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Effect of mitomycin C, verapamil, and hyperthermia on human gastric adenocarcinoma

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Abstract. The purpose of this study was to assess the efficacy of verapamil (20 μ*M*) and hyperthermia (42° C) as modifiers of mitomycin C (MMC), used at different concentrations, in inhibiting the growth of human gastric adenocarcinoma (AGS) cells. Combined verapamil and hyperthermia treatment resulted in a significant decrease in cell count by 72.2% as compared with the control value. Verapamil drastically enhanced the growth-inhibitory activity of MMC at high concentration against AGS cells by 67.5% and had no effect at intermediate and low concentrations. Hyperthermia did not enhance the effect of MMC on AGS cells. The modalities analyzed in this study require further investigation and may have potential for in vivo studies on gastric cancer therapy in the near future.

Key words: Mitomycin C – Verapamil – Hyperthermia

Introduction

Gastric cancer remains a significant problem in the United States, with 24,000 new cases and 13,600 deaths being reported in 1993 [4]. Multimodality therapy has not improved the survival of patients with gastric cancer, and current regimens have limited efficacy against advanced tumors [13].

Hyperthermia was "once thought to be one of the most promising new cancer therapies of the modern era" [31a] and remains one of the most important chemotherapeutic drug sensitizers identified to date [7, 31]. The possibility of enhancing the effect of cytotoxic drugs on tumor cells by means of hyperthermia has been clearly demonstrated both in vitro and in vivo [9, 22, 28, 32].

In addition to hyperthermia, verapamil has also been studied as a drug that can reverse the resistance of malignant cells to chemotherapeutic agents. Some clinical studies have shown no benefit for joint administration of chemotherapy and verapamil [24, 25]. The exact mechanism by which calcium channel blockers decrease drug egress remains unknown, but there is substantial evidence that it is not directly related to an alteration of calcium flux [10] or to an effect on calcium channels [26]. Other studies have also suggested that verapamil alters the binding of chemotherapy agents [3, 21] and their distribution [11] in tumor cells. Verapamil also reportedly alters cell membrane fluidity [14]. One of the mechanisms of the cytotoxic effect of hyperthermia is also alteration of the cell membrane [5, 15, 17]. Even small temperature changes can drastically alter the structure of biomembranes, thereby influencing many membrane-related cellular functions [15].

The combined effect on malignant cells of chemotherapeutic drugs, such as mitomycin C, and agents that can reverse tumor resistance to chemotherapy, such as hyperthermia and verapamil, has not been examined. This study was designed to evaluate the in vitro efficacy of mitomycin C applied in conjunction with hyperthermia and verapamil on the gastric cancer cell line AGS.

Materials and methods

Tumor cells. The human gastric adenocarcinoma cell line AGS was obtained from American Type Culture Collection (Bethesda, Md.). This line was isolated in 1979 from an adenocarcinoma of the stomach resected from a 45-year-old Caucasian woman. The patient had received no prior anticancer therapy. The cells were grown in RPMI 1640 medium (Biofluids, Bethesda, Md.) supplemented with 5% fetal bovine serum (FBS), L-glutamine (2 m*M*), penicillin (10 U/ml), and streptomycin (10 μg/ml). They were grown as a monolayer in 25-cm^2 tissue-culture flasks.

Reagents. Mitomycin C (MMC) was obtained from Sigma Chemical Co. (St. Louis, Mo.). On the basis of previously published data, MMC was used at the following concentrations: 0.01, 0.1, and 1.0 μg/ml [20]. MMC was dissolved in distilled water and filtered immediately before each experiment. Appropriate drug concentrations were made by dilution with fresh media.

Verapamil was also obtained from Sigma Chemical Co. (St. Louis, Mo.) and was used for experiments based on previously published data

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at 20 μ*M* concentration [18]. The drug was first dissolved in distilled water. The solution was then diluted with equal amounts of RPMI 1640 and filtered.

Treatment scheme. Cells were plated at 100,000 cells/flask in regular media and allowed to grow to 50% confluence while being in the logarithmic growth phase throughout the study. At this point, the cell count was $1.28 \pm 0.12 \times 10^6$ and treatment was started. All cells were grown at 37° C in standard media as described above. From day 1 through day 4, the respective treatments were carried out.

Four independent experiments were performed. The control for each experiment was AGS grown in standard conditioned media as described above without any treatment with MMC, hyperthermia, or verapamil. Each experiment involved the use of MMC, hyperthermia, or verapamil for a duration of 1, 2, 3, or 4 consecutive days, for a specified period of exposure each day. After treatment had been completed, standard growth conditions were restored and cell counts were performed on day 7.

Cells were exposed to verapamil for only 2 h each day, then washed with phosphate-buffered saline (PBS) and put in standard media. Hyperthermia was applied in a different incubator at 42° C under an atmosphere containing 5% CO_2 for 1 h on each day of treatment. Cells were heated with control or conditioned media to a target temperature of 42° C. The temperature of the media was monitored and the transient heating time required to achieve 42° C was 5 min. After completion of the hyperthermia session, all flasks were placed back in their regular incubator with a temperature of 37° C and a CO_2 concentration of 5%. The daily period of exposure of AGS cells to MMC was 2 h. When treatment has been completed, cells were washed twice with PBS and then regular media was added.

Experiment 1 involved treatment with hyperthermia alone, verapamil alone, and a combination of verapamil and hyperthermia, each being applied for a period ranging from 1 to 4 consecutive days. Experiments 2, 3, and 4 included treatment with MMC alone, MMC with hyperthermia, and MMC with verapamil and hyperthermia, each being applied for a period ranging from 1 to 4 consecutive days. Experiments 2, 3 and 4 differed only in the concentration of MMC used (experiment 2, 1.0 μg/ml; experiment 3, 0.1 μg/ml; experiment 4, 0.01 μg/ml).

Cells were grown in regular media under standard conditions from day 5 to day 7. On day 7, all cells were counted using a Coulter counter. All treatments were performed in duplicate and the standard deviation was calculated.

Results

After completion of a 4-day course of hyperthermia, no significant change was seen in the cell count as compared with the control value ($P > 0.05$, Fig. 1). However, after one and two hyperthermia sessions, a significant increase in cell survival was noted ($P < 0.05$), and this difference disappeared by the end of the hyperthermia treatment.

The administration of verapamil over 4 days did not cause any significant growth inhibition, regardless of the duration of treatment, in comparison with the control value ($P > 0.05$, Fig. 1). The combined effect of verapamil and hyperthermia on the growth inhibition of AGS cells was time-dependent and progressive over the 4 consecutive days of treatment. Joint administration of verapamil and hyperthermia did cause a significant decrease in cell survival in comparison with the control value and with the administration of hyperthermia alone or verapamil alone even after 1 day of treatment ($P < 0.05$, Fig. 1). After 1, 2, 3, and 4 consecutive treatments, the number of cells were decreased by 38.3%, 53.9%, 61.1%, and 72.2%, respectively, in comparison with the control value ($P < 0.05$, Fig. 1).

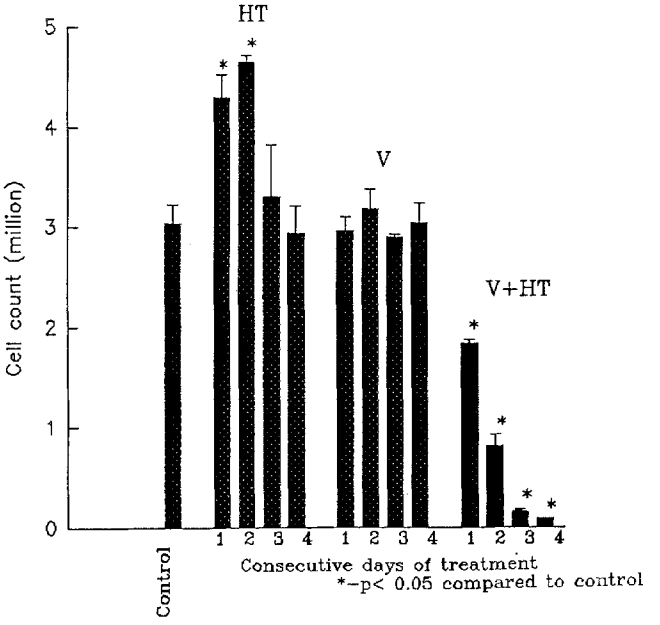

Fig. 1. Effect of verapamil (*V*) and/or hyperthermia (*HT*) on AGS cells – experiment 1

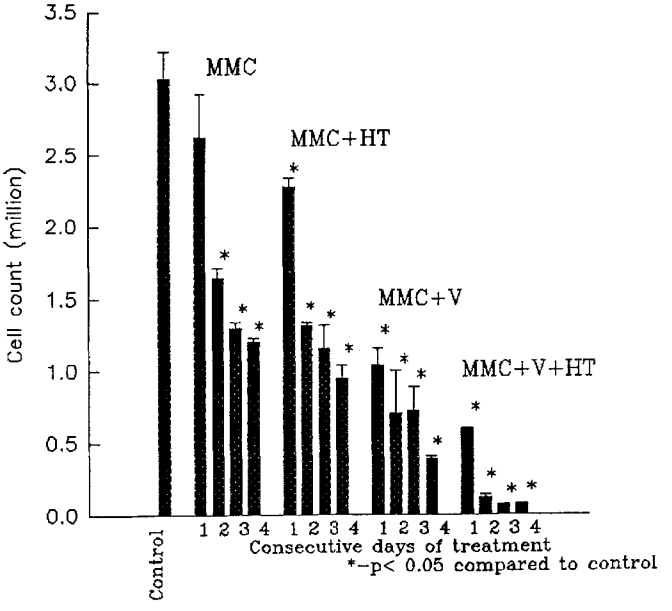

Fig. 2. Effect of MMC (1.0 μg/ml), verapamil (*V*), and hyperthermia (*HT*) on AGS cells – experiment 2

MMC treatment for 1 day at the high concentration (1.0 μg/ml) did not cause a significant decrease in the cell count in comparison with the control value ($P > 0.05$, Fig. 2). MMC treatment for 2, 3, and 4 days did cause a significant decrease in the number of cells by 50.6%, 61.6%, and 64.1%, respectively, in comparison with the control value ($P < 0.05$, Fig. 2). The cell counts obtained after 2 consecutive days of treatment differed significantly from those recorded after 1 day of treatment, but the results of 3 and 4 consecutive days of treatment were not signifi-

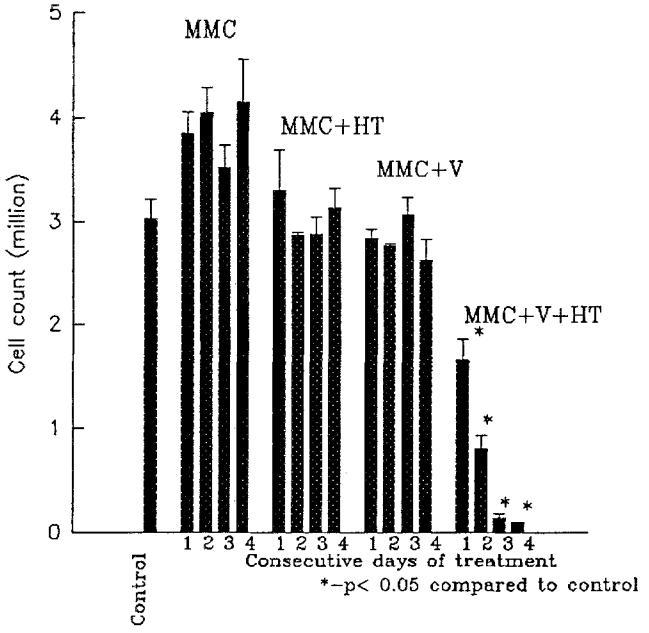

Fig. 3. Effect of MMC (0.1 μg/ml), verapamil (*V*), and hyperthermia (*HT*) on AGS cells – experiment 3

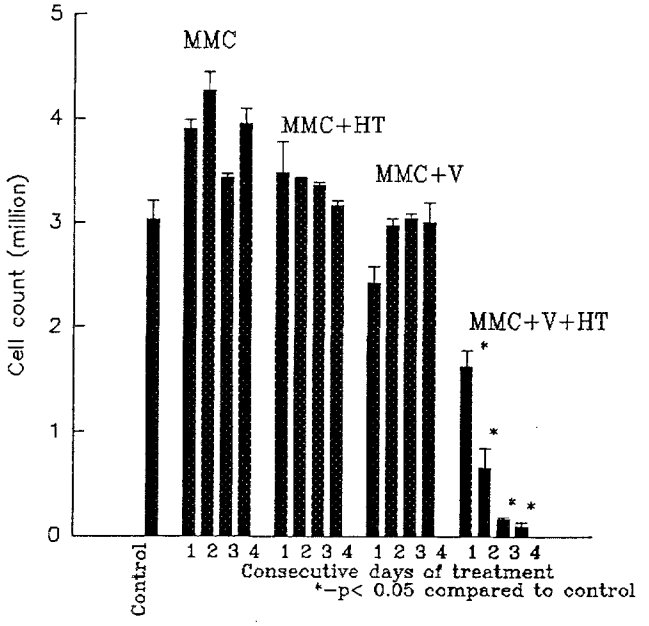

Fig. 4. Effect of MMC (0.01 μg/ml), verapamil (*V*), and hyperthermia (*HT*) on AGS cells – experiment 4

cantly different from the results of 2 days of administration of MMC at high concentration ($P < 0.05$, Fig. 2).

After 1 day, the joint administration of MMC (1.0 μg/ml) with hyperthermia resulted in a significant decrease in cell count of 31.8% in comparison with the control value ($P < 0.05$, Fig. 2). Moreover, 2, 3, and 4 days of combined MMC and hyperthermia treatment caused a significant decrease in cell count by 60.5%, 65.3%, and 71.6%, respectively, as compared with the control value. When the cell counts obtained after the joint administration of MMC with hyperthermia were compared with those recorded after MMC treatment only, a trend toward the enhancement of MMC's growth-inhibitory effect on AGS cell by hyperthermia was noted but was not significant ($P > 0.05$, Fig. 2).

Combined treatment with MMC (1.0 μg/ml) and verapamil for 1, 2, 3, and 4 consecutive days did cause a significant decrease in cell count by 68.9%, 78.8%, 78.5%, and 88.4%, respectively, in comparison with the control value ($P < 0.05$, Fig. 2). Verapamil also significantly enhanced the growth-inhibitory activity of MMC against AGS by 60.4%, 57%, 44.7%, and 67.5%, respectively, after 1, 2, 3, and 4 consecutive days of treatment as compared with MMC alone ($P < 0.05$, Fig. 2).

The joint administration of MMC at high concentration with verapamil in the presence of hyperthermia for 1, 2, 3, and 4 days did cause a significant decrease in the number of cells by 82.1%, 96.4%, 98%, and 97.8%, respectively, in comparison with the control value ($P < 0.01$, Fig. 2), but there was no difference when these results were compared with those obtained following the joint administration of verapamil and hyperthermia ($P > 0.05$, Fig. 1).

MMC at both intermediate (0.1 μg/ml) and low concentrations (0.01 μg/ml) did not cause any change in the cell count ($P > 0.05$, Figs. 3, 4). Verapamil or hyperthermia alone did not influence the effect of MMC at these concentrations on the AGS cell line. Combined employment of MMC with verapamil in the presence of hyperthermia did cause a significant growth-inhibitory effect on AGS cells, but the cell counts obtained did not differ from those recorded after the application of verapamil and hyperthermia alone ($P > 0.05$, Figs. 3, 4).

Discussion

Greater sensitivity to heat of different types of malignant tumors over normal tissues was first reported in 1903 by Jensen [12] and Loeb [19] independently. Later data based on utilization of rodent models have confirmed that hyperthermia alone can inhibit the survival of some experimental malignant cell lines in a time- and temperature-dependent manner [1, 30]. Results reported by other investigators have demonstrated that the response of human cells to hyperthermia is substantially different from that of rodent cells and that most human cells are more resistant to heat-induced killing when hyperthermia is applied at temperatures above 42° C [1a, 9a, 27].

Hyperthermia can enhance the cytotoxic effect of many chemotherapeutic agents in vitro [16, 29, 32]. The ability of hyperthermia to enhance the cytotoxic effect of MMC has previously been shown in vitro [2]. Intraperitoneal hyperthermic perfusion with MMC in patients with gastric cancer has resulted in the complete death of tumor cells in the abdominal ascites and of cell implants on the peritoneum [8]. Our study showed that hyperthermia did not enhance the cytotoxic effect of intermediate or low doses of MMC. When hyperthermia was used with a high concentration of MMC, a trend toward an enhancement of growth inhibition was noted.

Some in vitro studies have shown that the activity of MMC is potentiated by verapamil [6, 27], whereas other investigators have found no effect for this combination

[10]. It has also been shown that verapamil can completely reverse Adriamycin resistance, but reversal of MMC resistance was only partial. In vivo data have demonstrated a significant enhancement of the cytotoxic effect of various chemotherapeutic drugs by verapamil [23].

Our results showed that verapamil alone had no influence on the growth of AGS cells. A significant growth-inhibitory effect on AGS cells by verapamil and hyperthermia was noted, however, which appeared to be time-dependent (Fig. 1). In our study the growth-inhibitory effect of MMC at a high concentration on AGS cells was dramatically enhanced by verapamil (Fig. 2). When a combination of MMC and verapamil was applied in the presence of hyperthermia, a significant inhibition of the growth of AGS cells was achieved (Figs. 2–4), but there was no difference when these results were compared with those obtained using verapamil with hyperthermia alone (Fig. 1). Neither verapamil nor hyperthermia showed any cytotoxic effect when used in combination with intermediate or low doses of MMC.

This study did not show any significant enhancement of the antiproliferative effects of MMC, but the interaction of verapamil and hyperthermia in inhibiting the growth of AGS cells was noteworthy. In the future, in vivo studies will examine the therapeutic potential of this combination therapy in animal models.

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