The Effect of Megestrol Acetate on Growth of HepG2 Cells In vitro and In vivo

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ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) is generally considered as a sex hormone-dependent tumor, and hormonal therapy has been proposed as a strategy for the treatment of HCC. The aim of the study is to investigate the effect of megestrol acetate, a synthetic progesteronal agent, on growth of HepG2 cells in vitro and in vivo.

Experimental Design: Cell growth in vitro was assessed by a colorimetric method, and cell growth in vivo was assessed by tumor volumetrics.

Results: Megestrol acetate was shown to inhibit the growth of HepG2 cells in vitro in dose- and time-dependent manners with an IC50 of 260 μM (24-h incubation). The growth of HepG2 cell-transplanted tumors in nude mice was also inhibited by i.p. injection of megestrol acetate (10 mg/kg/day). The tumor volumes of the megestrol acetate-treated group regressed to 59% of controls by week 6 and to 41% of controls by week 13. Apoptosis following G1 arrest was observed in megestrol acetate-treated cells and may be a mechanism through which megestrol acetate inhibits HepG2 cells. Megestrol acetate was also demonstrated to have a beneficial effect on the weight gain of tumor-bearing nude mice, and the mean weight of the megestrol acetate-treated animals was higher than that of controls from week 4 of the treatment period, and the differences were statistically significant in week 5 and 6 (P < 0.05, compared with controls). No significant survival advantage was, however, demonstrated in the treatment group.

Conclusions: This study showed that megestrol acetate inhibited the growth of HepG2 cells grown in vitro and in vivo. These data provide useful information for clinical study of megestrol acetate for the treatment of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world (1). Surgery currently remains the only modality of treatment that consistently prolongs survival. However, only 10–15% is operable at diagnosis (2, 3), and the prognosis for inoperable HCC remains dismal (4, 5). Attempts such as radiotherapy (6), chemotherapy (6–8), and immunotherapy (9) have not been proven effective in the majority of patients.

There has been sustained interest in hormonal therapy in HCC. Both clinical observations and laboratory investigations have suggested that HCC is a sex hormone-dependent tumor, namely its male predominance (10), an apparent relationship to steroid hormones (11, 12) and the altered status of sex hormone receptors (13, 14). New therapeutic approaches with hormonal agents have thus been attempted in the treatment of HCC. Glucocorticoid antagonists such as progesterone and RU486 suppress α-fetoprotein expression in human hepatoma cell lines (15, 16). Octreotide has also been shown to be effective in inhibiting growth of HCC cell lines grown in vitro and in vivo through an unknown mechanism (17). The data supporting the use of these agents in clinical practice have however remained weak.

Tamoxifen has similarly generated a great deal of interest as a possible therapeutic modality in HCC, and a number of randomized controlled trials have been carried out. Although estrogen receptor (ER)-positive HCC is known to respond to tamoxifen (18, 19), there is increasing evidence that many HCCs are ER negative (20, 21). Although initial results were conflicting, later and larger multicenter trials have shown that tamoxifen has no role in the treatment of HCC (4, 22–25).

Megestrol acetate is a synthetic progesteronal agent with multiple drug actions. As a potent antiestrogen agent that acts at the postreceptor level and thus independent of ER, megestrol acetate is used in the second-line management of carcinoma of the breast. It has been reported to cause minor reduction of tumor size and prolonged survival time in HCC (26). A small controlled trial has shown that megestrol acetate favorably influence the course of advance HCC and improve patient survival (27). Quite apart from its antiestrogen mechanisms, megestrol acetate impacts positively on the quality of life in patients with advanced malignancy (28).

The direct effect of megestrol acetate on the growth of HCC cells has, however, not been previously reported, although such studies are crucial to provide experimental support for the clinical studies of megestrol acetate in the treatment of HCC. We report here the results of a study aimed at investigating the effects of megestrol acetate on human HCC cells (HepG2) in vitro and in vivo.

MATERIALS AND METHODS

Materials. Megestrol acetate was purchased from Sigma Chemical Co. (St. Louis, MO). Human HCC cell line, HepG2, was obtained from American Type Culture Collection (American Type Culture Collection, Manassas, VA). Six-week-old
Results in Vitro. The addition of megestrol acetate in cultures of HepG2 cells showed dose-dependent inhibition on growth of the cells. The dose-survival curve is shown in Fig. 1A. The IC\textsubscript{50} for megestrol acetate was calculated by a linear regression. The IC\textsubscript{50} of 260 μM megestrol acetate also showed time-dependent inhibition on the growth of the human liver tumor cells (Fig. 1B).

Effects of Megestrol Acetate on Growth of HepG2-Transplanted Tumors in Vivo. As shown in Fig. 2, tumor volumes in nude mice were not affected by treatment of megestrol acetate in the first 5 weeks. However, from week 6 of megestrol treatment, a significant suppression of tumor growth was observed. The tumor volumes of the megestrol acetate-treated group regressed to 59% of controls at week 6 and to 41% of controls at week 13 (P < 0.05 compared with controls). The megestrol acetate-induced reductions of tumors are progressive during the period of treatment.

Effects of Megestrol Acetate on Cell Cycle Progression. Cell growth and inhibition are mediated by cell cycle progression (33, 34). On the basis of the above results showing the
inhibitory effects of megestrol acetate on the human liver tumor cells grown in vitro and in vivo, the effect of megestrol acetate on cell cycle progression was also examined as a sequential step to understand the possible mechanisms involved in the inhibitory effects of megestrol acetate. As shown in Fig. 3, treatment by 50–400 μM megestrol acetate had no significant effects on cell cycle progression at 6 h. However, G1 arrest of the cells was demonstrated at the incubation time of 12 h in the megestrol acetate-treated group, and this megestrol acetate-induced G1 arrest was more obvious at 24 h when 50 μM megestrol acetate was used. When higher concentrations of megestrol acetate were used, megestrol acetate-treated cells revealed apparent sub-G1

Fig. 1  Dose- and time-dependent inhibition of HepG2 cells by megestrol acetate. HepG2 cells were subcultured in each well of 96-well plates and incubated overnight for their attachment. For the dose-dependent study, aliquots of a stock solution of megestrol acetate (20 mM) were added to the wells to make final concentrations of 25, 50, 100, 200, 400, 600, and 800 μM, respectively, and the incubation was carried out in 37°C for 24 h (A). For the time-dependent study, 200 μM megestrol acetate were used, and incubation was carried out at 37°C for 6, 12, 24, 36, and 48 h, respectively (B). Cell survival was then determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method as described in “Materials and Methods.” Points are means ± SD of at least triplicates.

Fig. 2  Effects of megestrol acetate on HepG2-transplanted tumors in nude mice. HepG2 cells were transplanted as a solid tumor in nude mice, and volumes of the tumors were measured weekly as described in “Materials and Methods.” The tumor-bearing nude mice received peritoneal injections of megestrol solution (10 mg/kg/day) and injection water for control. Points are means (±SD) of tumor volumes in megestrol acetate-treated and control groups (n = 9). *, P < 0.05, compared with control.

Fig. 3  Flow cytometric analysis of HepG2 cells treated with megestrol acetate. Exponentially growing cells were incubated with 50–400 μM megestrol acetate for 6–48 h. Cells were fixed, stained with propidium iodide, and analyzed by flow cytometry as described in “Materials and Methods.” Cells in each phase were analyzed using WinMDI multicycle software. Results were from a representative one of three independent experiments.
peaks at 24 h. The cell numbers in these sub-G₁ peaks were increased by megestrol acetate treatment in both dose- and time-dependent manners (Fig. 3).

**Determination of Apoptosis in Megestrol Acetate-Treated Cells.** Apoptotic cells with fragmented DNA should appear in the sub-G₁ peak, and this has been considered as a marker of apoptotic cells (35). However, the sub-G₁ peak does not necessarily indicate DNA oligonucleosomal fragmentation because cells with diminished amount of DNA and some cellular debris may also accumulate in the sub-G₁ region (36, 37). Therefore, to confirm the presence of apoptotic cells in the sub-G₁ region as showed in Fig. 3, ligation-mediated PCR was performed to detect DNA oligonucleosomal fragments, which are typical features of apoptotic cells. As shown in Fig. 4, DNA laddering was observed in the HepG2 cells treated with 300 and 400 µM megestrol acetate for 24 h. These results agreed with the flow cytometry data and showed that megestrol acetate could induce apoptosis of HepG2 cells, and this appears to be a mechanism for the inhibitory effects of megestrol acetate on the growth of the cells.

**Effects of Megestrol Treatment on the Hosts of Transplanted Tumors.** As shown in Fig. 5, the mean of body weights of the megestrol acetate-treated animals were higher than that of controls from week 4 of treatment. However, significant differences in weights were only observed in weeks 5 and 6 of treatment (P < 0.05, compared with controls). The means of body weights of both control and treatment groups increased gradually until week 6 of treatment and then decreased progressively until the death of the animals.

The survival time of individual animal was recorded, and survival curves of the test and the control groups were shown in Fig. 6A. No obvious changes were observed in the survival curve of the megestrol acetate-treated group when compared with the control group. There was neither any significant difference in average survival time between the two groups (Fig. 6B).

**DISCUSSION**

Megestrol acetate has been used clinically for the treatment of malignancies, including endometrial carcinoma, ovarian cancer, breast cancer, prostate cancer, renal cell carcinoma, and malignant melanoma for >30 years (28, 38, 39). Treatment with
megestrol acetate is most relevant in sex hormone-related cancers such as breast and ovarian cancers (40–42) and gives the best results. HCC has also been shown to be sex hormone dependent (10–14), and megestrol acetate may potentially be a useful treatment modality for HCC. However, when compared with ovarian and breast cancers, much fewer studies have been done on HCC with respect to treatment with hormonal agents. The results obtained from the present study will be useful for consideration of clinical studies on the therapeutic effects of megestrol acetate on HCC. We have shown that megestrol acetate inhibited the growth of HepG2 cells in vitro in dose- and time-dependent manners (Fig. 1, A and B). These effects were also observed on HepG2 cell-transplanted tumors grown in vivo (Fig. 2). Consistent with our results, megestrol acetate has been shown to inhibit growth of endometrial carcinoma and ovarian cancer cells grown as xenograft models (43–45). The importance of sex hormone receptors in growth of sex hormone-dependent tumors and the value of hormonal manipulations for the treatment of these cancers are, at present, not well defined.

Clinical studies relating the expression of progesterone receptors (PgRs) to the clinical features of tumors and their response to hormone therapy and prognosis have yielded varying results (46–50). In experimental animal models, however, it has been shown that megestrol acetate could only inhibit the growth of PgR-positive tumors but not PgR-negative tumors (43–45). These results suggested the roles of PgR in the regulation of tumor growth, and megestrol acetate may affect the growth of cancer cells through PgRs. In this study, however, PgR protein could not be detected in HepG2 cells by Western blotting using an anti-PgR antibody (sc-810; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and nor could PgR mRNA be detected by reverse transcription-PCR in three independent experiments (data not shown). These data suggested that PgR is not involved in action of megestrol acetate in the HepG2 cells. Megestrol acetate is known to exhibit multiple actions in human cancer cells (51) and other mechanisms such as affinity to androgen and glucocorticoid receptors, and action through these receptors (52, 53), interactions with growth factors (54), and a direct cytostatic influence on tumor cells (55) have been proposed to account for the antitumor action of megestrol acetate. Megestrol acetate has also been shown to alter the metabolism of estrogen in tissue and to suppress plasma estrogens. This antiestrogen activity may also play roles for antitumor effects of megestrol acetate (56, 57). Apoptosis may be a consequential important step of these effects because megestrol acetate has been reported to inhibit the growth of prostatic tumors by induction of apoptosis of the tumor cells (58). In our study, apoptosis was demonstrated in megestrol acetate-treated cells by induction of apoptosis of the tumor cells (58). In our study, apoptosis was demonstrated in megestrol acetate-treated cells by analysis of DNA fragments using a ligation-mediated PCR method. However, DNA ladders were not observed at lower concentrations of megestrol acetate (100 and 200 μM), whereas a significant sub-G1 peak appeared in flow cytometry analysis at these concentrations (Figs. 3 and 4). The inconsistency may be due to limitation of sensitivity of the ligation-mediated PCR DNA ladder analysis. Particles in the sub-G1 peaks could also resulted from cell debris or dead cells from necrosis or other death pathways (36, 37). These results suggest that induction of apoptosis may be a mechanism through which megestrol acetate inhibits the human liver tumor cells. As shown in Fig. 3 in dose- and time-dependent studies, megestrol acetate-treated cells were found to be arrested in G1 phase at incubation time points of 12 and 24 h when lower megestrol acetate concentrations were used. When higher concentrations of megestrol acetate were used or when incubation time was increased, cell numbers in G1 phase decreased with a progressive increase of cell numbers in sub-G1 peak, which has been shown to represent apoptotic cells. The relationship between cell cycle arrest and apoptosis is best understood in the context of G1 arrest (59, 60). Many common molecules can affect cell cycle progression and apoptosis (60). The antineoplastic phospholipid, ET-18-OCH3, has been shown to arrest BAC1.2 cells irreversibly in G1 phase by preventing their progression to S phase. This was followed by triggering of DNA fragmentation and morphological changes associated with apoptosis (61). It appears in this study that megestrol acetate arrests HepG2 cells in G1 phase and consequently causes these G1-arrested cells to go to apoptosis instead of entering into S phase.

Clinical studies have also been designed to investigate the effect of megestrol acetate treatment on appetite, body weight, quality of life, and survival (28, 42). Although no significant survival advantage was demonstrated (62), beneficial effects on appetite and body weight have been observed in megestrol acetate-treated cancer patients in the majority of studies (28, 38, 42, 63, 64). In this study, as shown in Fig. 5, the means of the body weight of megestrol acetate-treated animals were higher than that of controls from week 4 of treatment, and the differences were statistically significant in week 5 and week 6. In this aspect, the effect of megestrol acetate on nude mice appear to be similar to that of human studies (28, 38, 42, 63, 64). Preliminary studies suggest that progesterone agents down-regulate interleukin-6, an inflammatory cytokine widely implicated in cancer-associated anorexia and weight loss. Decreased interleukin-6 may be the reason for improvement of cachexia and anorexia in cancer patients, although this was not supported in a later study (65). The mechanism through which megestrol acetate improves appetite and abrogate weight loss remains unclear. In our study, the beneficial effects of megestrol acetate on weight gain in nude mice could be partially attributed to the regression of tumors.

As discussed above, in the nude mice model, megestrol acetate could inhibit growth of tumors and improve the overall condition of the hosts. However, as shown in Fig. 6A, survival of tumor-bearing nude mice was not affected by megestrol acetate treatment, and the difference of average survival time (weeks) in megestrol acetate-treated and control groups was not significant (Fig. 6B). Because the life span of nude mice is only 4–6 month, the limitation of the model is that the late stages of the follow-up period overlaps with the naturally declining body conditions of the hosts as the animals approach the end of their natural life spans. Tumor necrosis occurred in some of the treated animals from week 15 of treatment (data not shown). Therefore, the effects of megestrol acetate treatment on the survival of the hosts may not be reflected because of the relatively short follow-up period, which is attributed to short life span of the host animals.

In this study, the IC50 of 260 μM for megestrol acetate was much higher when compared with 42.8 μM for cisplatin (29), which is a widely used anticancer drug for treatment of human
malignancies, including HCC. This suggests that the direct effect of megestrol acetate on the growth of HepG2 cells can only be achieved at higher dosages, and high dosages are needed clinically for inhibition of growth of HCC in patients. The high IC50 for megestrol acetate value may also explain the equivocal and discrepant results of the limited number of studies using megestrol acetate for the treatment of inoperable HCC (26, 27).

In conclusion, this study showed that megestrol acetate could inhibit growth of HepG2 cells in vitro and in vivo. The induction of apoptosis preceded with the G1 arrest of these cells by megestrol acetate is a possible mechanism for this inhibition. These data provide useful information for clinical studies using megestrol acetate in the treatment of HCC.

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