Endocrine factors influencing melanoma progression

PhD thesis booklet

Judit Dobos

Semmelweis University
PhD School of Pathological Sciences

Project leader: Andrea Ladányi PhD

Official reviewers: Borbála Vincze PhD
László Buday PhD

Examination committee members: Prof. Tibor Kerényi
Beáta Somlai PhD
Gábor Réz PhD

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INTRODUCTION

According to recent findings, beside cancers traditionally considered as hormone-dependent, several other tumor types show different behavior in the two sexes, indicating the possible role of endocrine factors in the course of these diseases. There are gender differences in the biology of lung cancer. Estrogen receptor is present in non-small cell lung cancer (NSCLC) and estrogen has an effect on NSCLC cell lines *in vitro* and in animal models as well. Gender is a significant prognostic factor in colon tumors and in hepatocellular carcinoma. Thyroid neoplasms occur more often among women, but the prognosis is worse in men. In these diseases and in a number of others as well estrogen and androgen receptors are present, the epidemiological data show survival advantage in women and an increasing number of experiments suggest the influence of estrogen on tumor cells.

The incidence of melanoma has been increased in the last few decades. The localization is different in the two sexes. In women it appears mostly on the extremities, while in men rather on head, neck and trunk. Among men the nodular type occurs more often. In women the skin and the lymph nodes, in men viscera are more often the first sites of metastasis. These

factors may all contribute to the poorer outcome of the disease observed in men. The role of age in prognosis is not clear, but a shorter survival of women after menopause has been described.

Previous experiments of our team showed that intrasplenic injection of human melanoma cells resulted in a significantly higher number of liver metastases in male than in female SCID mice.

Studies applying biochemical methods for the detection of estrogen receptors gave positive results in some cases, while approaches using specific monoclonal antibodies generally failed to demonstrate the presence of estrogen receptor in melanoma tissues. Presumably a number of melanoma cases express the functional receptor at concentrations too low to detect by immunohistochemical methods.

Steroid receptors are members of the nuclear receptor family. After binding their specific ligand they act as transcription factors. After phosphorylation the receptor dissociates from the chaperon protein and undergoes conformational changes. As dimers they bind to specific DNA sequences of the promoters of steroid-responsive genes and activate or inhibit transcription in complex with regulator proteins. This is the classic route of the genomic effects of steroids. Through the non-classic route they recruit other

transcription factors and act as stabilizators of the complex. Beside genomic ones, steroid hormones also have non-genomic effects, when the membrane receptors are activated and act through second messengers.

One possible mechanism behind the observed female superiority in survival of melanoma patients is the tumor growth inhibitory effect of 2-methoxyestradiol ($2ME_2$). This is the only estradiol metabolite devoid of estrogenic activity *in vivo* and has no known physiological function. Its activity is independent of the presence of estrogen receptors.

$2ME_2$ is a known antiangiogenic and antitumor agent. It has been demonstrated to induce G_2/M cell cycle arrest and apoptosis in a variety of tumor cell lines *in vitro*, and to inhibit the growth and vascularization of tumors. Its antiproliferative activity has been attributed to the disruption of microtubule function. It has also been suggested that $2ME_2$ regulates apoptosis by influencing caspase activation, upregulation of the death receptor 5 protein or p53, or phosphorylation and inactivation of Bcl-2.

AIMS

- Analysis of sex hormone receptor expression at protein and mRNA level on human melanoma cell lines
- Examination of the *in vitro* effects of sex steroids on melanoma cells
- Analysis of the expression and function of glucocorticoid receptor
- Examination of the pattern of colony formation of human melanoma cells in SCID mice with regard to the gender of the host
- Studies on the biological effects of $2ME_2$ *in vivo* and *in vitro*

METHODS

To detect sex hormone receptors in human melanoma cell lines and frozen tumor samples, we used immune and molecular techniques (immunocytochemistry, immunohistochemistry, flow cytometry, nested PCR, quantitative PCR). We studied the *in vitro* effects of sex steroids and the glucocorticoid dexamethasone on proliferation, adhesion and migration of human melanoma cells.

The organ preference of *in vivo* colony formation of melanoma cells was determined in spleen-liver metastasis model, lung colonization assay and after intracardiac injection.

We measured the growth inhibitory effect of $2ME_2$ in proliferation assay, and the rate of apoptotic cells and those arrested at the G_2/M phase of the cell cycle by flow cytometry. The mechanism of apoptosis was mapped using caspase inhibitors and measuring the changes in mitochondrial membrane potential. The effect of $2ME_2$ on the microtubules was studied by the immunohistochemical detection of α-tubulin. We examined the effect of $2ME_2$ on the growth, colony formation and apoptosis of human melanoma cells *in vivo* in spleen-liver metastasis model.

RESULTS

With flow cytometric analysis we showed the presence of ERα in a few percent of human melanoma cells, while AR was undetectable in the studied cell lines. We failed to detect steroid receptors with immunocytochemistry both in cell lines and in frozen sections from xenograft models. At mRNA level there were orders of magnitude differences in ERα expression among our melanoma cell lines, but it did not reach the

expression level of MCF7, the positive control breast cancer cell line. We found the same results in 5 cell lines in the case of PR. ERβ was present in all of our melanoma lines, though the expression was weaker compared to that of ERα in the positive controls. AR was detectable only at very low level. Sex hormones did not influence the *in vitro* features of the human melanoma cells considerably.

We examined the presence and function of glucocorticoid receptor, another member of the nuclear receptor family. We detected GCR both at protein and at mRNA level in the case of all melanoma lines studied. The presence of GCR has not yet been demonstrated by immunocytochemistry in human melanoma lines, though it was detected in mouse melanoma cells (B16). With quantitative PCR we showed that the expression level of GCR in melanomas is significantly lower compared to normal skin or nevus. Dexamethasone was effective in influencing the *in vitro* features of melanoma cells (proliferation, adherence, migration) only at high doses or using prolonged treatment.

Despite the lack of significant effects of sex steroids on melanoma cells *in vitro*, our previous experiments showed that intrasplenic injection of human melanoma cells resulted in a much higher number of liver colonies in male than in female

SCID mice. There was no gender difference in lung colonization after injection of melanoma cells into the tail vein. Intracardiac injection resulted in colonies in different organs, but we observed difference between the two sexes only in the case of the liver. In males there were more colonies than in females, and orchiectomy decreased, while ovariectomy increased their number.

As this gender difference appeared only in the case of the liver, we examined the role of 2-methoxyestradiol, an endogenous metabolite of estradiol that is produced mainly in the liver, has a known antitumor effect and exerts its activities independently of the steroid receptors.

In all of the 8 human melanoma lines studied $2ME_2$ caused inhibition of proliferation by cell cycle arrest at the G_2/M phase and apoptosis induction, which implies that sensitivity to this metabolite is a general characteristic of melanoma cells. This observation might be important in the light of the inherent resistance of melanomas to drug-induced apoptosis. In melanoma cells we found that mitochondria, caspase-9 and, to a lesser extent, caspase-8 were involved in the process of $2ME_2$-induced apoptosis. We found bundling of microtubules after $2ME_2$ treatment at the same concentration effective in growth inhibition.

These findings have *in vivo* relevance as well, since $2ME_2$ treatment significantly increased the number of apoptotic cells in liver colonies after intrasplenic injection of human melanoma cells and reduced the primary tumor weight and the number of liver colonies.

The fact that sex steroids failed to directly influence melanoma cells suggested that they may exert their effect on the host. Estradiol has a known effect on the endothel of capillaries and on angiogenesis. In our preliminary results, however, we found no difference between the two sexes in the density of the vasculature either in the primary tumors of the spleen or in the liver colonies.

The gender difference observed in the spleen-liver metastasis model emerged on the first day, when the number of melanoma cells in the liver showed a 3- and 8-fold decrease in male and female mice, respectively. Immune mechanisms of the host may play a role in the rapid elimination of disseminated tumor cells. The elements of the natural immune system are present even in SCID mice. According to the literature, IL-6 production by Kupffer cells and, in correlation with this, drug-induced hepatocarcinogenesis showed gender differences. Our preliminary experiments showed that selective

inhibition of Kupffer cells results an increase in the number of liver colonies in both sexes.

Malignant melanoma is the most aggressive form of skin cancer with rapidly increasing incidence rate. Despite the wide variety of therapeutic approaches tested over the years, metastatic disease is still associated with dismal prognosis due to the minimal success of systemic therapy. Therefore, there is a need for more effective treatment regimes for the management of both high-risk and metastatic melanoma. Because of its efficacy in experimental tumor models and its limited toxicity, $2ME_2$ has emerged as an attractive drug candidate evaluated in recent Phase I and Phase II clinical trials against a variety of human cancers including breast and prostate carcinoma. Based on its *in vitro* and *in vivo* antitumor effects on melanoma cells, we propose that clinical studies testing the efficacy of $2ME_2$ in patients with malignant melanoma should also be considered.

CONCLUSIONS

1. We failed to detect sex hormone receptors in considerable amount in human melanoma cell lines, and sex steroids did not significantly influence the *in vitro* features of the cells.
2. We proved the presence of glucocorticoid receptor in human melanoma cells, and showed a markedly lower expression level in melanomas compared to normal skin. Dexamethasone at high doses was effective in inhibiting proliferation.
3. In preclinical models the pattern of colony formation of human melanoma cells showed gender differences in the case of the liver but not in other organs. This phenomenon evolves at an early phase of colony formation.
4. 2-methoxyestradiol effectively inhibited melanoma cell proliferation by inducing apoptosis and cell cycle arrest at the G_2/M phase. The mechanism of action involved microtubules, mitochondrial damage and caspase activation as well.

PUBLICATIONS

Connected to thesis:

1. **Dobos J**, Tímár J, Bocsi J, Burián Z, Nagy K, Barna G, Peták I, Ladányi A. (2004) In vitro and in vivo antitumor effect of 2-methoxyestradiol on human melanoma. Int J Cancer, 112: 771-6. (IF: 4.416)
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Not connected to thesis:

1. Döme B, **Dobos J**, Tóvári J, Paku S, Kovács G, Ostoros G, Tímár J. (2007) Circulating bone marrow-derived endothelial progenitor cells: characterization, mobilization and therapeutic considerations in

malignant disease. Cytometry, Part A (accepted, IF: 3.293)

2. Döme P, Teleki Z, Rihmer Z, Péter L, **Dobos J**, Kenessey I, Tóvári J, Tímár J, Paku S, Kovács G, Döme B. (2007) Circulating endothelial progenitor cells and depression: a possible novel link between heart and soul. Mol Psychiatry (accepted, IF: 11.804)
3. Lövey J, Kenessey I, Rásó E, **Dobos J**, Vágó A, Kásler M, Futosi K, Döme B, Tímár J, Tóvári J. (2007) [Human recombinant erythropoietin-alpha increases the efficacy of irradiation in preclinical model] Hung Oncol, 51: 53-61. (in Hungarian)
4. Döme B, Tímár J, **Dobos J**, Mészáros L, Rásó E, Paku S, Kenessey I, Ostoros G, Magyar M, Ladányi A, Bogos K, Tóvári J. (2006) Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. Cancer Res, 66: 7341-7. (IF: 7.656)