

A natural herbal remedy modulates angiogenic activity of bronchoalveolar lavage cells from sarcoidosis patients

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Abstract

Sarcoidosis is a systemic inflammatory disease with abnormally high angiogenic activity of inflammatory cells. Reumaherb preparation consisting of three herbs: Echinacea purpurea, Harpagophytum procumbens, and Filipendula ulmaria, and it exerts anti-inflammatory, antioxidant, and analgesic activity and stimulates regenerative and immunological processes.

The aim of this paper was to estimate the effect of Reumaherb on immunological angiogenesis induced by bronchoalveolar lavage (BAL) cells collected from six patients with sarcoidosis and grafted into Balb/c mice skin. After grafting, the animals were fed for three days with 0.6 or 1.2 mg of Reumaherb (calculated from recommended human daily dose) daily, suspended in 40 µl of water, or 40 µl of water alone (control group).

A significant reduction of newly formed blood vessels was obtained in four cases for 1.2 mg and in three cases for 0.6 mg daily dose of this remedy. Thus, we hypothesise that Reumaherb promotes anti-angiogenic activity and may potentially be used in diseases associated with excessive blood vessel formation.

Key words: sarcoidosis, bronchoalveolar lavage cells, BAL, herbal remedy, leukocyte-induced angiogenesis, LIA, murine skin.

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Introduction

Therapy of chronic inflammatory diseases is often difficult and long-lasting, so it may be associated with serious side effects (especially in the case of corticosteroids and nonsteroidal anti-inflammatory drugs). There is a need for safer medicines that could be applied in disorders with prolonged activation of the immune system. Sarcoidosis, a chronic systemic disease, belongs to the group of the most frequent interstitial lung diseases. Its pathogenesis involves immunological disturbances with characteristic granulomas forming in affected organs. The disease affects mainly young people [1].

Chronic inflammation is always accompanied by angiogenesis. Many key stimulatory mediators for angiogenesis, such as vascular endothelial growth factor (VEGF), fibroblast growth factor b (bFGF), transforming

growth factor (TGF)- α , IL-1, IL-6, and IL-8, platelet-derived growth factor (PDGF), and tumour necrosis factor α (TNF- α) show pro-inflammatory activity, while angiogenic inhibitors [e.g. IL-10, IL-12, interferon (IFN)- α , IFN- γ , and TGF- β] have anti-inflammatory potential. In normal conditions, both processes are induced locally for a short time to destroy harmful factors, e.g. pathogens, and then subside, before regeneration of affected tissue. The long-term and systemic character of both processes usually has serious medical consequences. Such pathological conditions can be observed in sarcoidosis. Elevated levels of angiogenesis have been shown in sarcoidosis patients [2, 3].

Natural medicine offers many preparations of plant origin, with anti-inflammatory and anti-angiogenic action. *Echinacea purpurea* is one of the herbs with anti-angio-

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genic, antioxidant, anti-inflammatory, and immunostimulatory properties [4].

Filipendula ulmaria, besides its anti-inflammatory and antioxidative action, reveals hepatoprotective properties. *Harpagophytum procumbens*, a herb used in South African medicine, exhibits anti-rheumatic, analgesic, and anti-inflammatory activity and was successfully used for alleviation of pain and mobility improvement in musculoskeletal conditions. These three herbs are components of Reumaherb preparation. Reumaherb displays anti-inflammatory, antioxidant, and analgesic activity and stimulates regenerative and immunological processes. It was successfully used in the treatment of degenerative disorders of joints and musculoskeletal disorders, as well as diseases with inflammatory pathogenesis [5, 6].

Formulations with anti-angiogenic properties could have a favourable therapeutic effect in diseases with elevated levels of angiogenesis, including sarcoidosis.

In order to test this hypothesis, in this paper we evaluate the effect of Reumaherb on immunological angiogenesis induced by bronchoalveolar lavage (BAL) cells collected from patients with sarcoidosis.

Material and methods

Patients' characteristics

The study population consist of seven patients (three women and four men), with diagnosed pulmonary sarcoidosis, who underwent bronchoscopic bronchoalveolar lavage (BAL) for clinical reasons. The diagnosis of the disease was based on clinical, radiological, and histopathological examination. According to X-ray findings, sarcoidosis was classified as stage I process (acute phase) in four patients, and stage II/III in three patients (chronic phase).

None of the patients was receiving oral or inhaled steroids at the time of BAL or during the previous three months.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed in all patients for diagnostic purposes, with informed consent. The control group consisting of healthy subjects was not formed for ethical reasons, instead literature data of healthy control group presented by John *et al.* [7] were used for comparison purposes. Bronchoalveolar lavage was performed by standard procedure [8]. Fifty millilitres of sterile 0.9% saline solution (Natrium chloratum 0.9% inj., Polfa, Lublin) at room temperature was instilled through a flexible fiberoptic bronchoscope four times, to a total volume of 200 ml, with harvesting of the fluid under immediate gentle vacuum. Recovered BAL was filtered through sterile gauze, and the cells were counted. The fluid was then centrifuged at 400 g for 10 minutes. Cells were > 90% viable as assessed

by trypan blue exclusion. Cytospinned smears of BAL cells were stained with May-Grunwald-Giemsa differential staining method and evaluated by counting a minimum of 600 cells. CD4- and CD8-positive cells were identified by specific monoclonal antibody (LSAB+ Kit, DAKO, Denmark), according to the detailed description provided by the manufacturer. The cells were counted under a light microscope at a final magnification of 1000 \times .

Angiogenesis assay

Angiogenesis test was performed according to Sidky and Auerbach [9] with Skopińska-Różeńska *et al.* modification [10]. Angiogenesis test was performed based on BAL cells achieved from six sarcoidosis patients. Balb/c inbred mice (eight weeks old) from the Polish Academy of Sciences breeding colony were anaesthetised intraperitoneally with 3.6% chloral hydrate (Sigma-Aldrich, USA; 0.1 ml per 10 g of body mass) before performing injections. Both flanks of each mouse were shaved with a razor and then 2-3 intradermal injections of 0.05 ml of BAL cells suspension (10×10^6 /ml) were performed on each flank. Cell suspensions were supplemented with 0.05 ml/ml of 0.01% trypan blue in order to facilitate subsequent recognition of injection sites.

Tested animals were fed Reumaherb preparation (Herbapol, Poznań; tablets 100 mg) (0.6 or 1.2 mg suspended in 40 μ l of water) or 40 μ l of water (controls) for three days with use of an Eppendorf pipette. After 72 hours, the mice were treated with a lethal dose of Morbital (Biowet, Puławy, Poland).

All newly formed blood vessels (thin, with ramifications, and extending to the injection site) were identified and counted under a dissection microscope on the inner skin surface, at magnification of 6 \times , in the 1/3 central area of the microscopic field.

All experiments were accepted and supervised by the local Ethical Committee.

Statistical analysis

All data are presented as mean \pm SEM.

Angiogenic activity was calculated as the mean number of newly formed blood vessels. Differences between the groups were calculated by two-way ANOVA followed by Bonferroni post-test (GraphPadPrism software, Inc.; version 5). The differences were considered significant at p value < 0.05.

Results

Bronchoalveolar lavage characteristics

Sarcoidosis patients presented BAL lymphocytosis and increase of CD4/CD8 ratio when compared to the control group of healthy subjects presented by John *et al.* [7]. The results are shown in Fig. 1 and Table 1.

Angiogenic activity of bronchoalveolar lavage cells

Analysis of angiogenesis test was performed based on BAL cells originating from six sarcoidosis patients.

Reumaherb preparation used at the dose of 1.2 mg decreased angiogenic activity of BAL cells from four patients, and no effect was observed in two patients (Fig. 2, Table 2).

Angiogenic activity of BAL cells was also modulated when 0.6 mg of remedy was used. BAL cells from three patients presented decreased the level of angiogenic activity, while angiogenic properties of cells from one patient were increased. BAL cells from another two patients from this group showed angiogenic response that did not differ from the control level (Fig. 2, Table 2).

Discussion

Sarcoidosis is a systemic inflammatory disease with abnormally high angiogenic activity of inflammatory cells [2]. The morphological pattern of BAL cells obtained from our patients (lymphocytosis and increase of CD4/CD8 ratio, Fig. 1, Table 1) is usually observed in the course of sarcoidosis [8]. The phenomenon of increased angiogenesis observed in BAL fluid from patients with sarcoidosis is probably caused by changes of macrophage phenotype/function. According to Chorostowska-Wynimko *et al.* [11], non-CD4⁺ and non-CD8⁺ cells are the main source of angiogenic activity of BAL cells from sarcoidosis patients. Recent years have brought new insights into the role of macrophages in sarcoidosis [12-14]. It has been shown that M2 type macrophages are present within the sarcoidosis nodules, which, unlike M1 macrophages (IFN- γ -activat-

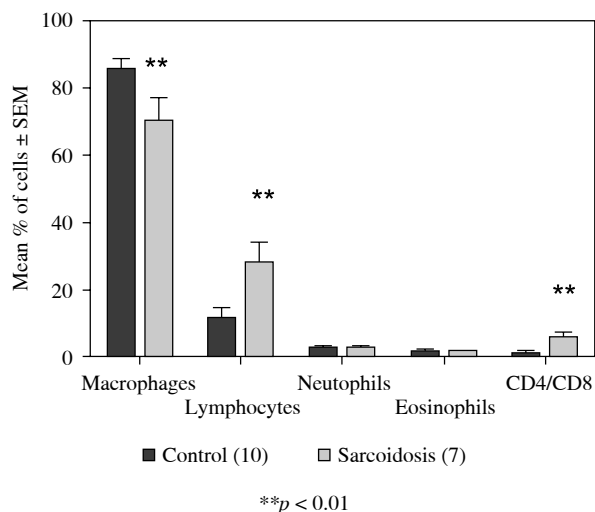


Fig. 1. Morphological characteristic of BAL cells derived from pulmonary sarcoidosis patients and control group of healthy subject (data of healthy subjects according to John *et al.* [5])

ed), are activated alternatively by IL-4, IL-13, and release VEGF. Elevated expression of VEGF in granulomas and macrophages isolated from sarcoidosis patients was observed by Tolnay *et al.* [15]. Sera isolated from non-pulmonary sarcoidosis patients also show increased levels of VEGF [16]. Our previous studies have shown that the levels of pro-angiogenic cytokines, matrix metalloproteinase (MMP-9), and IL-8 are elevated in BAL of patients with sarcoidosis. Furthermore, a strong correlation between these cytokines and IL-10 in the BAL of these patients

Table 1. A comparison of BAL characteristics of studied and control group – statistical analysis

Two-way ANOVA				
Source of variation	% of total variation	P-value	P value summary	Significant?
Interaction	2.68	< 0.0001	***	yes
Group of people	0.03	0.5471	NS	no
BAL cells %	90.28	< 0.0001	***	yes
Bonferroni posttests				
Control (10) vs. sarcoidosis (7)				
BAL cells %	Difference	t	P-value	Summary
Macrophages	-15.2	3.626	< 0.01	**
Lymphocytes	16	3.816	< 0.01	**
Neutrophils	0.1	0.02385	> 0.05	NS
Eosinophils	-0.1	0.02385	> 0.05	NS
CD4/CD8	4.870	1.162	< 0.01 unpaired t test	**

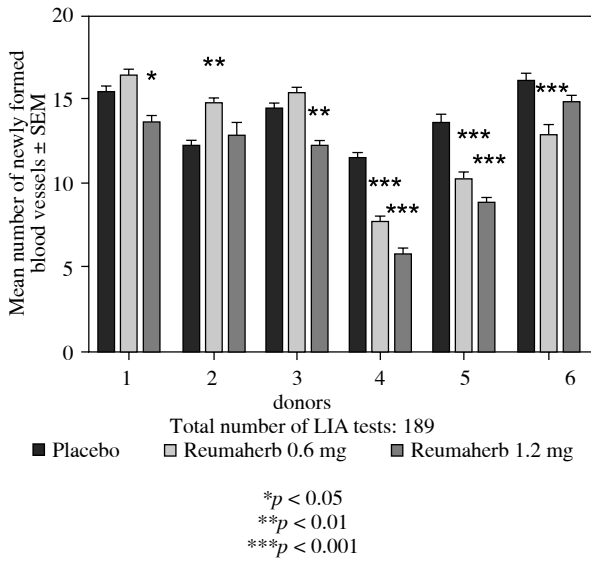


Fig. 2. The influence of Reumaherb on angiogenic activity of BAL cells derived from sarcoidosis patients

implicates the significance of angiogenesis in the pathogenesis of sarcoidosis [8]. Therefore, formulations with anti-angiogenic properties could have favourable therapeutic effect in the disease.

The results of the present paper demonstrate the anti-angiogenic activity of Reumaherb in some patients (Fig. 2, Table 2). A dose of 1.2 mg showed stronger anti-angiogenic properties than 0.6 mg. The different neovascular reaction of BAL cells to Reumaherb seems to be related to sarcoidosis stage (four patients in the acute phase, two patients in the chronic phase). Zielonka *et al.* [3] demonstrated the differences between neovascularisation levels induced by sera from sarcoidosis patients at various stages of disease. That is why the present study should be expanded to a larger cohort of sarcoidosis patients to confirm the anti-angiogenic properties of Reumaherb.

An anti-angiogenic effect of Reumaherb applied orally (1.2 mg) to mice was seen also when mononuclear cells (MNC) from healthy donors were preincubated for 24 hours with sera from sarcoidosis patients and then injected into murine skin to induce neovascular reaction [5]. Furthermore, Reumaherb (1.2 mg, *p.o.* fed to mice) exerted

Table 2. A comparison of the Reumaherb influence on angiogenic activity of BAL cells derived from sarcoidosis patients – statistical analysis

Two-way ANOVA				
Source of variation	% of total variation	P-value	P-value summary	Significant?
Interaction	15.61	< 0.0001	***	yes
Drug	10.91	< 0.0001	***	yes
Donors of BAL	58.1	< 0.0001	***	yes
Bonferroni posttests				
Placebo vs. Reumaherb 0.6 mg				
Donors of BAL	Difference	t	P-value	Summary
Donor 1	1.0	1.878	> 0.05	NS
Donor 2	2.5	5.035	< 0.01	**
Donor 3	0.9	1.624	> 0.05	**
Donor 4	-3.9	7.226	< 0.001	***
Donor 5	-3.4	5.451	< 0.001	***
Donor 6	-3.3	5.561	< 0.001	***
Placebo vs. Reumaherb 1.2 mg				
Donors of BAL	Difference	t	P-value	Summary
Donor 1	-1.8	3.095	< 0.05	*
Donor 2	0.6	1.127	> 0.05	NS
Donor 3	-2.2	3.97	< 0.01	**
Donor 4	-5.8	10.75	< 0.001	***
Donor 5	-4.7	7.828	< 0.001	***
Donor 6	-1.3	2.566	> 0.05	NS

anti-inflammatory and anti-angiogenic activity on MNC from rheumatoid arthritis patients. These observations encourage the use of Reumaherb as adjuvant medicine in the group of diseases with high level of angiogenesis [5, 6].

Furthermore, our previous studies have shown that Reumaherb and Immunal Forte (dry extract of *Echinacea purpurea*) decrease neovascular reaction in murine skin after grafting human kidney cancer cells or their homogenates. Also, neovascular reaction induced in murine skin by syngeneic L-1 sarcoma cells, as well as VEGF concentration in L1 sarcoma tumour tissue, were diminished by the preparation derived from *Echinacea purpurea* [17-19]. Elevated angiogenic activity of BAL cells from sarcoidosis patients was also reduced by natural preparation containing coastal salt-lake mud distillate with cinnamic acid and coumarin (FIBS). In this experimental model, mice were injected homogenates of BAL cells or supernatants from 48-hour cultures of these cells with FIBS [20].

Both Reumaherb and FIBS belong to natural plant antioxidants that downregulate angiogenesis *in vitro* and *in vivo* [5, 20, 21]. Another antioxidant synthetic compound, N-acetylcysteine, reduced expression of pro-angiogenic factors (IL-8, MMP-9 and intercellular adhesion molecule 1) by BAL cells isolated from interstitial lung disease patients, including sarcoidosis [22, 23].

Taken together, Reumaherb, as well as its anti-inflammatory activity, may also display anti-angiogenic properties. Considering its capability to decrease neovascularisation, this remedy could probably be effective as an adjuvant medicine in the treatment of disorders with abnormally high angiogenesis. However, in order to confirm the usefulness of Reumaherb in anti-angiogenic treatment of sarcoidosis patients, our preliminary findings should be extended to a larger number of cases.

The authors declare no conflict of interest.

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