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Estrone and progesterone inhibit the growth of murine MC38 colon cancer line

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ABSTRACT

The unsatisfactory effectiveness of reference chemotherapy in colon cancer (fluorouracil – FU) results in continuous search for agents, which could enhance the action of FU. Some epidemiological data such as a decreased risk of colorectal cancer among menopausal women receiving hormonal replacement therapy indicate the role of female sex hormones in the pathogenesis of this disease.

The aim of this study was to examine the direct effects of various concentrations of estrone and progesterone (10^{-4} to 10^{-12} M) applied alone or together with FU on the growth of murine MC38 colon cancer *in vitro*.

Estrone inhibited MC38 cancer growth in a wide range of concentrations (10^{-12} to 10^{-4} M) with similar potency and at some concentrations (10^{-6} and 10^{-4} M) augmented also the cytotoxic action of FU. Progesterone induced MC38 cancer growth inhibition at high concentrations (10^{-5} to 10^{-4} M) in dose- and time-dependent manner but it did not intensify antineoplastic effect of FU. A weak inhibitory effect of progesterone was also observed for lower concentrations (10^{-5} to 10^{-10} M) in long lasting cultures (72 h).

The results indicate that estrone and progesterone inhibit the MC38 cancer growth and that estrone increases also the cytotoxic effect of FU, what confirms the role of female sex steroids in modulation of colon cancer growth.

1. Introduction

In the developed countries colorectal cancer is one of the most common cancers [1]. Chemotherapy is an important part of treatment in this neoplasm, because approximately 30% of all patients with colon cancer have metastatic disease at diagnosis, and 50% of early-stage patients will eventually develop metastatic or advanced disease [2]. For about 50 years, fluorouracil (FU) has remained the main chemotherapeutic agent in this cancer. Unfortunately, the response to FU is observed only in 10–15% patients [3]. Its combination with leucovorin (LV) enhanced the mean response ratio to 23% and became the standard regimen in adjuvant and palliative chemotherapy of this disease [4]. During the last decade FDA (Food and Drug Administration) has approved six new drugs for treatment of advanced stages of colon cancer. Three of them belong to cytotoxic agents: irinotecan (1996), oxaliplatin (2002), oral formulation of fluorouracil – capecitabine (1998), and the other three to monoclonal antibodies: bevacizumab targeting vascular endothelial growth factor (2004), cetuximab (2004) [5] and panitumumab (2006) [6], both directed against the epithelial growth factor receptor. Although the new drugs prolonged the median survival from 12 months (FU + LV) to about 21 months, the effectiveness of the therapy is still unsatisfactory and the treatment of metastatic disease remains palliative [5]. Therefore, there are still researches carried on for new substances enhacing the antineoplastic effect of FU, including cytostatic drugs as well as other biomodulators.

The role of estrogens in colon carcinogenesis has been discussed for many years. This association was suggested by some epidemiological data such as an age-specific occurrance of colorectal cancer in women [7], protective influence of increasing parity and sex differences in site specific incidences of the neoplastic lesion in bowels [8]. Moreover, a lot of observational studies [9] and the last randomized primary prevention trial – the Women's Health Initiative (WHI) [10] showed a decreased risk of colorectal cancer among menopausal women receiving hormonal replacement therapy (HRT). Although generally most of these protective effects are attributed to the action of estrogens, it could not be excluded that the positive effects are connected also with the other female sex hormone – progesterone. Such a hypothesis could be supported by the fact that in the WHI study the reduction in colon cancer risk was observed only in users of combined estrogen and progestin HRT [10], whereas in women after hysterectomy, receiving only estrogens, the protective effect was not noticed [11].

In spite of many facts implying involvement of female sex steroids in colorectal carcinogenesis, the number of studies examining the direct effect of these hormones on the colon cancer growth is limited and gives often opposing results. Studies *in vitro* [12] and experiments with ovariectomized animals [13,14] have shown

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that estradiol (E2), which is the most often examined estrogen, can inhibit as well as stimulate the growth of this neoplasm. The influence of the weaker estrogen – estrone (E1) and progesterone on the colon cancer growth has been hardly ever a matter of study.

Thus, the aim of this paper was to examine the direct effects of estrone and progesterone applied alone or together with FU on the murine MC38 colon cancer growth *in vitro* assessed by two colorimetric methods reflecting changes in proliferation and apoptosis.

2. Materials and methods

Murine Colon 38 cancer cells were used in the study. The cells were routinely grown in a humidified incubator at 37 °C with 5% CO_2 in RPMI 1640 medium (Sigma), supplemented with: 25 nM Hepes buffer (Sigma), 4 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin solution (Sigma), 2 g/l sodium bicarbonate (Sigma) and 5% fetal calf serum (FCS, Biochrom). The cells were passaged every 7 days with 0.05% trypsin/0.02% EDTA (Trypsin-EDTA, Sigma) and the medium was changed every 3–4 days.

After one of trypsinisation procedures, the cells were plated (15 000–60 000/well depending on time of culture and colorimetric method; e.g. 20 000/well for 72 h culture in Mosmann method) into 96-multiwell plates (Nunc). To avoid the influence of estrogens and estrogen-like substances, the cells were cultured in phenol red-free RPMI 1640 medium (Sigma) supplemented with 5% charcoal-treated hormone free FCS (Biochrom). After preincubation (24 h) the cells were cultured for further 4, 12, 24 or 72 h in the presence of various concentrations of the examined substances (fluorouracil, estrone, progesteron) applied alone or jointly.

To assess the interaction with sex steroids, fluorouracil (Fluorouracil, Roche) was used at the concentration of 1 μM, which was chosen from a wide range of examined concentrations (1–1024 μM; data not shown) as inducing the minor cancer growth inhibition. The control group for FU received medium. Estrone (Estrone 3-hemisucinate, Sigma) and progesterone (Progesterone cell culture tested, Sigma) were dissolved in absolute ethanol in proportion of 1 mg/1 ml and were examined in the range of concentrations from 10^{-4} to 10^{-12} M. The control group for sex steroids received an adequate concentration of ethanol vehicle without the examined substance (maximum ethanol concentration: 3.7 vol%).

Cancer growth was assessed by the two colorimetric methods:

- Mosmann method (Easy for You, The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica Poland) based on the measurement of total metabolic activity of cultured cells, which reflects changes in proliferation and cell death;
- Method based on bromodeoxyuridine incorporation into cell nuclei (Cell Proliferation ELISA, BrdU; Roche Applied Science) directly correlating with cell proliferation.

In the BrdU incorporation method, BrdU was added to each well 4 h prior to the experiment termination.

The intensity of reaction was estimated via measurement of optical density (OD) using an ELISA reader (λ = 450 nm). The statistical significance was determined using one-way ANOVA with post hoc LSD test (Least Significant Difference). $P < 0.05$ was considered as a statistically significant difference. The correlation between cancer growth inhibition and concentrations of the examined substances or duration of cultures was determined by *r* Pearson coefficient and then the significance of differences was analyzed with the Student's *t*-test.

The results obtained in the BrdU incorporation and Mosmann methods were presented as the percentage of OD of the adequate control group.

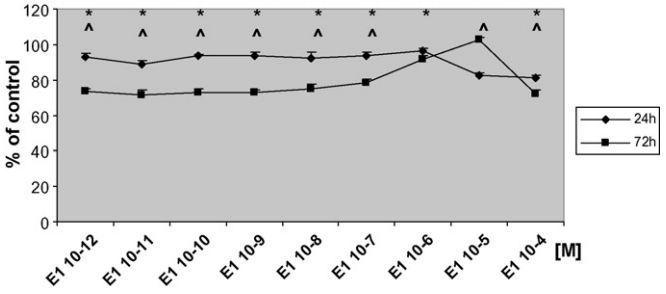

Fig. 1. The effect of estrone (E1) on the growth of MC38 cancer assessed by Mosmann method in 24 and 72 h culture. $X \pm$ S.E.M., ^$p < 0.05$ vs. control in 24 h culture; *$p < 0.05$ vs. control in 72 h culture.

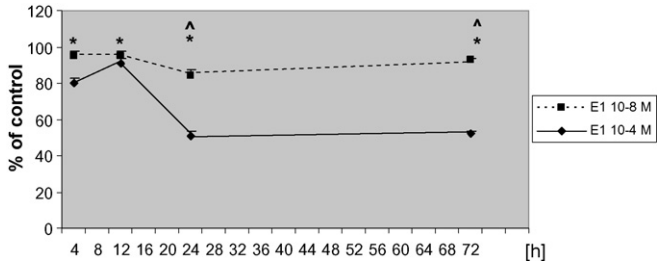

Fig. 2. The effect of estrone (E1) on the growth of MC38 cancer assessed by BrdU incorporation method in 4, 12, 24 and 72 h cultures. $X \pm$ S.E.M., *$p < 0.05$ vs. control for E1 10-4 M; ^$p < 0.05$ vs. control for E1 10-8 M.

3. Results

Estrone in a wide range of concentrations (10^{-12} to 10^{-4} M) induced similar, moderately strong inhibition of the MC38 cancer growth, which was slightly more potent after 72 h (up to 72% of control group) than after 24 h incubation (up to 81% of control group) (Fig. 1). The inhibitory effect of estrone was not observed only for the concentration of 10^{-6} M in 24 h culture and for the concentration of 10^{-5} M in 72 h culture (Fig. 1). The beginning of action was noticed as early as after 4 h incubation in BrdU method for the high estrone concentration (10^{-4} M) (Fig. 2) and in 12 h culture in Mosmann method for both studied concentrations (10^{-4} and 10^{-8} M) (data not shown). Estrone at some concentrations (10^{-6} and 10^{-4} M) enhanced the cytotoxic action of FU used at the concentration of 1 μM in 72 h culture (Fig. 3).

Progesterone inhibited MC38 cancer growth in a wide range of concentrations (10^{-10} to 10^{-4} M) in 72 h culture. Its inhibitory effect was strong at high concentrations (8×10^{-5} and 10^{-4} M; up to 17% of control group) (Figs. 4 and 5) and less potent at lower concentrations (10^{-5} to 10^{-10} M; up to 92% of control group) (Fig. 4). In 24 h

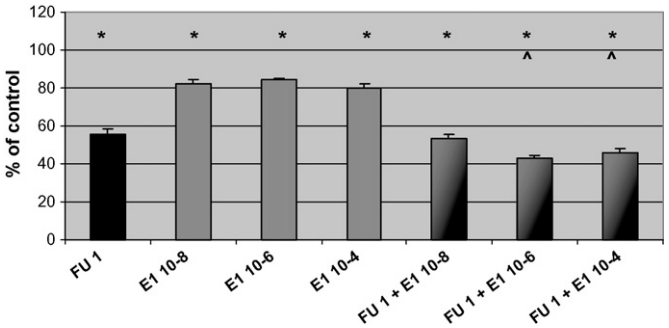

Fig. 3. The effect of estrone (E1) applied alone or jointly with fluorouracil (FU) on the growth of MC38 cancer assessed by Mosmann method in 72 h culture. $X \pm$ S.E.M., *$p < 0.05$ vs. control, ^$p < 0.05$ vs. FU 1. FU 1 – fluorouracil 1 μM; E1 10-8, E1 10-6, E1 10-4 – estrone 10^{-8} M, estrone 10^{-6} M, estrone 10^{-4} M.

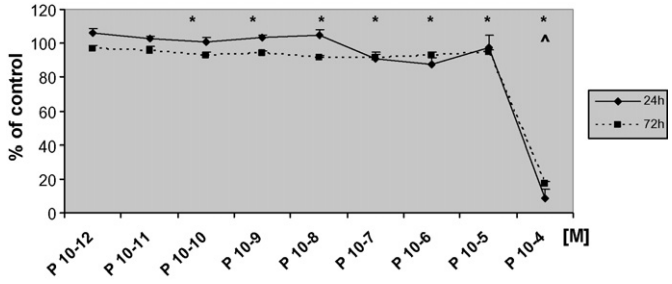

Fig. 4. The effect of progesterone (P) on the growth of MC38 cancer assessed by Mosmann method in 24 and 72 h culture. $X \pm$ S.E.M., $\hat{}p < 0.05$ vs. control in 24 h culture; $^*p < 0.05$ vs. control in 72 h culture.

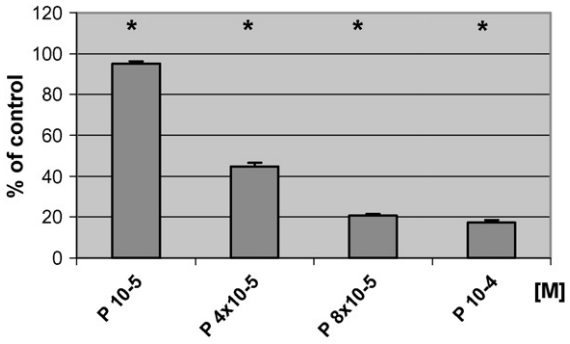

Fig. 5. The effect of progesterone (P) on the growth of MC38 cancer assessed by Mosmann method in 72 h culture. $X \pm$ S.E.M., $^*p < 0.05$ vs. control.

culture progesterone evoked the cancer growth inhibition only at the highest concentration (10^{-4} M) (Fig. 4). The beginning of its action (10^{-4} M) was observed as early as after 4 h incubation in both methods (Mosmann method – data not shown; BrdU method – Fig. 6). The inhibitory effect of the lower concentration of progesterone (4×10^{-5} M) was revealed just after 12 h incubation by Mosmann method (data not shown). Using progesterone in a narrow range of high concentrations (10^{-5} to 10^{-4} M) we observed time- and dose-response effect (Figs. 5 and 6), with r Pearson coefficient ranging from −0.67 to −0.95 (depending on the studied correlation) and $p < 0.001$. In contrast to estrone, progesterone (10^{-5}–10^{-4} M) did not intensify the cytotoxic action of FU in 72 h culture (Fig. 7).

4. Discussion

In the present study we have shown that estrone and progesterone inhibited MC38 colon cancer growth. To our knowledge, we have also revealed for the first time that estrone can potentiate the cytotoxic action of FU. In our study, estrone induced MC38 growth inhibition in a wide range of concentrations (10^{-12}–10^{-4} M) with

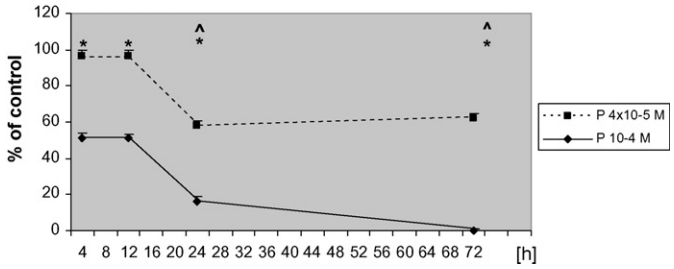

Fig. 6. The effect of progesterone (P) on the growth of MC38 cancer assessed by BrdU incorporation method in 4, 12, 24 and 72 h cultures. $X \pm$ S.E.M., $^*p < 0.05$ vs. control for P 10^{-4} M; $\hat{}p < 0.05$ vs. control for P 4×10^{-5} M.

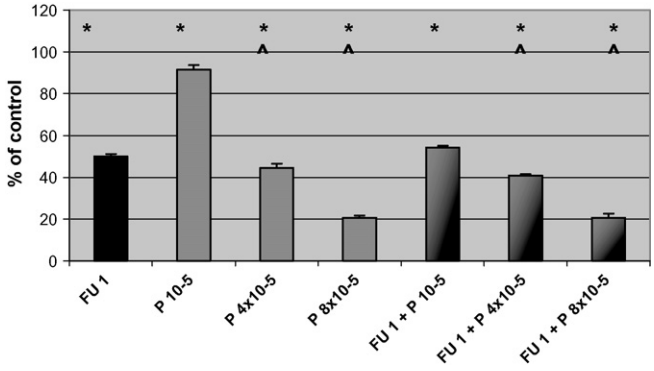

Fig. 7. The effect of progesterone (P) applied alone or jointly with fluorouracil (FU) on the growth of MC38 cancer assessed by Mosmann method in 72 h culture. $X \pm$ S.E.M., $^*p < 0.05$ vs. control; $\hat{}p < 0.05$ vs. FU 1. FU 1 – fluorouracil 1 μM; P 10-5, P $4 \times$ 10-5, P $8 \times$ 10-5 – progesterone 10^{-5} M, progesterone 4×10^{-5} M, progesterone 8×10^{-5} M.

the exception of 10^{-6} M in 24 h culture and 10^{-5} M in 72 h culture. Such an effect could be connected with a different mechanism of action for the lower (i.e. under the ineffective concentration) and the higher (i.e. above the ineffective concentration) concentrations.

Our results are consistent with reports demonstrating the inhibitory influence of estrone on some other colon cancer lines: SW620 (10^{-7} M) [15], HCT116, DLD-1 and LoVo (10^{-9}, 10^{-8} and 10^{-6} M) [16]. Interestingly, in all those studies estradiol did not evoke such an effect, except for the LoVo line. Moreover, some authors did not reveal any influence of estrone on the other colon cancer lines such as HCT8 and Caco-2 [16,17]. In our study we observed a moderate inhibitory effect of estrone used in a wide range of concentrations (from 10^{-12} to 10^{-4} M). However, in our previous study, we had demonstrated that estradiol, which is structurally very similar to estrone, evoked a strong inhibitory effect on the growth of the MC38 colon cancer line but only at high concentrations (between 10^{-5} and 10^{-4} M), being ineffective in other concentrations (10^{-12}–10^{-6} M) [18]. Moreover, some reports concerning the local estrogen metabolism, suggested even a different action of the both estrogens on colon carcinogenesis and indicated estrone as the protective factor. The key role in this process seems to play a change in 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, which is responsible for the interconversion of estradiol to estrone. According to the authors of some papers, changes in expression of 17β-HSD isoforms 2 and 4, resulting in the loss of estradiol inactivation and in the diminished protective action of estrone, can be involved in colon carcinogenesis [15,19]. As mentioned above, in our present and previous studies [18] both estrogens inhibited the growth of the examined colon cancer line, but at different concentrations and with a different potency.

In agreement with our study remains also an *in vivo* experiment, which showed that the incidence and weight of azoxymethane-induced colon tumor in ovariectomized ERαKO and wild-type female mice fed with diets containing soy protein with estrone (in a dose extrapolated from a typical human HRT dose) were lower than in animals receiving only soy protein [20].

The preventive influence of estrone on colon carcinogenesis is also suggested by many studies which revealed a risk reduction of this neoplasm among women receiving HRT [9]. It is worth mentioning that estrone is the most popular estrogen used in majority of those studies. Premarin administered in WHI trial [10] contains conjugated equine estrogens in which estrone sulfate makes up about 45% (equiline sulfate – 25% and the remaining 30% – other estrogens) [21]. Moreover, even in the case of orally administered estradiol, only its small part (5%) is systemically bioavailable, and

most of it is metabolized in the liver to estrone and subsequently converted and stored as estrone sulfate [21]. Therefore, it can not be excluded that the protective effect of HRT on colon cancer, which is commonly attributed to the action of estradiol, is connected also with the influence of estrone. Moreover, some data indicates that only combined (estrogen and progestin) HRT reduced the risk of colon cancer [10,11,22,23], which suggests the protective effect of progestin compounds. This is in accordance with our study, which showed a strong antiproliferative action of progesterone at high concentrations (8×10^{-5} and 10^{-4} M) and a less potent effect at the lower concentrations (10^{-5} to 10^{-10} M). On the contrary, in other studies progesterone (10^{-5} to 10^{-9} M) did not induce the growth inhibition of colon cancer lines such as DLD-1 [24], SW620 [15] and LoVo [25]. Such opposing results in the literature concern also the other sex hormones such as estrone (mentioned above) and estradiol, which was shown to inhibit as well as stimulate the growth of various colon cancer lines [12]. These conflicting data may be due to different types of cell lines, different estrogen/progesterone receptors pattern and various concentrations of sex hormones.

As mentioned above, our study investigated two sex steroids, which are not only hormones produced by an organism but also the most common ingredients of HRT. Among many estrone and progesterone concentrations inhibiting the MC38 cancer growth in our study, one was a concentration of 10^{-10} M, which corresponds to the physiological serum concentration of these steroids during the reproduction period or during the use of HRT (40–300 pg/ml) [26]. Their inhibitory effect at this concentration was moderately strong for estrone and weak for progesterone. However, estrone, as the less potent estrogen, seemed to be a safe agent as a potential antineoplastic drug.

Additionally, in our study were also performed experiments examining the combined effect of FU and the steroids (estrone and progesterone), which so far has not been studied. It was shown that estrone in some concentrations increased the cytotoxic effect of FU. Since our study was only a preliminary one in this matter, the mechanism of this interaction (synergistic or additive) was not precisely determined. Moreover, in our experiments, progesterone was not found to intensify the action of FU, but at high concentrations (4×10^{-5} and 8×10^{-5} M) inhibited MC38 cancer growth more effectively than FU (1 μM). Therefore, although the combined effect of FU with progesterone at the concentrations $4 \times 10^{-5}$and 8×10^{-5} M was more potent than FU alone, it was not treated as the intensification of FU action induced by progesterone. In this case the effect of progesterone at a concentration of 8×10^{-5} M given alone was stronger than the action of FU alone and there was no significant difference between their combined effect and the strongest factor alone (progesterone 8×10^{-5} M). A similar result was found in the case of the combination of FU with progesterone at a concentration of 4×10^{-5} M.

In our study we have also tried to explain the mechanism of steroid action using two different colorimetric methods for the assessment of cancer line growth, of which one (the Mosmann method) reflected cell viability depending on both – cell proliferation and apoptosis, while the other method (based on BrdU incorporation) correlated directly with cell proliferation. Unexpectedly, in our experiments, the hormones showed a similar effectiveness in cancer growth inhibition in both methods, and existing differences between these methods did not seem to have any specific tendency. This could confirm that the inhibitory influence of estrone and progesterone on the MC38 cancer growth is connected exclusively with their antiproliferative effect. However, it should be emphasized that a decrease in BrdU incorporation might be at least in part also due to a decline in cell number caused, for example, by cell apoptosis. Thus, both methods used in our experiments for the assessment of cancer growth were not sufficient to determine precisely the mechanism of sex steroid action, but the similarity of obtained results confirmed their credibility.

5. Conclusions

Summing up, we have shown in this paper that estrone and progesterone inhibited MC38 colon cancer growth and that estrone intensified cytotoxic effect of FU. Our results indicate that female sex steroids are involved in modulation of colon cancer growth. It suggests the potential use of these hormones, especially estrone, in colon cancer therapy, which could enhance the efficacy of FU. Further studies are needed to elucidate whether this hypothesis is true in human colon cancer and whether it is worth clinical application.

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