Original Contribution

Hyperthermia Promotes Verapamil Induced Apoptotic and Cytotoxic Activities in FM3A Murine Mammary Cancer Cells

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Abstract: Verapamil (VEP) is a calcium channel blocker that has been used in cancer therapy. Previous reports have indicated that VEP proved to be ineffective in inducing apoptosis in some cancer cells at normothermic temperature (37°C). We therefore investigated whether exposure of FM3A murine breast cancer cells to 20 μ M VEP at 43°C as opposed to 37°C will intensify anticancer effects. To do this, we exposed the cells as follows: (i) 20 μ M VEP for 1 h at 37°C, (ii) 20 μ M VEP 43°C for 1 h, (iii) 43°C heat for 1 h without VEP and (iv) 37°C for 1 h without VEP which served as control. Exposure to 43°C heat alone or 20 μ M VEP at 37°C independently resulted in diminished apoptotic and cytotoxic activities compared to simultaneous exposure to 20 μ M VEP and 43°C heat. The apoptotic cell death occurred in a time-dependent manner as follows: (i) concurrent effects of 43°C heat and 20 μ M VEP ($5.2 \pm 1.5\%$, $15.1 \pm 2.4\%$ and $35.7 \pm 4.4\%$), (ii) 43°C heat only ($4.3 \pm 1.0\%$, $8.6 \pm 1.0\%$ and $12.8 \pm 2.0\%$), and (iii) 20 μ M VEP only ($4.6 \pm 0.2\%$, $5.9 \pm 1.0\%$ and $8.0 \pm 0.7\%$) at 1, 6 and 24 h post exposure respectively compared to the control value of $2.0 \pm 0.1\%$. Concurrent exposure, of cells to 20 μ M VEP and 43°C heat also resulted in a 96% suppression of cell proliferation compared to 53% when cells were exposed to 20 μ M VEP at 37°C or 56% to 43°C heat alone. The increased cell killing by 20 μ M VEP at 43°C revealed a hyperthermic enhancement of VEP-induced apoptotic mechanisms.

Key Words: FM3A cells, verapamil, hyperthermia, apoptosis, cytotoxicity

Introduction

The combined therapy with hyperthermia and anti-cancer drugs have been demonstrated for various compounds including doxorubicin hydrochloride ¹⁾, cisplatin ^{2, 3)}, quercetin ⁴⁾, cisplatin and peplomycin ⁵⁾. Verapamil (VEP) is a phenylalkylamine that binds to the L type voltage-dependent and-independent

calcium channels at normothermic (37°C) temperature, blocking calcium influx. At hyperthermic temperatures, however, VEP is reported to promote increases in intracellular $[Ca^{2+}]$ concentration⁶⁻⁹⁾. Furthermore, it is also well established that in cells undergoing apoptosis, the earliest detectable change is a rapid, sustained increase in $[Ca^{2+}]$ concentrations¹⁰⁾. The aim of this study was to investigate whether the apoptotic and cytotoxic activities of VEP against FM3A cells are intensified when the cells are exposed to 20 μ M VEP at hyperthermic (43°C) as opposed to normothermic (37°C) temperature.

Materials and Methods

In vitro culture treatment / induction of cell death

RPMI 1640 and foetal calf serum (FCS) were obtained from Life Technologies (Grand Island, NY, USA); verapamil, propidium iodide and ribonuclease A were purchased from the Sigma Chemical Co. (St. Louis MO, USA). FM3A mouse mammary cancer cell line, obtained from Riken Cell Bank (Tsukuba, Japan), were maintained in RPMI 1640 media supplemented with 20% FCS, 200 U/ml penicillin and 200 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. For experiments, FM3A cells ($\sim 2 \times 10^6$ cells/ml) were cultured in RPMI medium at 37°C (control), 43°C or medium containing 20 μ M VEP at 37°C or 43°C (tests). The dose of VEP chosen is near the lower range (20-100 μ M) earlier used on different cancer cells¹¹⁾. In treatments involving VEP, prior to exposure to the respective temperature, cells were incubated in VEP for 1 h, then exposed to the heat for additional 1 h.

Determination of percentage of apoptotic cells and cell cycle phases

Flow cytometric analyses were used for the assessment of the numbers of hypodiploid (apoptotic) cells and cells with diploid DNA content, as well as cell cycle phases. After VEP and/or hyperthermia (HT) exposures, 1 ml of medium containing $\sim 2 \times 10^6$ cells were transferred to 1.5 ml sterile eppendorf tubes in triplicates for each of the test conditions. Eppendorf tubes were centrifuged at 1000 rpm for 5 min. Cell pellets were then washed twice in ice cold phosphate buffered saline (PBS) and resuspended in 400 μ l ice cold (4°C) PBS. Cells were further diluted in 400 μ l of 70% ethanol (by dropwise addition while vortexing). The resulting cell suspensions were allowed to fix for 24 h at 4°C. After fixing, cells were spun at 2000 rpm, and washed twice in ice cold (4°C) PBS. The pellets were resuspended in 400 μ l of 0.1% (w/v) sodium citrate containing 1 ml/ml RNase A. Cells were then incubated at 37°C for 20 min. Cell suspensions were stored at -20°C until required for flow cytometric analysis. Immediately before flow cytometry, cells were spun at 2000 rpm for 5 min and the pellets were resuspended in 400 μ l 0.1% (w/v) sodium citrate containing 100 μ l/ml propidium iodide. Cells were incubated in the dark at 21°C for 15 min. Samples were then examined with Becton Dickinson FACScan using excitation wavelength of 488 nm, gating out doublets and clumps using pulse processing and collecting fluorescence (emission wavelength) of 585 ± 21 nm. Cells with DNA content less than diploid cells in the G₀/G₁ phase are considered as apoptotic. DNA histograms and cell cycle phases were analyzed using Sfit and Rfit software.

Cytotoxicity assays

Cytotoxic effects VEP were assayed using cell proliferations and clonogenic efficiencies after cells were exposed to VEP at 37°C and 43°C. Cell proliferation assays were performed by inoculating 2×10^5 cells each sample exposed to VEP at 37°C and 43°C for 1 h into 10 ml of culture media (2×10^4 cells/ml) and

incubated for 5 days. Changes in cell number from each sample was estimated every 24 h using the new improved Neubauer Hemocytometer. The percentage of cellular inhibition was calculated as follows:

$$\% \text{ of cellular inhibition} = 100 - \frac{100 (\text{Number of cells in treated samples})}{(\text{Number of cells in control samples})}$$

To estimate clonogenic efficiency, 2×10^3 cells from each sample were seeded in 3 ml RPMI media containing 0.33% agar and layered over 4 ml of RPMI containing 0.5% agar in 60 mm tissue culture dishes (Iwaki Glass, Funabashi, Japan), in triplets. Culture dishes were then incubated under regular culture condition for 11 days. Colonies were stained with Giemsa stain, and the aggregates consisting of 20 or more cells were counted as a colony. Results are expressed as the mean number of colonies from 3 plates. The number of colonies in test samples were calculated as follows:

$$\% \text{ of colony suppression} = 100 - \frac{100 (\text{Number of colonies in treated samples})}{(\text{Number of colonies in control samples})}$$

Statistical analysis

Statistical significance of differences in apoptosis and cell proliferation between control and test groups were analyzed by ANOVA using the statistical package STATVIEW (Abacus concepts, Inc, Berkeley, CA, 1992).

Results

As shown in Table I, exposure of FM3A cells to 20 μ M VEP at normothermic (37°C) temperature induced no significant change, whereas exposure to 20 μ M VEP at hyperthermic (43°C) temperature induced an accumulation of cells in the S and the G₂/M phases. These changes were significantly different from the control, HT only or VEP only. The increased cell population in the S phase following treatment with 20 μ M VEP at 43°C resulted in a reduction of cells in the G₀/G₁ phase, with a distinct reduction of cells in the G₀/G₁ phase occurring after 24 h. Thus, at 43°C VEP exerted a prominent blocking action of

Table I. Cell cycle distributions after heat and verapamil treatments at 1, 6 and 24 hours post treatment.

Treatment group	Exposure time (h)	G ₀ /G ₁ (%)	S(%)	G ₂ /M(%)	G ₂ /G ₁ ratio
Control	1	57.4	32.9	9.6	0.16
	6	54.7	34.1	11.2	0.2
	24	54.2	33.7	12.1	0.22
VEP at 37°C	1	56.3	31.2	12.5	0.22
	6	56.5	30.6	12.9	0.23
	24	55.2	31.8	13	0.24
43°C alone	1	57.6	33.6	8.8	0.15
	6	53.3	35.5	14.2	0.27
	24	52.6	30.4	17	0.32
VEP at 43°C	1	57.6	37.6**	4.8**	0.08
	6	53.3	36.5**	20.2**	0.19
	24	47.3**	41.9***	20.8**	0.23

Values are means $\pm$ SEM of 8 observations in 2 separate experiments.

, $P < 0.01$; *, $P < 0.001$.

Fig. 1. Apoptosis histograms showing response of FM3A cells to hyperthermia alone (HT), verapamil alone (VEP) or combination of the two treatments (VEP + HT) at 1, 6 and 24 h post treatments. Values are means $\pm$ SEM of 12 observations in 4 separate experiments. CON, control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 2. Cell growth curves of FM3A cells treated with hyperthermia (43°C heat) alone (HT), verapamil alone (VEP) or a combination of the two treatments (VEP + HT) followed by incubation at 37°C for 5 days. Cell numbers were determined every 24 h. Values are means $\pm$ SEM of 12 observations in 4 separate experiments. CON, Control.

Fig. 3. Clonogenic assay of FM3A cells treated with hyperthermia (43°C heat) alone (HT) verapamil alone (VEP) or a combination of the two treatments (VEP + HT) followed by incubation at 37°C for 11 days. Aggregates of 20 cells or more were counted as a colony. Values are means $\pm$ SEM of 12 observations in 4 separate experiments. CON, Control. *, $P < 0.05$; ***, $P < 0.001$.

cell transition from the S phase of the cell cycle. The increase in cells in the S phase coincided with significant induction of apoptosis (Fig. 1). There was comparatively reduced apoptosis when the cells were exposed to 20 μ M VEP at 37°C, or 43°C heat alone 24 h after treatment (Fig. 1). On the other hand the magnitude of apoptosis was nearly doubled when cells were treated with 20 μ M VEP and 43°C heat. Contrary to the independent treatments with 20 μ M VEP or 43°C heat, the apoptotic effects of simultaneous exposure to the two treatments was observable as early as 1 h post treatment. At 24 h after the simultaneous treatment, there was a significant increase in the number of apoptotic cells (Fig. 1).

The cytotoxic effects of the respective treatments were equally evaluated via growth inhibitory effectiveness and clonogenicity. Fig. 2 shows the proliferation of cells over 5 days period after cellular exposure to 20 μ M VEP at 37°C, 20 μ M VEP at 43°C heat, or 43°C heat alone for 1 h each. Treatment of cells with 20 μ M VEP at 37°C or 43°C heat alone revealed a relative ineffectiveness (even though significant at $P = 0.001$) in inhibiting the *in vitro* growth of FM3A cells as well as colony formation as compared to the effect of 20 μ M VEP and 43°C heat simultaneously applied (Figs. 2 and 3). Exposure of FM3A cells to 20 μ M VEP and 43°C heat simultaneously resulted in a significant inhibition of cell growth and colony formation.

Discussion

The important findings in this study are as follows: (i) flow cytometric analyses revealed that exposure of cells to 20 μ M VEP at 37°C caused an insignificant change in cell cycle phase; (ii) exposure of cells to 20 μ M VEP at 43°C resulted in the accumulation of cells in the S phase and the G₂/M phases of the cell cycle, with a consequent decrease in the G₀/G₁ phase and increased apoptosis, particularly after 24 h; and (iii) hyperthermia enhances the apoptotic and growth inhibitory activities of VEP as revealed by the response of cells exposed at 43°C compared to 37°C.

From the above findings, it can be presumed that the mechanism of VEP action at the cell level is associated with disturbances of cell division which obviously reflected in increased of apoptotic activities. In addition, although 20 μ M VEP at normothermic (37°C) temperature was comparatively less effective than the hyperthermic treatment in inducing apoptosis, there was a visible effect when compared to control. This is not surprising since other studies, including *in vivo* experiments have shown that verapamil (40 mg/kg ip) induced apoptosis of 4.3 index compared to 0.5 in the saline treated control rats¹². Even though some previous reports have indicated that VEP proved ineffective in inducing apoptosis in some cancer cells at normothermic temperature (37°C). It is clear from this study that VEP has a remarkable degree of effectiveness in FM3A cells under this condition. Interestingly, when the treatment with VEP was performed under hyperthermic (43°C) conditions, the anti-cancer effects of VEP are further intensified, thus supporting our hypothesis. On account of the fact that 43°C heat on its own provided evident anticancer benefits, it may be logical to think that the mechanism of hyperthermic potentiation of VEP involved some degree of additivity between 43°C heat and VEP or on the other hand, the effect of 43°C occurred entirely via heat induced increased Ca²⁺ influx. Further studies are therefore required for scrutinization of these effects.

Addressing closely the mechanism involving increased Ca²⁺ influx, it is known that calcium channels maintain [Ca²⁺] homeostasis which is critical for cell proliferation^{13, 14}, and VEP appear to disrupt this

homeostasis. VEP does this by binding to the L type voltage-dependent and -independent Ca^{2+} channels blocking calcium influx, thus promoting anti-proliferative, anti-invasive and anti-metastatic activities^{11, 15)}. On the other hand, at higher temperature, VEP appears to act through a different mechanism, *i.e.* increasing calcium influx⁶⁻⁹⁾. The role of VEP in cellular apoptosis may therefore appear to be paradoxical. Shchepotin *et al.* studied the mechanisms responsible for the growth inhibitory effect of hyperthermia and VEP in human colon cancer cell line HT-29, and showed that apoptosis of HT-29 cells produced by hyperthermia in the presence of VEP is a p53-independent process¹⁶⁾. They also hypothesized that HT-29 cells must have Bcl-2 independent pathways to protect cells against death-inducing signals, since HT-29 cells constitutively express apoptosis promoting proteins, such as Bax and c-Myc, while they fail to produce Bcl-2. Furthermore, there are several downstream mechanisms by which cellular Ca^{2+} influx induce apoptosis. Transient increase in cytosolic Ca^{2+} and subsequent activation of the Ca^{2+} dependent protease calpain as well as proteases of the caspase family is one mechanism¹⁷⁾. Other mechanisms involve the production of reactive oxygen species¹⁸⁾ and the release of intermediate metabolites of arachidonic acid¹⁹⁾. It is not clear at point which of downstream mechanisms by which VEP induces apoptosis. This is a question we intend to further address in subsequent experiments.

To conclude, this study demonstrated that hyperthermia enhanced the cytotoxic effect of verapamil. From the view point of thermoenhancement, verapamil may provide superior benefit against cancer when used at hyperthermic temperatures.

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マウス乳がん FM3A 細胞におけるベラパミルによるアポトーシス誘導 および殺細胞効果の温熱による増強

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要 旨：ベラパミール (VEP) はカルシウムチャネルブロッカーであるがガンの治療にも用いられる。これまで正常温度 (37℃) ではある種のガン細胞ではアポトーシスを誘導しないとの報告がある。そこで我々は、VEP の抗腫瘍効果の温熱による増強の有無を FM3A 細胞を用いて、1) VEP (20 μ モル) 1 時間暴露 (37℃), 2) VEP (20 μ モル) 1 時間暴露 (43℃), 3) 43℃ 暴露 1 時間, 4) 37℃ 1 時間 (コントロール) の各条件にて検討した。43℃ 温熱のみあるいは VEP のみでは、アポトーシス誘導、殺細胞効果ともに 43℃ 温熱と VEP の併用条件にくらべ低かった。アポトーシスは以下のように処理後 1, 6, 24 時間と時間依存性に誘導された: 1) 43℃ 温熱と VEP の併用 ($5.2 \pm 1.5\%$, $15.1 \pm 2.4\%$, $35.7 \pm 4.4\%$), 2) 43℃ 温熱のみ ($4.3 \pm 1.0\%$, $8.6 \pm 1.0\%$, $12.8 \pm 2.0\%$), 3) VEP のみ ($4.6 \pm 0.2\%$, $5.9 \pm 1.0\%$, $8.0 \pm 0.7\%$)。コントロール値は $2.0 \pm 0.1\%$ であった。増殖抑制効果についても、43℃ 温熱のみでは 56%, VEP のみでは 53% であったが、43℃ 温熱と VEP との併用により 96% と高い増殖抑制効果を認めた。43℃ 温熱による VEP の殺細胞効果の増強にはアポトーシス誘導の増強が関与することが示唆された。