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Effect of Fraxiparine and Heparin on Experimental Tumor Metastasis in Mice

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Abstract. *Background: Low molecular weight heparins (LMWH) have become increasingly important in anticoagulant therapy. Antitumor and antimetastatic activity of heparin and LMWH-s have also been reported. Materials and Methods: Fraxiparine, a new modified LMW-H, was tested for antimetastatic effect using 3LL-HH intravenous, B_{16} intra-foot pad and 3LL-HH intrasplenic models in C_{57} Bl/6 mice. The dose of Fraxiparine was 38, 57 and 172 IU/kg, respectively. Heparin (100 IU/kg) was used as a positive control. Both pre-treatment (starting 6 hours before tumor inoculation) and post-treatment (starting 24 hours after tumor inoculation), followed by daily injections, were applied in the intra-foot pad and intrasplenic models. In the intravenous model, only a single dose was administered one hour after tumor cell injection. Results: Fraxiparine at the dose of 57 IU/kg was significantly antimetastatic in the intravenous model. Continuous treatment, starting 6 hours before tumor inoculation, with 173 IU/kg Fraxiparine resulted in a strong inhibition of lung metastases in the intra-foot pad model, but was ineffective in the intrasplenic model. Heparin did not influence the metastasis number in any of the metastasis models. Conclusion: These data may be of importance in the anticoagulant treatment of human cancer patients.*

Factors influencing blood flow are deeply involved in the complications accompanying malignant neoplasms. Increased risk of thrombosis is well known in various tumors, such as carcinoma of the exocrine pancreas (1, 2, 3). On the other hand, hemorrhage may be caused by exulcerating tumors, tumor toxicity or as a side-effect of cytostatic therapy (4). Although anticoagulation increases the possibility of hemorrhage, the higher risk seems to be for thrombosis.

Tumor cells exert pro-coagulant activity. Experimental data have shown that cancer cell heparanase activity is associated with invasion and metastasis (5). Factors causing platelet adherence to tumor cells (6) and platelet activating factor augment experimental metastasis (7).

On the other hand, experimental metastasis could be inhibited by anticoagulants (8), such as heparin and the vitamin K antagonist Warfarin (9). Inhibitors of platelet aggregation (prostacyclin, dipyridamole) have also been reported to decrease the number of metastases in experiments (10, 11).

In recent years, low molecular weight heparins (LMW-H) have become increasingly important in anticoagulant therapy (12). Decrease of lung nodules in mice caused by intravenous injection of B_{16} melanoma cells has been reported as an effect of such chemically-modified heparins (13).

The aim of this study was to investigate the possible antimetastatic activity of a widely used potent LMW-H, Fraxiparine (14, 15), using experimental mouse metastasis models.

Materials and Methods

Experimental animals. Eight to ten-week-old inbred male C_{57} Bl_6 mice, (Charles River Ltd, Gödöllö, Hungary) weighing 20±2 g, were used. The animals were kept in plastic cages (5 per cage) in a temperature and humidity controlled animal facility (20±2˚C, 55±5% humidity). Mice were fed with rodent pellets (Charles River Ltd) and provided with tap water *ad libitum*.

Tumors. The highly metastatic variant of Lewis Lung Tumor (3LL-HH) and B_{16} melanoma, maintained in our Laboratory, were used. The number of inoculated tumor cells was $5x10^4$ intravenously in the case of 3LL-HH, $5x10^5$ into the foot pad, in the case of B_{16} melanoma, and $5x10^3$ into the spleen in the case of 3LL-HH tumor. The inoculations were performed under Nembutal (Phylaxia, Budapest, Hungary) anesthesia.

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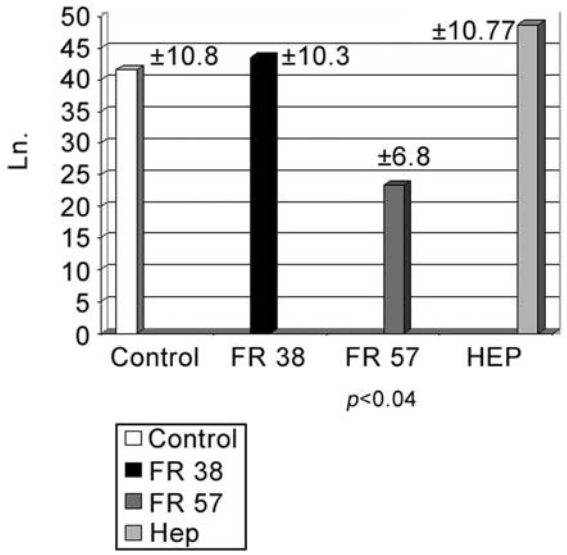

Figure 1. *Lung colony number in mice inoculated i.v. with 3 LL-HH cells and treated s.c. with a single dose of Heparin (100 IU/kg), Fraxiparine (38 and 57 IU/kg), at sacrifice.*

Table I. *Lung metastasis number in mice inoculated into foot pad with B_{16} cells and treated 24 hours after tumor inoculation with Heparin (100 IU/kg), Fraxiparine (38 and 57 IU/kg) (Experiment 2a), at sacrifice.*

	Control	Fraxiparine 38 IU/kg	Fraxiparine 57 IU/kg	Heparin 100 IU/kg
	45	217	24	47
	105	215	196	13
	228	171	78	136
	147	208	144	45
	173	62	62	47
	178	39	68	83
	62	172	96	151
	21	31	90	120
	6	81	111	138
		198		135
Mean	107.2222	139.4	96.55555	91.5
SD	78.34822	76.86235	50.01277	50.27093
$p <$		0.379201	0.735911	0.615467

Treatment. The anticoagulants, Fraxiparine (Sanofi Synthelabo, Budapest, Hungary) and Heparin (Richter G. Ltd, Budapest, Hungary) were applied subcutaneously. The dose of Fraxiparine was 38 IU/kg (human preventive dose for medium-risk patients), 57 IU/kg (human preventive dose for high-risk patients) and 172 IU/kg (human therapeutic dose), respectively, while that of Heparin was 100 IU/kg.

Experimental design.

Experiment 1 (Intravenous model). 3LL-HH cells were injected into the tail vein of the mice. One hour after tumor cell injection, 38 IU/kg, 57 IU/kg Fraxiparine or 100 IU/kg Heparin was administered as a single injection.

The mice were sacrificed 20 days after tumor cell inoculation by exsanguination under Nembutal anesthesia. Lung weight, lung colony number as well as body weight were registered.

Experiment 2a (Intra-foot pad model). B_{16} melanoma cells were injected into the left hind foot pad of the mice. Fraxiparine (38 IU/kg and 57 IU/kg) and Heparin (100 IU/kg) treatment were started 24 hours after tumor inoculation and repeated daily until day 21 after tumor inoculation. The left hind extremity bearing the primary tumor was amputated under Nembutal anesthesia, 10 days after tumor inoculation.

Experiment 2/b. The anticoagulant treatment started 6 hours before tumor inoculation and daily doses of Fraxiparine were 57 IU/kg and 172 IU/kg, while that of Heparin was 100 IU/kg. Amputation was performed 17 days after tumor inoculation and the treatment finished 47 days after tumor inoculation.

The mice were sacrificed by exsanguination under Nembutal anesthesia on day 21 (Experiment 2a) and on day 47 (Experiment 2b). Primary tumor weight, lung weight, lung metastasis number and changes in body weight were registered.

Experiment 3a (Intrasplenic model). 3LL-HH tumor cells were injected into the spleen. Anticoagulant treatment (38 IU/kg, 57 IU/kg Fraxiparine and 100 IU/kg Heparin) was started 24 hours after tumor inoculation and was repeated daily until day 14 after tumor inoculation.

Experiment 3b. Doses of Fraxiparine were 57 IU/kg and 172 IU/kg and that of Heparin was 100 IU/kg.

The treatment started 6 hours before tumor inoculation and was repeated daily for 11 days. Animals were sacrificed by exsanguination under Nembutal anesthesia on day 14 (Experiment 3a) and day 11 (Experiment 3b) after tumor inoculation. Liver weight, liver metastasis number and changes in body weight were registered.

The number of animals was 10/group. Ten control mice were included in each experiment, receiving 0.1 ml physiological saline subcutaneously as a single (Experiment 1) or daily (Experiments 2 and 3) injection. All animal experiments were performed following the requirements of the Hungarian Government, (243/1988.XII.31) and after permission of the Animal Welfare Controlling Office, Budapest, Hungary (No 25-133/2001), meeting the standards required by the UKCCCR guidelines (16).

Statistical analysis. Student's *t* probe was applied to determine SD and significance.

Results

Experiment 1. A slight, non-significant decrease in body weight of control and treated mice was registered during the course of the experiment. No significant difference was found between the body weights of control and treated animals. Similarly, no significant difference was calculated between the lung weight of the control and treated mice. The number of lung colonies of the mice treated with 57 IU/kg Fraxiparine was significantly

Table II. *Lung metastasis number in mice inoculated into foot pad with B_{16} cells and treated 6 hours before tumor inoculation with Heparin (100 IU/kg), Fraxiparine (57 and 172 IU/kg) (Experiment 2b), at sacrifice.*

	Control	Fraxiparine 57 IU/kg	Fraxiparine 172 IU/kg	Heparin 100 IU/kg
	7	28	1	31
	19	7	2	2
	2	116	3	4
	1	2	12	3
	14	11	1	6
	4	21	5	74
	6	26	13	8
	39	2	1	2
	23	4	0	
	81		0	
Mean	19.60	24.11	3.80	16.25
SD	24.57	35.91	4.83	25.23
$p<$		0.76	0.07	0.78

Table III. *Liver metastasis number of mice inoculated into the spleen with 3LL-HH tumor and treated 6 hours before tumor inoculation with Heparin (100 IU/kg) and Fraxiparine (57 IU/kg and 172 IU/kg) (Experiment 3b), at sacrifice.*

	Control	Fraxiparine 57 IU/kg	Fraxiparine 172 IU/kg	Heparin 100 IU/kg
	90	48	98	63
	118	63	156	151
	135	64	158	156
	151	118	175	178
	166	124	183	183
	188	134	186	190
	198	158	202	191
	205	160	203	214
	212	227	212	216
	239			263
Mean	170.20	121.78	174.78	180.50
SD	46.62	57.28	34.72	52.36
$p<$		0.058294	0.812924	0.647817

fewer compared to the control. No significant changes in lung colony numbers were found in the case of 38 IU/kg Fraxiparine on 100 IU/kg Heparin treatment (Figure 1).

Experiment 2a. A slight, but statistically significant decrease in body weight of the control and all treated groups was experienced in the course of the experiment. No difference in primary tumor weight, between the control and treated groups was found at the time of amputation. The lung weights of the control and treated mice did not differ significantly from each other (data not shown here). However, lung metastasis number decreased slightly in the 57 IU/kg Fraxiparine and 100 IU/kg Heparin treatment, but this decrease was not significant (Table I).

Experiment 2b. The weight of control and treated animals did not decrease in this experiment. A slight, but statistically significant increase of body weight was registered in the 172 IU/kg Fraxiparine treatment, compared to the control. The weight of primary tumors of the same group was slightly, but not significantly, lower than the control (data not shown here).

The number of lung metastases did not differ significantly from the control with the 57 IU/kg Fraxiparine and 100 IU/kg Heparin treatment, although the average number of metastases was slightly elevated in the 57 IU/kg Heparin group. However, the number of lung metastases in animals treated with 172 IU/kg Fraxiparine was, on average about one-fifth of that of the controls. Due to the high SD, this value was just below significance (Table II).

Experiment 3a. The body weight of the control and all treated groups decreased slightly, but not significantly, in the course of the experiment.

Neither the liver weight nor the liver metastasis number showed significant differences between the control and Fraxiparine- or Heparin-treated animals (data not shown here).

Experiment 3b. The body weight of the control and treated animals did not change significantly in the course of the experiment. No difference was revealed between the control and treated groups regarding liver weight (data not shown here), and no significant effect on liver metastases of either Fraxiparine or Heparin treatment could be observed (Table III).

Complications of treatment. An important difference was observed between the mice treated repeatedly with Heparin and those treated repeatedly with Fraxiparine. The Heparin-treated mice regularly showed suffusions at the site of the subcutaneous injections. No suffusions were seen in the site of the injections in the Fraxiparine-treated animals, even at the higher (172 IU/kg) dose.

Discussion

Anticoagulant prophylaxis and therapy in patients with cancer is a well-established and accepted therapeutic measure (17), and clinical trials on animals have been reported (18). Heparin has proven to have not only antimetastatic properties, but also to exert an effect on tumor growth and survival (19). It is of major interest that cancer patients treated for venous thromboembolism with LMW-H have a better survival rate than patients treated with the unfractionated heparin (20).

Although some data in the literature have cited a lack of effect of heparin or modified heparin on experimental

metastasis (21, 22), our results, in accordance with others (23), indicate that the effectiveness of these compounds may depend on the experimental model, dose of the anticogulant and timing of the application. According to our data, heparin, administered in human preventive dose, did not influence the metastasis number in the three (intravenous, intra-foot pad, intrasplenic) metastasis models. On the other hand, the antimetastatic effect of Fraxiparine was evident when 3LL-HH cells were injected intravenously. In the extremity-lung model this effect became apparent in the case of 6-hour pre-treatment before tumor inoculation with a relatively higher dose of Fraxiparine. The dose-range of Fraxiparine applied here is within the range of prophylactic and therapeutic doses used in clinical practice.

Both heparin and Fraxiparine were ineffective in the spleen-liver metastasis model, regardless of dose and timing of the treatment.

The lack of serious weight loss of the anticoagulant-treated tumor-bearing animals points to the absence of serious side-effects. The observation that repeated heparin-injections caused suffusions at the site of injections, while Fraxiparine did not exert such a side-effect, may also be of clinical importance.

Metastasis is multifactorial, but hemostatic activation can be considered as one of the factors which facilitates tumor progression. In this respect, anticoagulant and especially LMW-H therapy seems to have not only antihemostatic activity, but may also contribute to the retardation of tumor metastasis.

Further experimental and controlled prospective randomized human studies are needed in this respect.

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