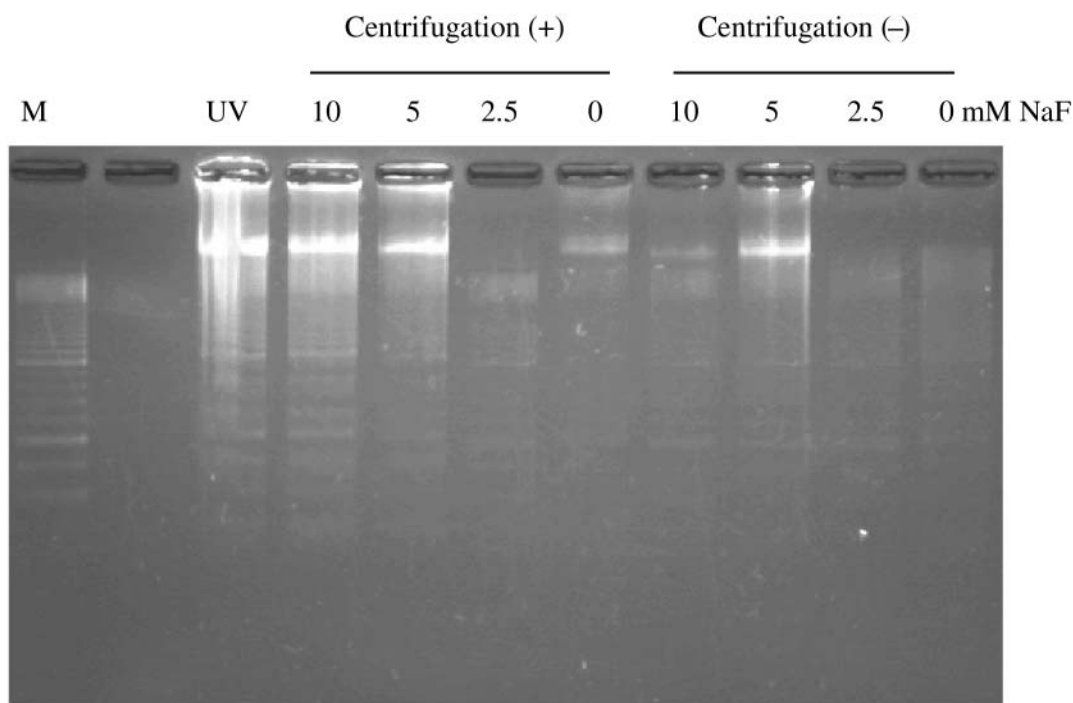
Centrifugation (+)   Centrifugation (–)

M   UV   10   5   2.5   0   10   5   2.5   0 mM NaF

Figure 5. *Effect of centrifugal force on NaF-induced DNA fragmentation. HL-60 cells ($1x10^6$/mL) were left to stand for 20 min at room temperature (26˚C) or were subjected to centrifugation at 780 xg with the indicated concentrations of NaF. The medium was replaced with fresh drug-free medium and the cells were incubated for an additional 6 h to assay the DNA fragmentation by agarose gel electrophoresis (Method III in Figure 1). M, marker DNA; UV, DNA from apoptotic HL-60 cells by UV irradiation.*

Table I. *Effect of centrifugal force on the cytotoxicity induction by apoptosis inducers.*

	CC_{50} (mM)	
Treatment	(–) Centrifugal force	(+) Centrifugal force
NaF	>32.0	13.7±10.1
H_2O_2	0.93±0.59	1.48±0.64
Sodium ascorbate	0.67±0.27	0.47±0.17
Gallic acid	1.40±0.53	1.70±1.04
	CC_{50} (μM)	
Daunorubicin	0.93±0.56	0.87±0.11
Doxorubicin	0.32±0.04	0.33±0.05
Idarubicin	1.09±0.31	0.80±0.13
Mitoxantrone	0.48±0.13	0.42±0.20
Peplomycin	0.26±0.22	0.21±0.16
5-FU	>2000	>2000

Near confluent HSC-2 cells were incubated for 20 min with various concentrations of apoptosis inducers with or without centrifugal force (145 xg) (Method II in Figure 1). After changing with fresh medium, the cells were incubated for an additional 24 h to determine the relative viable cell number by the MTT method. The CC_{50} value was determined by the dose-response curve. A representative example of these experiments is shown in Figures 3 and 4.
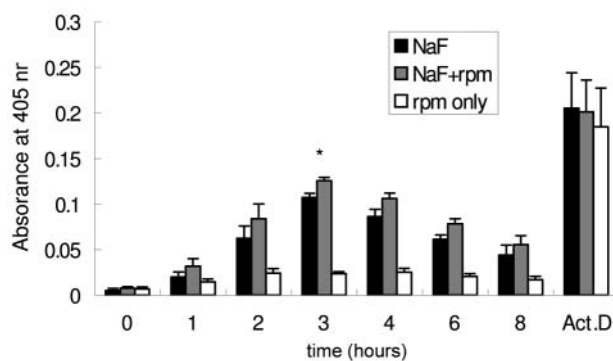
Figure 6. *Stimulation of NaF-induced caspase-3 activation by centrifugal force. HL-60 cells ($5x10^6$/mL) were left to stand for 20 min (26˚C) (black bar) or were subjected to centrifugation at 780 xg (grey bar). The cells were then resuspended in fresh medium containing the indicated concentrations of NaF. The cells were harvested at the indicated times thereafter and caspase-3 activity was measured by the substrate cleavage assay (Method IV in Figure 1). Cells that were incubated without NaF, but subjected to centrifugation, were used as the control (white bar). Each point represents the mean±S.D. from 4 independent experiments. $*<0.01$ vs. control (Student's t-test). Act.D: actinomycin D.*

Discussion

Pressure has been reported to induce apoptosis (characterized by the appearance of the sub G1 population and caspase-3 activation) (5, 6) *via* an unknown mechanism. The present study demonstrated that pressure is an additional factor that modifies the apoptosis induction by NaF. The mechanism by which centrifugal force and NaF additively induce cytotoxicity and apoptosis remains to be elucidated.

We demonstrated for the first time that centrifugal force (either 145 xg or 780 xg) enhanced the cytotoxic or apoptosis-inducing activity of NaF in HSC-2 and HL-60 cells. On the other hand, centrifugal force did not stimulate the cytotoxic activity of 3 redox compounds or 6 chemotherapeutic agents. Since NaF is an inhibitor of enolase, a glycolytic enzyme (19), the observed phenomena may be due to the additivity of centrifugal force and inhibition of glycolysis. Further study is required to test this possibility. RUNX2 is a transcription factor that is involved in bone development and homeostasis. It has recently been shown that treatment of rats with orthodontic force induced the expression of VEGF at the pressure site (20). VEGF is one of the growth factors, transcription of which is stimulated by hypoxia-inducible factor (HIF)-1α (21). HIF-1α is a master gene that controls glycolysis, inflammation and cell survival (21). It remains to be clarified whether centrifugal force (model of orthodontic force) affects the expression of HIF-1α.

We here investigated the effect of centrifugal force on the cytotoxic effect induced by NaF and found that cytotoxic and apoptosis activities were enhanced. The effects of centrifugal force on the biological activity of drugs affecting glycolysis should be studied in the future.

Acknowledgements

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