The influence of herbal remedies on cutaneous angiogenesis induced in mice after grafting of human kidney cancer tissue

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Abstract

The aim of the work was to study the effect of 5 herbal remedies with known immunostimulatory activity (Padma 28, Immunal forte, Reumaherb, Argoeleuter, Bioaron C) on neovascular reaction induced in the skin of Balb/c mice after grafting of human kidney cancer tissue homogenate or isolated tumor cells. Mice were fed remedies for 3 days following tumor grafting. Then, mice were sacrificed and newly formed blood vessels were counted in dissection microscope on the inner skin surface. Results: All tested remedies decreased cutaneous angiogenesis induced by human kidney cancer homogenate. In the case of cells isolated from tumor tissue, only Immunal forte diminished neovascular reaction.

Key words: *angiogenesis, mice, human kidney cancer, herbal remedies.*

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Introduction

Immunomodulation is an important strategy for overcoming various acute and chronic infections. The results of experimental and clinical studies supported that herbal medicines may be a valuable, additional treatment in some diseases.

PADMA 28 is a Tibetan plant remedy, made up of 22 defined plant ingredients. It possess immunomodulatory properties and might be beneficial for the prevention of immunity deficiencies induced by infection [1].

IMMUNAL forte is a dry extract of *Echinacea purpurea* succus, stabilized with alcohol. Extracts of *Echinacea purpurea* plants, traditional drugs of American Indians, are now widely used for the treatment of viral respiratory tract infections [2].

REUMAHERB is a complex remedy, containing extracts of *Echinacea purpurea*, *Filipindula ulmaria* and *Harpagophytum procumbens*. *Harpagophytum procumbens* (Hp), commonly known as Devil's Claw is a traditional South African herbal remedy used for rheumatic conditions. In Europe it has become a popular antiinflammatory and analgesic preparation for supportive treatment of degenerative joint diseases and various pains. Clinical trials support Hp as a beneficial treatment for the alleviation of pain and improvement of mobility in a variety of musculoskeletal conditions. Thymomimetic and immunomodulatory effects of REUMAHERB were also described [3-6].

ARGOELEUTER contains extract of *Eleutherococcus senticosus* (Siberian Ginseng). Siberian Ginseng is native to the Russian tajga and the northern regions of China, Korea and Japan. Its adaptogenic properties are well known, immunomodulatory properties of this plant were also reported [7-10].

BIOARON C syrup is a complex remedy, composed of biostymin (aloe extract), succus aroniae and vitamin C, used for improving immunity in children and for the treatment of upper respiratory tract infections. This remedy contains water extract of aloe leaf (*Aloe arborescens Mill*), chokeberry fruit juice (*Aronia melanocarpa Elliot*) and vitamin C [11-13].

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The first aim of the present study was to exclude the possible unwanted effect of these remedies (stimulation) on the early steps of angiogenesis process induced in mice skin after grafting of cancer cells or cancer tissue homogenates (safety test). The second aim was to select remedies with immune-stimulatory and angio-inhibitory effects, for use as safe additional treatment in immuno-compromised patients with cancers.

Materials and methods

The study was performed on 7-8 weeks old inbred Balb/c mice, weighing about 20 g, females, delivered from the Polish Academy of Sciences breeding colony. Herbal remedies were administered to mice *per os* in daily doses calculated from the doses recommended for humans (applying the counter 7 for differences between mouse and human in relation of the surface to body mass). That were: 5.8 mg of PADMA28, 0.64 mg of IMMUNAL forte, 1.2 mg of REUMAHERB, 3 mg of ARGOELEUTER, 25 µl of BIOARON C. Mice received drugs by Eppendorff pipette, in 40 µl of 10% ethyl alcohol, for 3 days after tumour homogenate or cells grafting. Control animals received 10% alcohol.

Preparation of renal tumour tissue homogenate

Tumour tissue was obtained surgically from 5 patients with kidney clear cell carcinoma. 2.5 g of tissue were suspended in 5 ml of phosphate-buffered saline (PBS), homogenized with an ultrasonic disrupter VirSonic (Virtis) for 2 minutes, at frequency 22.5 KHz and stored at –70°C, in 1 ml aliquots.

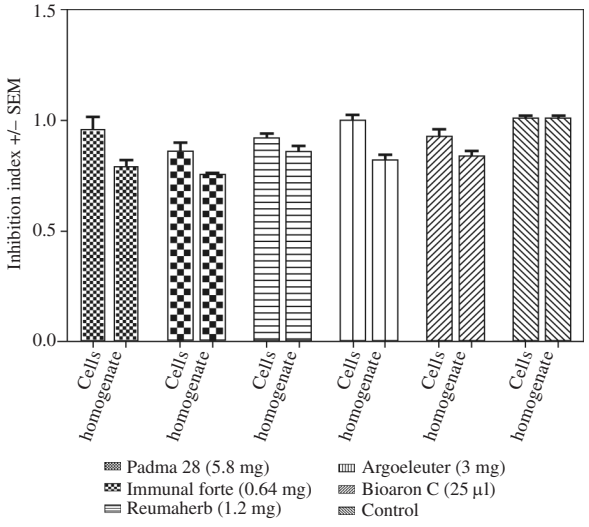

Fig. 1. The effects of herbal remedies on neovascular reaction in mice skin after grafting of human kidney cancer cells or homogenate

Preparation of tumour cells suspension

Material was obtained surgically from 5 patients with kidney clear cell carcinoma. About 5 g of tumour tissue was dispersed mechanically and subjected to enzymatic digestion by use of collagenase 0.1 mg/ml (Sigma) and DNAse 0.001 mg/ml (Serva) dissolved in PBS for 45 min on magnetic shaker in room temperature. Then, obtained suspension was filtered through a sieve, washed twice in PBS and suspended in Parker medium in concentration of 10×10^6 per ml. Viability of tumour cells was assessed by 0.5% trypan blue exclusion method.

Cutaneous angiogenesis assay was performed according to [14] with own modifications [15]. Briefly, multiple 0.05 ml samples of homogenate or cell suspension were injected intradermally into partly shaved, narcotised Balb/c mice (3-4 mice per group, 4-6 injections per mouse). In order to facilitate the localisation of injection sites later on, the suspension was coloured with 0.1% of trypan blue. On the day of grafting and on the following two days mice were fed tested substances in 10% ethyl alcohol, or 10% ethyl alcohol as a control. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6×, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cells injection, differ from the background vasculature in their tortuosity and divarications. All experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

For all experiments animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

Statistical evaluation of the results was performed by two-way ANOVA and the significance of differences between the groups was verified with a Bonferroni Multiple Comparison PostTest (GraphPadPrism software package).

Results

The results are presented in Fig. 1. and on the Table 1. All tested remedies decreased cutaneous angiogenesis induced by human kidney cancer homogenate. The highest inhibitory effect was presented by IMMUNAL forte. In experiments with cells isolated from tumour tissue, only IMMUNAL forte diminished neovascular reaction.

Performed analysis of variance revealed, that variation among column means is highly significantly greater than expected by chance (Table 1). In the case of mice grafted with tumour homogenate, Bonferroni Multiple Comparison Test indicated highly statistically significant differences between the controls and mice fed herbal remedies. In the groups of mice grafted with tumour living cells, only

Table 1

Two-way ANOVA				
Source of Variation	% of total variation	*p*-value		
Interaction	3.57	0.1404		
Drug	13.64	< 0.0001		
Material	9.58	< 0.0001		
Source of Variation	*p*-value summary	significant?		
Interaction	NS	No		
Drug	***	Yes		
Material	***	Yes		
Source of Variation	Df	sum-of-squares	mean square	F
Interaction	5	0.1392	0.02785	1.686
Drug	5	0.5325	0.1065	6.447
Material	1	0.3741	0.3741	22.64
Residual	173	2.858	0.01652	
Number of missing values	295			
Bonferroni posttests				
Control vs. Padma28 (5.8 mg)				
Material	difference	t	*p*-value	summary
Cells	–0.05000	1.182	> 0.05	NS
Homogenate	–0.2200	5.117	< 0.001	***
Control vs. Immunal forte (0.64 mg)				
Material	difference	t	*p*-value	summary
Cells	–0.1500	3.163	< 0.01	**
Homogenate	–0.2600	5.413	< 0.001	***
Control vs. Reumaherb (1.2 mg)				
Material	difference	t	*p*-value	summary
Cells	–0.09000	2.193	> 0.05	NS
Homogenate	–0.1500	3.593	< 0.001	***
Control vs. Argoeleuter (3 mg)				
Material	difference	t	*p*-value	summary
Cells	–0.01000	0.2506	> 0.05	NS
Homogenate	-0.1900	4.277	< 0.001	***
Control vs. BioaronC (25 µl)				
Material	difference	t	*p*-value	Summary
Cells	–0.08000	1.607	> 0.05	NS
Homogenate	–0.1700	3.539	< 0.01	**

IMMUNAL forte exerted statistically significant effect ($p < 0.01$).

Discussion

It is a general agreement, that inhibition of angiogenesis may become a promising strategy in cancer therapy [16]. We previously reported, that extracts from various plants and some substances of animal origin, may influence the development of angiogenic reaction induced in mice skin by syngeneic L-1 sarcoma cells, or by cells isolated from human tumors [17-22]. The question arose, whether this suppression was dependent on inhibition of angiogenic factors production by transplanted cells, or, whether the reading of angiogenic signals by endothelial cells is disturbed. For this purpose we compared in the present paper the effect of 5 herbal remedies on both types of grafts:

- sonicated homogenate of human kidney cancer tissue, representing cocktail of angiogenic factors,
- living cells isolated from kidney tumor.

Important finding of our present investigation is that no stimulation of neovascular reaction was observed in all experimental groups. That opens the possibility for safe use all presented herbal remedies in patients with cancers, or with high risk of cancer, for improving their immunity.

On the contrary, all remedies decreased neovascular reaction when applied after intradermal injections of sonicated tumor tissue homogenates. It means, that some substances present in remedies may influence efferent arc of the process of new vessel formation (blockade of receptors or their synthesis on endothelial cells?). Tested remedies contain substantial amounts of various polyphenols, triterpenes and other chemical compounds which may be potential candidates as inhibitors of endothelial cells activation [23, 24]. Previously, we observed such effects for various methyloxantines, epigallocatechin, epigallocatechin gallate, ursolic acid, convallamaroside, salidroside, rosavin and some phenolic acids [25-31].

Suppression of angiogenic factors production and(or)release by transplanted living tumor cells were observed only in mice fed IMMUNAL forte. This support the results obtained in our other experimental study, where we observed the effect of *Echinacea*-containing remedies IMMUNAL forte and ECHINAPUR on the morphology, angiogenic activity and vascular endothelial growth factor (VEGF) concentration of murine L-1 sarcoma tumors. Both remedies reduced VEGF concentration in tumor tissue and diminished the number of small blood vessels at a margin of tumor [32].

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