Use of Natural Products in Anticytokine Therapy

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This work was designed to study direct anticytokine activity of natural products on the cell model of inflammation. We developed a combination of medicinal plants (calendula, elder, and pansy) with maximum antiinflammatory activity. This combination can be used for pathogenetic therapy of various inflammatory diseases, including atherosclerosis.

Key Words: *Inflaminat; atherosclerosis; proinflammatory cytokines; cell model of inflammation*

Local inflammatory reaction is considered as a possible cause for the development and progression of atherosclerotic lesions in human arteries [4,6,7]. Anticytokine therapy reducing the signs of inflammation can be used for creation of new drugs for the prevention and therapy of atherosclerosis. Modern antiinflammatory drugs (*e.g.*, nonsteroid antiinflammatory drugs) are not prescribed in atherosclerosis, because of the absence of data on their antiatherosclerotic efficiency and because of the risk of side effects during long-term treatment with these pharmaceutics.

This work was designed to study anticytokine activity of several medicinal plants on *in vitro* and *ex vivo* cell models of inflammation. These plants hold much promise for the development of a natural preparation for the prevention and adjuvant therapy of atherosclerosis.

MATERIALS AND METHODS

Monocytes were obtained from freshly isolated citrate blood by centrifugation in a Ficoll gradient. The cells were incubated in medium 199 containing 10% fetal bovine serum (FBS), antibiotics (penicillin, streptomycin, and fungizone), and glutamine (Gibco Europe). The incubation was performed in a CO_2 incubator (5% CO_2) at 37°C and 100% humidity for 14 days (until maturation to macrophages) [10].

In *in vitro* experiments, bacterial lipopolysaccharide (LPS, 1 μg/ml, Sigma) and aqueous extract of the test components (0.1 mg/ml) were added to the incubation medium. Expression of interleukin-1 (IL-1) in cultured cells was measured after 24-h incubation by the method of enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against IL-1 (Biogenesis).

Proinflammatory activity of human blood serum (10%) was estimated by its ability to induce overexpression of IL-1 and TNF-α in cultured cells during 24-h incubation. Blood serum with high proinflammatory activity was added *ex vivo* to the incubation medium. Blood serum samples were obtained from healthy donors before and 2, 4, and 8 h after treatment with the extract of medicinal plants. Diclofenac served as a reference preparation. Control cells were incubated in medium 199 containing 10% FBS (Gibco Europe). After incubation, expression of IL-1 and TNF-α was measured by ELISA with the corresponding monoclonal antibodies. Cell protein content was estimated by the method of Lowry.

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The results were analyzed using SPSS 10.0.1 software (SPSS Inc.). Variations in mean values were statistically significant at $p<0.05$.

RESULTS

During primary *in vitro* screening we evaluated anticytokine activity of 31 medicinal plants (black chokeberry, Amur barberry, hawthorn, European elder, common heather, common speedwell, common oak, ginseng, St. John's wort, white willow, Kalanchoe pinnata, calendula, meadow clover, willow herb, European cranberry, stinging nettle, silverweed, giant burdock, coltsfoot, lungwort, common juniper, common mint, greater plantain, couch grass, chamomile, licorice, bearberry, common yarrow, pansy, beggar ticks, and sage). Pansy, elder, calendula, hawthorn, and St. John's wort were most potent in inhibiting the expression of IL-1 induced by bacterial LPS. The decrease in IL-1 expression by 15% from the control was considered significant. Aqueous extracts of calendula, pansy, elder, St. John's wort, and hawthorn decreased IL-1 expression by 19.3±3.1, 17.8±2.4, 20.1±4.9, 22.3±6.3, and 18.4±4.4% from the basal level, respectively.

Anticytokine efficiency of natural components exhibiting maximum activity in *in vitro* model was studied *ex vivo*. We studied the effect of aqueous extracts from medical plants on induction of the expression of proinflammatory cytokines by human serum in cell culture. We revealed a decrease in proinflammatory activity of blood serum (Table 1). Elder and calendula produced a potent integral effect on the expression of IL-1 and TNF-α, respectively (cytokine expression was suppressed for 8 h after single treatment with the extract). The extract of hawthorn and elder berries did not modulate TNF-α expression. Study of the integral effect on expression of IL-1 and TNF-α showed that calendula exhibits maximum anticytokine activity and hawthorn berries possesses minimum anticytokine activity.

In the next series we verified the assumption that combined treatment with medicinal plants can led to potentiation of their anticytokine activity. Calendula, pansy, St. John's wort, and elder were administered in various combinations (Table 2).

TABLE 1. Average Decrease in Proinflammatory Activity of Blood Serum after Single Administration of the Extract from Natural Products ($M\pm m$)

Extract	Time after administration	Expression of proinflammatory cytokines, % of basal level	
		IL-1	TNF-α
Hawthorn berries	2 h	87.3±3.4*	94.3±2.7*
	4 h	84.0±2.1*	96.3±5.8
	8 h	88.7±9.4	113.3±6.7
	Integral effect	86.6±5.0	101.3±5.0
Elder berries	2 h	68.0±2.6*	80.3±2.0*
	4 h	70.7±2.4*	98.0±14.1
	8 h	88.3±2.0*	121.7±21.6
	Integral effect	75.7±2.3	100.0±12.6
St. John's wort herb	2 h	90.3±4.7*	84.0±1.7*
	4 h	83.0±2.1*	87.3±1.8*
	8 h	95.7±5.4*	97.3±3.3*
	Integral effect	89.7±4.1	89.5±2.3
Calendula flowers	2 h	78.3±9.2*	72.7±9.0*
	4 h	77.3±13.2*	71.0±11.5*
	8 h	81.7±11.4	70.3±21.2
	Integral effect	79.1±11.3	71.3±13.9
Pansy herb	2 h	68.7±3.8*	85.3±10.3
	4 h	84.7±5.2*	83.3±14.6
	8 h	85.3±9.4	80.3±4.1*
	Integral effect	79.6±6.1	83.0±9.7

Note. Here and in Table 2: *significant decrease in the proinflammatory response, $p<0.05$.

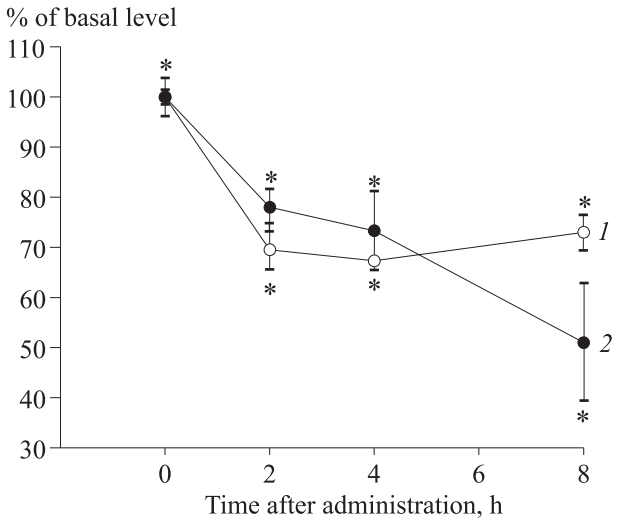

Fig. 1. IL-1 expression after single administration of Inflaminat (*1*) and diclofenac (*2*).

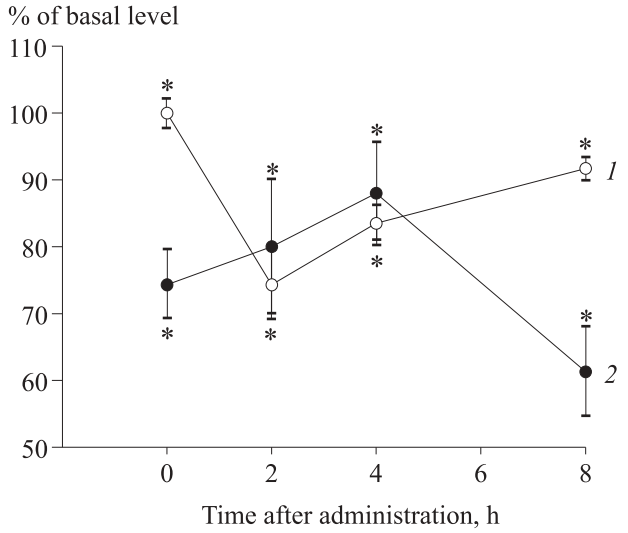

Fig. 2. Decrease in TNF-α expression after single administration of Inflaminat (*1*) and diclofenac (*2*).

Administration of St. John's wort in combination with various plants led to suppression of anticytokine activity. Expression of proinflammatory cytokines decreased most significantly under the influence of blood serum taken after treatment with the extract of elder, calendula, and pansy: 2, 4, and 8 hours after administration of 500 mg mixture (1:1:1) we revealed a decrease in induced expression of IL-1 (by 30.5±4.0, 32.7±0.3, and 27.0±3.6%, respectively) and TNF-α (by 25.7±5.0, 16.5±2.7, and 8.3±1.8%, respectively; Figs. 1 and 2). The integral decrease in IL-1 and TNF-α expression was 23.5±2.2 and 16.8±1.8%, respectively.

The nonsteroid antiinflammatory drug diclofenac was used as the reference preparation in this cell model. Proinflammatory activity of the serum was studied at equal intervals after administration of 100 mg diclofenac. IL-1 expression 2, 4, and 8 h after diclofenac administration decreased by 22.0±5.5 ($p<0.05$), 26.7±7.9 ($p<0.05$), and 49.0±11.8% ($p<0.05$), respectively. TNF-α expression decreased by 20.0±10.0 ($p<0.05$), 12.0±7.5 ($p<0.05$), and 38.7±6.6% ($p<0.05$) 2, 4, and 8 h after diclofenac administration, respectively. The integral effect of diclofenac on IL-1 and TNF-α expression was 32.6±8.4 and 23.6±8.0%, respectively.

The effectiveness of diclofenac was taken as 100%. The relative anticytokine effectiveness of combined treatment with medicinal plants for expression of IL-1 and TNF-α was 95 and 81%, respectively.

An antiinflammatory preparation Inflaminat was synthesized from this combination of medicinal plants. This preparation was registered as a bioactive additive. One capsule of Inflaminat contains the following active ingredients: 165 mg calendula flowers, 165 mg elder berries, and 165 mg pansy herb.

These medicinal plants enter the composition of antiinflammatory teas in traditional medicine.

TABLE 2. Integral Decrease in Proinflammatory Activity of Blood Serum over 8 h after Single Administration of Medi-cinal Plants in Various Combinations (*M*±*m*)

Combination	Expression of proinflammatory cytokines, % of basal level	
	IL-1	TNF-α
Calendula, pansy, and elder	76.5±2.2*	83.2±1.8*
Calendula, elder, pansy, and St. John's wort	85.2±4.8*	90.9±2.5*
Pansy, St. John's wort, and elder	87.6±5.2*	87.2±3.3*
Calendula, pansy, and St. John's wort	85.8±3.9*	90.0±1.8*
Pansy and elder	85.2±14.1	75.9±7.2*
St. John's wort and elder	89.8±4.1*	88.0±10.4

Our results suggest that the antiinflammatory effect of these medicinal plants is mediated by the anticytokine mechanism.

In vitro studies showed that ethanol and chloroform extracts of elder leaves significantly decreased TNF-α expression and slightly reduced LPS-induced expression of IL-1 in cultured human blood monocytes [13], while bioactive additives from elder extracts with antiviral activity *in vitro* increased the production of proinflammatory (IL-1, IL-6, IL-8, and TNF-α) and antiinflammatory cytokines (IL-10) in the primary culture of human blood monocytes [2,3]. It was found that extract of elder flowers inhibited proinflammatory cytokine expression, prevents integrin activation, and decreases oxidative burst in cultured monocytes and macrophages and human blood neutrophils during LPS-induced inflammatory response *in vitro* [5]. Contradictory data on *in vitro* effects of elder extracts on cytokine expression can be explained by differences in the composition of the test preparations and by the use of different methods for evaluation of their activity. Our results indicate that aqueous extracts of elder berries produce an anticytokine effect on proinflammatory cytokines IL-1 and TNF-α (*e.g.*, under *ex vivo* conditions).

We also demonstrated that the extracts of pansy herb and calendula flowers *in vitro* and *ex vivo* inhibit proinflammatory cytokine expression. The antiinflammatory effect of pansy herb is probably related to the presence of triterpenoid saponins [8]. Moreover, extracts of pansy herb exhibit direct antimicrobial activity [12], but the mechanism of this effect remains unknown. The antiinflammatory effect of calendula preparations is also associated with high content of triterpenoid alcohols [1].

The *in vitro* cell model is extensively used for evaluation of anticytokine activity of medicinal preparation and natural components [2,3,8,13]. We developed the *ex vivo* model on the basis of primary culture of human blood monocytes and macrophages. This model allowed us to evaluate anticytokine activity of various substances and their metabolites after assimilation and biotransformation in the human organism. We hypothesized that the antiinflammatory effect of some medicinal plants is mediated by a common mechanism associated with inhibition of proinflammatory cytokine production in cells. It should be emphasized that anticytokine activity of a complex natural preparation developed by us is comparable to that of nonsteroid antiinflammatory drugs (*e.g.*, diclofenac).

Long-term treatment with garlic preparation prevents the development of early atherosclerotic lesions in human carotid arteries [9]. This effect is primarily related to inhibition of cholesterol accumulation in cells of the arterial wall. These changes are accompanied by a decrease in proinflammatory activity of blood serum, which correlates a decrease in the thickness of the intima-media layer of the carotid arteries [11]. It can be hypothesized that prolonged antiinflammatory (anticytokine) therapy with complex natural preparation Inflaminat will led to regression of atherosclerosis. Further studies are required to estimate antiinflammatory and antiatherosclerotic activity of this preparation.

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