Inhibition of adhesion breast cancer cells by anticoagulant drugs and cimetidine*

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Recent studies suggest that anticoagulant drugs and cimetidine therapy in malignancy may improve cancer survival and inhibit the metastatic process.

In this study we investigated and compared the effects of anticoagulant drugs (unfractionated heparin, warfarin, acetylsalicylic acid, low-molecular-weight heparins – nandroparinum, enoxaparinum, dalteparinum and reviparinum), cimetidine and combination of cimetidine with anticoagulants on adhesion of highly invasive breast cancer cells lines – BT 549 and MDA-MB-231 (MDA 231) – *in vitro*.

High antiadhesion effect was observed with cimetidine, warfarin and acetylsalicylic acid. Low-molecular-weight heparins had a small antiadhesion effect in independent use. In combination with cimetidine, a potential effect of cimetidine on the antiadhesion was observed. The antiadhesion effect was dependent on the type of the cancer cell line. Different effects between cell lines BT 549 and MDA 231 were observed. The strongest antiadhesion effect was obtained using the combination of cimetidine with acetylsalicylic acid. In the majority of applications of the drugs and their combinations, a proportional antiadhesion effect was dependent on the concentration and time.

We suppose that anticoagulant drugs might have higher antimetastatic effect in combination with cimetidine. The choice of anticoagulants can decrease the adhesion, decrease tumor angiogenesis and cause the shortening of blood clotting time. Cimetidine can decrease the adhesion of cancer cells and increase the activity of NK cells. Indeed, according to our results, application of cimetidine and anticoagulant drugs intensifies the antiadhesion effect together with other antimetastatic effects.

Key words: Breast cancer, metastasis, adhesion, anticoagulation, cimetidine, heparin.

The metastatic cascade is a very complicated process. It can be divided into four stages [14]: 1st stage – escape from the primary tumor mass and invasion of surrounding tissue, 2nd stage – intravasation, 3rd stage – adhesion to endothelial wall and extravasation, 4th stage – invasion, angiogenesis and growth at a distant site. Very important is the ability of metastatic cells to adhere to the endothelial wall. Long-term circulation in vascular systems decreases the probability of survival of metastatic cells and increases the probability of attack by natural defense system cells.

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Advanced breast cancer is frequently associated with hematogenous metastases that are accompanied by serious complications. An increase in morbidity and mortality in breast cancer patients has been frequently observed. Out of 186 examined patients who died of breast cancer, 64% had reported bone metastases at autopsy [23]. This study presented the observation that bone was the second most common site of breast cancer next to lung. The Walther's data demonstrate that the three common sites of distant tumor metastasis are lungs, liver and bone. Weiss [24] autopsied 1 060 breast cases and detected bone metastases in 62%. This frequency was equivalent to that of lung metastases. Cifuentes and Pickern [4] found bone metastases in

71% of 707 white women with breast cancer at autopsy. In a study of 587 patients [5] dying from breast cancers, it was observed that 69% had radiological evidence of skeletal metastases before death. This was comparable with 27% each for lung and liver metastases.

The main objective of the present study was to investigate the effect of common drugs on adhesion of breast cancer cells. We investigated cimetidine or anticoagulant drugs alone and the combination of cimetidine with anticoagulants. Cimetidine is famous by its antimetastatic effect [16] – it influences the expression of E-selectin [12]. In some clinical and experimental studies the antimetastatic effect of anticoagulant drugs, namely warfarin, unfractionated heparin and low-molecular-weight heparin were observed [18, 22].

Material and methods

Breast cancer cell lines: In the study highly invasive human breast cancer cell lines were used: BT 549 (invasive ductal carcinoma of 72-year old woman metastasized to 3 of 7 regional lymph nodes) and MDA 231 (adenocarcinoma of 51-year old woman from pleural effusion) – *in vitro* [3].

The cells were cultivated in DMEM supplemented with 10% FCS as described elsewhere. Cells were harvested in trypsin/EDTA, counted, and suspension of 2 x 10^5 cells/ml was used. Into each well of a 96-well plastic plate, 0.1 ml of cell suspension treated with different concentrations of drugs or untreated control cells, respectively, were pipetted. Plates were incubated for 12, 24 and 36 hours. For treatment of cells, the following drugs were used:

cimetidine (Primamet, Lek Lublana, Slovenia), unfractionated heparin (Heparin, Léčiva, Czech Republic), warfarin (Warfarin, Orion, Finland), acetylsalicylic acid (Aspegic, Sanofi-Synthelabo, France), enoxaparinum natricum (Clexane, Aventis, France), reviparinum natricum (Clivarin, Knoll, Germany), nandroparine calcium, (Fraxiparine Sanofi-Synthelabo, France) dalteparinum (Fragmin, Pharmacia-Up John, Sweden).

The drugs were used in three concentrations as follows: cimetidine 2, 4 and 6 mg/ml, heparin 20, 40 and 60 i.u./ml, warfarin 0.02, 0.04 and 0.06/ml, acetylsalicylic acid, Aspegic 2, 4 and 6 mg/ml, Clexane 0.1, 0.2 and 0.3 mg/ml, Clivarin 0.09, 0.18 and 0.27 mg/ml, Fraxiparine 30, 60 and 90 i.u./ml, Fragmin 10, 20 and 30 i.u./ml, at a single concentration or in various combinations.

For estimation of cell adhesivity, the MUH assay was used [22]. After cultivation, the tissue culture medium was removed, cells were washed with 0.2 ml of PBS at 37 °C, PBS was again removed and 0.1 ml PBS with MUH (methylumbelliferyl heptaonate) at the final concentration 100 μg/ml was added. After 30 min in 37 °C, the plates were removed and fluorescence activity was measured in a fluorometer FluoStar, excitation 355 nm, emission 460 nm. The results were analyzed using software Biolise.

Results

BT 549: In all experiments, inhibition of adhesion was proportional to the exposure time and concentration of drugs. The strongest antiadhesion effect was found when acetylsalicylic acid, warfarin and cimetidine were used. Small effects were found using the low-molecular-weight heparins (Fig. 1).

Significant effect was observed when combinations of cimetidine and anticoagulants were used. Potential effects of drugs were observed namely at smaller concentrations. Significant potential effects were also observed in combination of cimetidine and low-molecular-weight heparins (Fig. 1).

MDA 231: In this cancer cell line the most effective was a combination of acetylsalicylic acid and cimetidine. Low-molecular-weight heparins had a small or even adhesive stimulating effect (Fig. 2). The effect of heparin and reviparin in independent application was insignificant.

The best results in inhibition of adhesion were accomplished with the combination of cimetidine and heparin and the combination of cimetidine and acetylsalicylic acid (Fig. 2). Warfarin had no potentiating effect for antiadhesion activity of cimetidine. The combination of cimetidine with the same low-molecular-weight heparins (Clexane and Clivarine) showed a significant antiadhesion effect (Fig. 2). Among low-molecular-weight heparins, there were different antiadhesion results of their application in combination with cimetidine. In these combinations there was a direct proportion between the concentration and antiadhesion effect.

According to the results, the antiadhesion effect depends on the type of cancer cell line, and significant differences exist among anticoagulants.

Discussion

Data presented here are the results of an *in vitro* study of adhesive, highly invasive breast cancer cell lines. We investigated the effect of cimetidine, anticoagulant drugs and combinations of cimetidine with anticoagulants on the adhesion of highly invasive breast cancer cell lines. The antiadhesion effect of cimetidine is mediated by blocking of E-selectin expression [12]. The antiadhesion effect of cimetidine has been proved in tumors with high levels of sialyl Lewis antigents. The antimetastatic effect of cimetidine can be caused by the activation effect on NK cells [9, 10]. On the other hand, it was suggested that histamine might promote the growth of tumor cells [1]. The definite effect of cimetidine on the tumor growth has not been fully clarified,

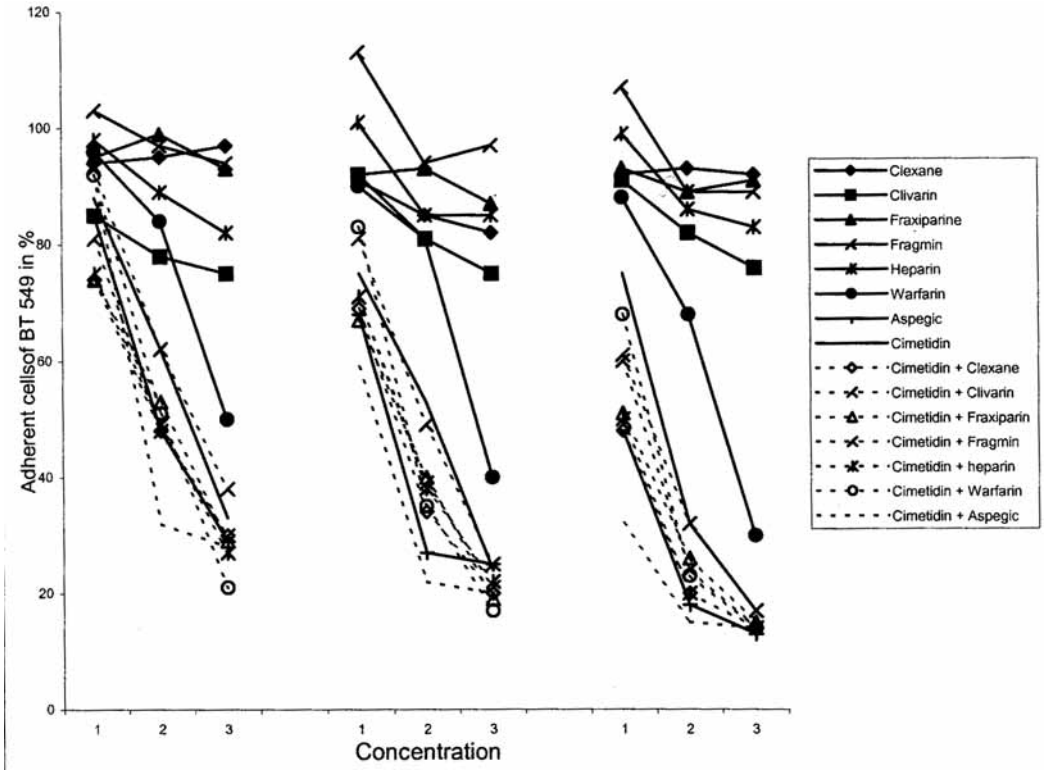

Figure 1. Number of adherent cells of BT 549 line. From left to right – 12, 24, 36 hrs. Interval 1, 2, 3 – growing concentrations (see Material and methods).

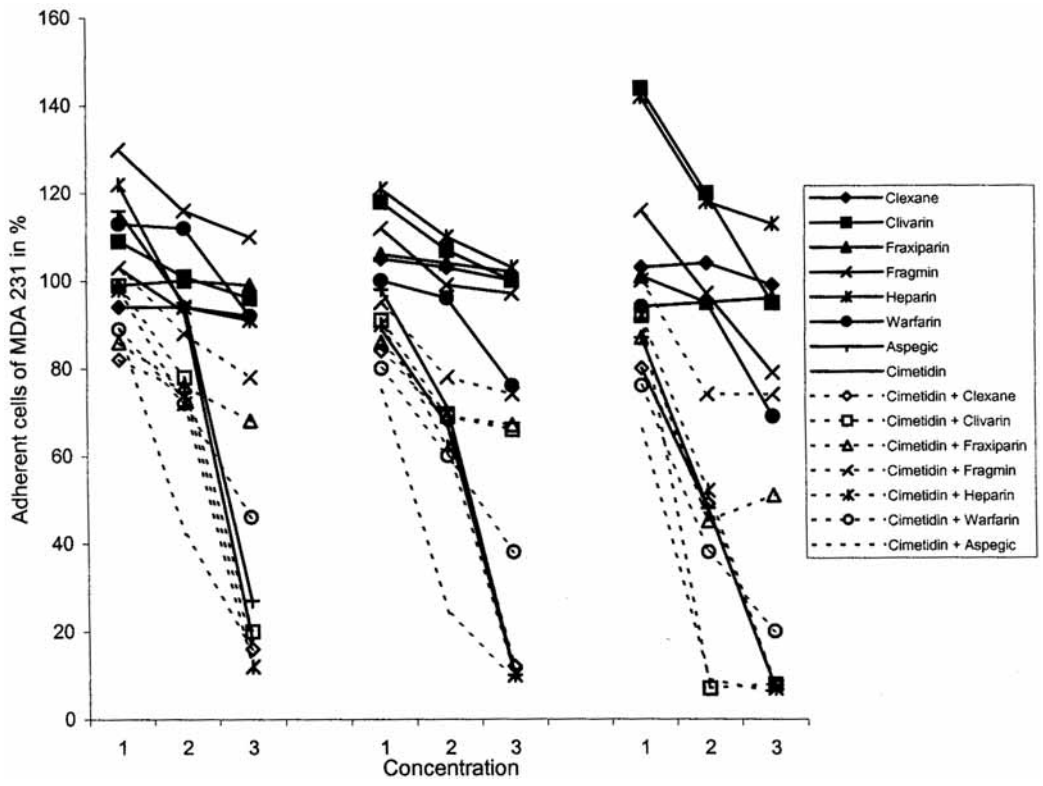

Figure 2. Number of adherent cells of MDA 231 line. From left to right – 12, 24, 36 hrs. Interval 1, 2, 3 – growing concentrations (see Material and methods).

but clinical trials have shown that it can improve survival of patients with colorectal cancer, melanoma and renal cell cancer [2, 20, 6, 17].

In clinical trials the strongest antimetastatic and antitumor effect was demonstrated in treatments with warfarin [25] and unfractionated heparin [11, 13]. The antimetastatic effect of acetylsalicylic acid was not observed [23]. Significant data about the effect of low-molecular-weight heparins in human patients are not usually presented. Otherwise, in animal models a significant antimetastatic effect was observed [18, 22].

The anticoagulant drugs are known to inhibit the metastatic cascade at more sites; adhesion of cancer cells is one of them. Anticoagulants inhibit the thrombin and fibrin formation induced by cancer cells [8]. Heparins may affect angiogenesis of new metastasis by modulating expression and function of angiogenic growth factor and inhibitors. Whereas unfractionated heparin and high-molecular-weight heparins appear to enhance the binding of these growth factors to their receptors, LMWH and small heparin fractions inhibit this binding [19].

The heparins did not affect the clearance of tumor cells from the lungs of mice when the activity of NK cells was depressed. Also antimetastatic effects of heparins were completely abrogated when NK cell reactivity in mice was suppressed [7]. We suppose that anticoagulant drugs might have a potentiating effect in combination with cimetidine. The choice of anticoagulants can decrease the adhesion of cancer cells, the coagulant effect of cancers, angiogenesis, and cimetidine can decrease the adhesion of cancer cells and increase the activity of NK cells. According to our results, application of cimetidine and anticoagulant drugs intensifies the antiadhesion effect together with other antimetastatic effects.

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