



EVALUATION OF ANTIPROLIFERATIVE AND ANTIMETASTATIC EFFECTS OF HEPARIN AND ERYTHROPOIETIN ON B16F10 MELANOMA CELL LINE

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Abstract

A number of chemotherapeutic agents and treatment strategies have been developed or designed to treat cancer patients. However, chemotherapeutic regimens frequently cause side-effects, one of which is anemia, a severe clinical problem for cancer patients. Erythropoietin is commonly used to treat anemia and reduce blood transfusions in cancer patients. Another agent which has potential use in cancer therapy is heparin, a glycosaminoglycan with a negative charge, known to increase the clearance of tumor cells from the blood in mice and also has anti-metastatic effects. In this current study, we investigated the effects of rEpo and heparin either as single agents or in combination on B16F10 melanoma cells. Contrary to our expectations based on the previous in vitro and in vivo studies, we have not found a significant growth-promoting effect of rEpo on B16F10 cells. We have also not observed a significant cytotoxic effect of heparin on B16F10 melanoma cell as assayed by MTT test ($p > 0.05$). However, heparin did significantly prevent the migration/proliferation of B16F10 cell in the wound assay as compared to the control cells after 24 h of incubation ($p < 0.001$). In addition, this effect of heparin was not prevented when rEpo was present in the medium in the wound assay ($p < 0.01$ as compared to the control). These results suggest that heparin may have a therapeutic potential as an anti-metastatic drug for cancer.

Key words: Cancer, Erthropoietin, Heparin, Melanoma, Metastasis.

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INTRODUCTION

Melanoma is a very aggressive cancer arising from melanocytes which are specialized pigmented cells found in the skin. Malignant melanoma is responsible for 60-80% of deaths from skin cancers. In patients with metastatic malignant melanoma, 5-year survival is about 14%. It is well-known that even a few millimeter thick tumor may have a high risk for developing distant metastasis within 5 years. This tumor type is highly resistant to chemotherapy; therefore, advanced pharmacological treatments are urgently needed to improve patient survival and quality of life. Recent studies indicate that the incidence of malignant melanoma has been increasing over the past two decades. Unfortunately, the mechanisms leading to malignant transformation of melanocytes and melanocytic lesions are not well-understood. During the malignant melanoma development, there are complex interactions of environmental and genetic factors, several of these are dysregulation of cell proliferation, apoptosis, and cell-to-cell interactions. Recent discoveries in cell signalling have provided greater insight into the molecular biology of melanoma, which would help provide novel targeted drugs and therapeutic approaches in the near future (4,5,13).

With the advances in molecular biology techniques, a number of antiproliferative and antineoplastic agents have been obtained or rationally designed. For example, heparin, a glycosaminoglycan with a negative charge, is known to increase the clearance of tumor cells from the blood in mice and also has antimetastatic effects. This effect was completely abolished in mice with depressed natural killer (NK) cell activity (12). Fibrin formation around tumor cells protected murine tumor cells from destruction by the cytotoxic cells. When heparin prevented fibrin formation,

killer cells were highly effective (14). Angiogenesis has an important role in metastasis; heparin minimizes angiogenesis via the inhibition of vascular endothelial growth factor and platelet activating factor. Heparin also decreases the effects of matrix metalloproteinases and serine proteases which are known to have important roles in metastasis. In addition, several reports indicate that heparin inhibits tumor cell attachment to heparansulfate proteoglycans (10).

Erythropoietin (Epo) is a glycoprotein hormone normally produced by the kidney and fetal liver. The principal function of Epo is the regulation of red blood cell production. Erythropoietin binds to Epo receptors (EpoR), then initiates signaling that stimulates growth and differentiation of erythroid progenitors to increase red blood cell mass. Recombinant human erythropoietin (rEpo) has been used for treatment of anemia in chronic kidney disease and chemotherapy-induced anemia in cancer patients (1,25). Recent experimental investigations have displayed the role of Epo as a strongly anti-apoptotic agent in non-erythroid and neoplastic tissues. However, the results of a few clinical trials show that rEpo-treatment may cause a negative impact on overall survival of cancer patients. These clinical data suggest a harmful role of Epo in cancer by a possible stimulation of tumor growth. Erythropoietin may act as a tumor growth factor and accelerate the growth of tumors. Signaling pathways of Epo and EpoR have been shown to influence numerous cellular functions in both normal and tumor cells, including proliferation, to apoptosis and drug resistance. In erythropoiesis, Epo exert its effects primarily by inhibiting apoptosis of erythroblast precursors and hence, increases red blood cell mass. Thus, the efficacy of chemotherapy regimes designed to target antiapoptotic pathways may be diminished by Epo that have been shown to increase protein levels of antiapoptotic genes, such as

Bcl-XL, and decrease protein levels of pro-apoptotic proteins, such as Bax (3,7,15,24,30).

In this study, we evaluated the antiproliferative and antimetastatic effects of heparin and also tumor growth-promoting effects of rEpo on B16F10 melanoma cell line, which is a highly metastatic cell line and is also commonly used in *in vivo* tumor formation assays. It was found that heparin exerts strong anti-migratory or anti-proliferative effects on B16F10 melanoma cell line; however, rEpo neither significantly promoted the migration and proliferation of B16F10 cells, nor inhibited the effects of heparin on B16F10 cells under the experimental conditions used here.

MATERIALS AND METHODS

Materials

RPMI-1640 cell culture media, fetal bovine serum (FBS), trypsin, penicillin/streptomycin and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] were obtained from Sigma-Aldrich Inc. (Steinheim, Germany). Heparin (Vasparin) and rEpo (Eprex) were obtained from Dumlupınar University, Evliya Çelebi Research and Education Hospital (Kütahya, Turkey).

Cell culture maintenance

B16F10 melanoma cell line was obtained from Dr. Nuray Erin of Akdeniz University (Antalya, Turkey). B16F10 cell line was generated from C57BL-6 mice. And it is commonly used to form and test the *in vivo* tumors (2). The cells were cultured in RPMI-1640 (plus 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin). The medium was supplemented with 10% FBS. Stock cultures were maintained in 25 cm² Corning flasks. For the experiments, the cells were grown in 35 x 10 mm Corning plates. The cells were subcultured when they reached to about 70% confluency. B16F10 melanoma cell passage number was between 51 to 55 during the experiments.

MTT-based cytotoxicity assay

Cells (25,000) were seeded in each plate (35 x 10 mm). In the logarithmic phase of the growth, cells were treated with various doses of heparin (10, 50, 75, 100, 150 200, 300 IU/ml) or rEpo (10, 50, 75, 100, 150 200, 300 IU/ml)

for 24 h. The cells were also treated with 250 IU/ml or 500 IU/ml heparin for 48 h in a separate experiment. After the exposure period, cells were treated with RPMI-1640 media containing 0.5% FBS + 0.5 mg/ml MTT at 37°C with 5% CO₂ for 4 h. After MTT treatment, cells were incubated with 3% SDS (200 µl) + 1 ml 40 mM HCl/isopropanol for 15 min in order to dissolve the MTT-formazan crystals. The homogenate was pipetted to dissolve the MTT-formazan crystals. The homogenate was diluted and the absorbance of each sample was recorded at 570 nm.

Wound assay

B16F10 cells were grown in 35 x 10 mm petri dishes. The cells were cultured to at least 90% confluence and then starved in low serum media (0.1% FBS in RPMI-1640) overnight. Using a 200 µl sterile pipet tip, three separate wounds were scratched in each plate. Then the cells were treated with isotonic solution (control), 50 IU/ml heparin, 75 IU/ml erythropoietin or a combination of 50 IU/ml heparin + 75 IU/ml rEpo for 24 h. The migration and proliferation of cells were recorded with an inverted microscope using a 4X objective.

Statistical analysis

Each experiment was run in duplicate (n = 4 to 6). Results were analyzed with GraphPad Prism 3.03 software. The statistical differences between the samples were evaluated by one-way ANOVA and the Bonferroni's multiple comparison post-test. A p value <0.05 was considered significant.

RESULTS

As discussed in the introduction, heparin and rEpo have opposite effects on the growth and metastatic potential of tumor cells. While heparin downregulates tumor growth and especially angiogenesis, rEpo is known to have tumor growth-promoting effects. Interestingly, erythropoietin is still used in cancer patients due to its well-documented benefits in anemic cancer patients having chemotherapy (11). We have therefore decided to re-examine the effects of heparin and rEpo in B16F10 melanoma cells as a single agent or in combination. First, we treated B16F10 cells with various concentrations of heparin (10-300 IU/ml) for 24 h and then determined the cell survival using MTT as-

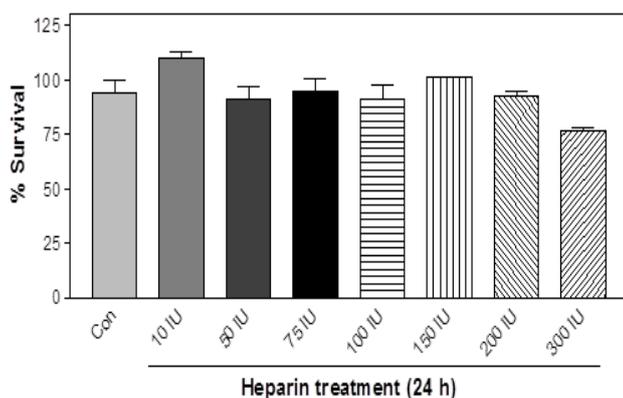


Figure 1. Effect of heparin on B16F10 cells. 25,000 cells were seeded on sterile 35 x 10 mm petri dishes. After 48 h of incubation, cells were treated with the indicated doses of heparin for 24 h. Cell survival was determined by MTT assay as described in the "Materials and Method" section. The results are presented as means ± SEM (n = 4).

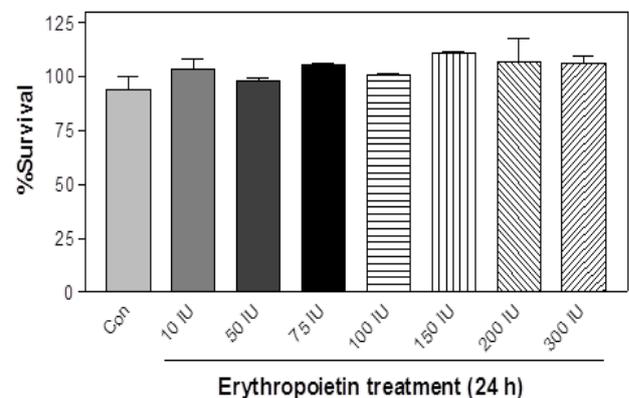


Figure 2. Erythropoietin effect on B16F10 cells. Similarly, B16F10 cells (25,000) were plated on sterile 35 x 10 mm petri dishes. And after 48 h of incubation, cells were treated with different doses of rEpo for 24 h. Then, cell survival was determined by MTT assay. The results are presented as means ± SEM (n = 4).

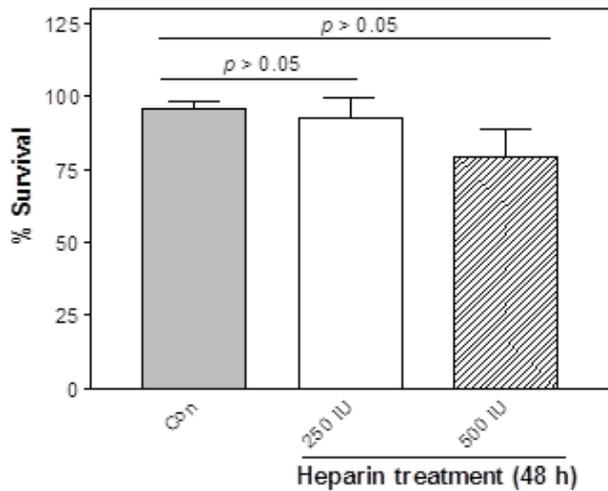


Figure 3. Effect of two different doses of heparin on B16F10 cells after 48 h treatment. Cells were plated in 35 x 10 mm dishes and treated at the logarithmic phase of the growth with 250 IU/ml or 500 IU/ml heparin for 48 h. Then cell survival was determined with MTT assay. The results are means \pm SEM (n = 6).

say. As can be seen in Figure 1, no significant effects on B16F10 cells were observed upon treatment with various doses of heparin. In the presence of 300 IU/ml heparin, a decrease of about 20% in cell viability was observed; however, this decrease was not statistically significant (Fig. 1). Afterwards, we similarly examined the effects of various concentrations of rEpo on B16F10 cells using MTT assay. Treatment with rEpo also did not cause a significant growth-promoting or inhibiting effects on the cell line used in this study (Fig. 2). Again, although an approximately 10% increase in the growth was detected in the presence of higher concentrations of rEpo, this increase was not significantly different (Fig. 2). To clarify the effect of heparin, the cells were also treated with two different doses of heparin (i.e., 250 IU/ml and 500 IU/ml) for a longer period of time (48 h). As seen in Fig. 3., further incubation of cells with two higher doses of heparin did not significantly reduced the cell number as compared to the control cell ($p > 0.05$); these results are in agreement with the above data. Then the effects of heparin and rEpo as single agents or in combination on B16F10 cells were tested by wound assay, which is commonly used in *in vitro* experiments to estimate the migration and/or proliferation rates of different cell lines or culture conditions. At $\geq 90\%$ confluency, B16F10 cells were treated 50 IU/ml heparin,

75 IU/ml rEpo or their combinations for up to 24 h. As can be seen in Fig. 4A and B, in contrast to our expectations based on our experiments with MTT assay, heparin treatment caused a significant reduction in cell migration/proliferation of B16F10 cells as compared to the control cells at 24h incubation period ($p < 0.001$). Nonetheless, rEpo treatment did not significantly influence the migration/proliferation of the cells as compared to the control cells when used alone ($p > 0.05$) (Fig. 4A and B). Furthermore, rEpo treatment did not prevent the anti-migratory or anti-proliferative effects of heparin when two agents were combined ($p < 0.01$) (Fig. 4A and B).

DISCUSSION

This study was undertaken to clarify the effects of heparin and rEpo on cell proliferation and cell migration using a highly metastatic cell line (i.e., B16F10 cells). As discussed in the Introduction, erythropoietin initiates a signaling cascade that stimulates growth and differentiation of erythroid progenitors to increase red blood cell mass (20). In early studies, erythropoiesis-stimulating agents (ESA) were shown to be safe and effective treatments for anemia in cancer patients (8). On the other hand, recent studies have provided conflicting data that ESAs may lead to increased mortality rates in cancer patients (6,32). The important clinical question is whether ESA-tumor interactions may lead to disease progression. Because of this uncertainty, the use of ESAs in oncology has recently been limited (16). Some investigators have reported that ESAs may have nonhematopoietic effects via direct activation of EpoR on nonhematopoietic cells, including tumor cells. Erythropoietin and EpoR expression is present not only in nonhematopoietic tissues, but also in malignant tumors (9,18). According to several studies, most tumors and cell lines expressed high levels of EpoR, and thus ESAs directly promoted tumor cell growth (23,31). In this current study, although we observed a 10% increase in growth of B16F10 cells using MTT assay, this increase was not statistically significant. The results may be partly due to lack or low rEpoR expression; however, Miller *et al.* showed that EpoR mRNA was detectable in B16F10 tumors (26). Therefore, we believe that this nonsignificant effect of rEpo on B16F10 cells is not due to lack of EpoR in B16F10 cells. The results of wound assay also confirmed the MTT assay by indicating that rEpo does not significantly promote the migration and/or proliferation of B16F10 cells. On the other hand, Kumar *et al.* reported that EpoR mRNA

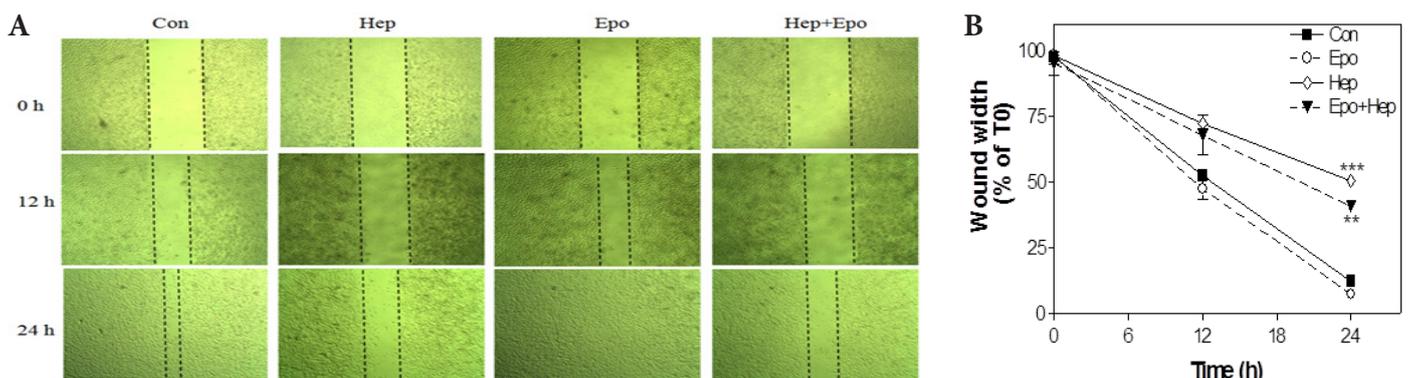


Figure 4. Effect of heparin and erythropoietin on cell migration. A) Cells were treated with heparin, rEpo or their combinations for 24 h. The migration and cell proliferation was recorded with an inverted microscope. B) Quantitation of the cell migration by measuring the relative distance in each wound. The results are means \pm SEM (n = 6). ** $p < 0.001$; ** $p < 0.01$.

expression was detectable in 90.1% of 65 melanoma cell lines. They found that Epo increased the migration capacity of melanoma cell, whereas EpoR knockdown significantly decreased the migration capacity of the most aggressive cell line 1205 Lu (metastatic melanoma). They concluded that the effect of Epo on tumor growth is likely dose- and time-dependent (19). We also tested the effects of heparin as a single agent or in combination with rEpo on B16F10 cells. It is known that heparin binds to growth factors and consequently affect proliferation and migration of cancer cells (10,17,22,29). Interestingly, we have not detected a statistically significant effect of heparin on B16F10 melanoma cells using MTT assay. Previously, Lee et al. showed that heparin could inhibit the interaction between B16F10 melanoma cells and activated platelets. In addition, they demonstrated that adhesion of the tumor cells to the activated vascular endothelium was also blocked, and hence the tumor growth was significantly delayed in response to heparin (21). Therefore, we then examined the effect of heparin by wound assay as well as the combined effect of heparin + rEpo. As presented in the Result section, the migration/proliferation of B16F10 cells was significantly blocked by heparin-treatment; in addition, rEpo was not able to overcome the effect of heparin, suggesting that heparin is a potent anti-metastatic and/or anti-proliferative agent under these experimental conditions. As mentioned above, the anti-metastatic effects of heparin may be due to its ability to bind growth factors. However, a detailed investigation of the mechanism of action of heparin is worthwhile prior to the clinical use in cancer patients. Lee et al. previously showed that when circulating tumor cells were deprived of their platelet cloak by LHD (a compound produced by the covalent bonding between the amine group of N-deoxycholyethylene diamine and the carboxylic acids of heparin), circulating tumor cells become more readily cleared by the immune system. In their study, they also showed that the heparin derivative could inhibit tumor cell residence in lung capillaries by blocking the interaction between tumor cells and platelets in the blood stream (21). Since our study was carried out in vitro, we suggest that heparin may inhibit cancer cell lines proliferation and migration through mechanisms other than simply making tumor cells susceptible to the immune system. Whether heparin exerts its anti-proliferation/anti-migration effects through a receptor-mediated mechanism would be interesting to examine in the future. In addition, several serious side-effects of heparin-administration are bleeding, bruising, and thrombocytopenia. And recently, it was also showed that heparin is a strong regulator of hepcidin expression, which is a major regulator of iron homeostasis. It is well-known that iron maldistribution is implicated in various diseases, including hemochromatosis, iron-loading anemias, and neurodegenerative disorders (27,28). The results presented here show that erythropoietin does not block the anti-metastatic/anti-migration activity of heparin on B16F10 melanoma cells. However, further in vivo studies need to be designed and carried out to determine whether erythropoietin-treatment would help annihilate or alleviate the side-effects of heparin-treatment. Consequently, heparin may have a therapeutic potential as an anti-angiogenic and anti-metastatic drug for cancer patients. And novel and better therapeutic strategies may be designed in combination with other cytotoxic agents.

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