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Mechanisms of Regeneratory Effects of Baikal Aconite Diterpene Alkaloids

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Regeneratory activities of Baikal aconite alkaloids were studied on the excision skin wound model. Manifest wound healing effects of songorine, napelline, and hypaconitine were detected. The therapeutic efficiency was based on activation of residual mesenchymal progenitor elements. The development of this phenomenon was explained by the direct effects of alkaloids on precursors and by higher production of growth factors by the skin stromal cells. Songorine exhibited the most pronounced specific activity due to more significant stimulation of progenitor cell differentiation associated with maximum activation of the secretory function of the microenvironment cells.

Key Words: *regenerative medicine; alkaloids; progenitor cells; skin wound; microenvironment*

Recent data on the properties and regularities of vital activities of poly(multi)potent precursor cells have opened a new trend in the treatment of many diseases: cell therapy. Pharmacological stimulation of postnatal endogenous stem cells is the most physiological approach to solution of the problems of regenerative medicine [1,3-5]. We have previously demonstrated regeneratory activity of a complex extract from Baikal aconite. Its effect on experimental excision skin wound manifested in sooner reparation and formation of the epithelialized regenerate [6]. Alkaloids, songorine, napelline, hypaconitine, 12-epinapelline N-oxide, and mesaconitine, were detected by thin layer chromatography in specimens of the studied material [6,7]. On the other hand, experiments demonstrated the important role of regional (resident) skin precursor cells in the regeneration of surface tissues by secondary intention [12].

We studied the regeneratory effects of individual diterpene alkaloids of Baikal aconite and the mechanism of these effects associated with resident mesenchymal progenitor cell functioning.

MATERIALS AND METHODS

The study was carried out on 2-month-old certified outbred male mice (n=136; 22-24 g) from Breeding Center of Institute of Pharmacology.

Excision skin wound served as the experimental model. It was created as follows. A skin fragment

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(10×10 mm) was cut out on the back free from hair in ether-narcotized mice. In order to prolong the healing process, the crust was regularly (every 24 h) removed from the wound.

The objects of our study were individually extracted diterpene alkaloids of Baikal aconite: songorine, napelline, hypaconitine, 12-epinapelline N-oxide, and mesaconitine (Irkutsk National Research State Technological University). The alkaloids were extracted with chloroform from raw aconite grass as free bases, separated, identified by the standard method [7], and daily applied as 0.00025% water solution (30 μl) starting from day 1 after experimental wound modeling throughout the entire period of wound healing. Controls received applications of the solvent in an equivalent volume according to the same protocol.

The wound healing effect was evaluated by the mean diameter of the wound, rate of the diameter reduction, and results of histological studies of skin biopsy specimens collected from the wound defect on day 5. Biopsy specimens of the skin wound defect were stained with hematoxylin and eosin. The content of mesenchymal precursor cells (CFU-F) in the wound [12] and the intensity of their differentiation [2] were evaluated by *in vitro* cloning on days 3 and 5 of the experiment after applications of songorine, napelline, or hypaconitine to the wound. In addition, the production of humoral growth factors stimulating CFU-F growth (colony-stimulating activity, CSA) by the stromal microenvironment cells were studied by the culture methods in these experimental groups (stromal cells were collected from the wound surface) and the direct effects of songorine, napelline, and hypaconitine on CFU-F (in response to addition of 100 nM of the substance to the culture) were evaluated. Bone marrow cells culture served as the test system [2,7].

The results were processed by the methods of variation statistics using the Student *t* test and non-parametric Mann–Whitney *U* test. The significance of differences in the results of the experiments (the parameters were expressed in fractures) was evaluated by Fisher angular transformation method. The differences were considered significant at $P \leq 0.05$.

RESULTS

In the controls, the wounds healed by day 18 of the experiment. Applications of songorine, napelline, and hypaconitine led to more rapid reparation of the tissue defect. Songorine applications resulted in complete regeneration of the skin by day 14, napelline and hypaconitine treatment led to regeneration on day 16 (Table 1). The wounds shrank in size in these groups, most markedly in mice treated with songorine. The parameter decreased by 11, 13, and 18% of control on days 3, 5, and 7, respectively, in this group. Moreover, songorine significantly accelerated (by 86%) wound healing during the first days (1-3; Table 2) of the experiment. On the other hand, 12-epinapelline N-oxide had virtually no effects on tissue regeneration parameters evaluated by visual metrological methods (Tables 1, 2).

Histological studies on day 3 after wound modeling showed a leukocytic necrotic layer containing fibrin on the wound surface and a thin layer of granulation tissue with numerous cells (mainly neutrophils and macrophages) under it in all groups. The inflammatory process involved the sublayer of striated muscles. Intermuscular laminas were edematous and infiltrated with leukocytes. We observed edema and hyperemia of the derma and growth of the epidermis at the wound edges; the epidermis consisted of 8-10 layers of homogeneous undifferentiated large round cells and a thick horny layer. On day 5, the newly formed epithelium by the wound edges represented a cell layer of varying thickness without vertical anisomorphism. The frontal edge of the epithelium was

TABLE 1. Effects of Baikal Aconite Alkaloids on the Dynamics of Skin Wound Healing in Outbred Male Mice (cm; $\bar{X}\pm m$)

Group	Day of observation							
	1	3	5	7	9	12	14	16
Control	1.09±0.01	0.98±0.02	0.90±0.02	0.76±0.03	0.57±0.02	0.28±0.03	0.10±0.04	0.06±0.03
Songorine	1.08±0.02	0.87±0.03*	0.78±0.03*	0.62±0.02*	0.53±0.01	0.21±0.02		
Napelline	1.05±0.03	0.92±0.05	0.78±0.05*	0.67±0.07	0.51±0.08	0.23±0.06	0.08±0.04	
Hypaconitine	1.03±0.03*	0.92±0.03	0.79±0.02*	0.66±0.03*	0.50±0.04	0.16±0.04*	0.07±0.03	
12-Epinapelline N-oxide	1.09±0.03	0.96±0.03	0.81±0.04	0.69±0.04	0.54±0.05	0.29±0.05	0.12±0.04	0.06±0.04
Mesaconitine	1.11±0.02	0.99±0.02	0.89±0.03	0.74±0.03	0.61±0.03	0.23±0.04	0.11±0.03	0.01±0.01

Note. Here and in Table 2: *$P<0.05$ in comparison with the control.

TABLE 2. Effects of Baikal Aconite Alkaloids on the Dynamics of Skin Wound Healing (Wound-Healing Activity) in Outbred Male Mice ($\bar{X}\pm m$)

Parameter		Control	Songorine	Napelline	Hypaconitine	12-Epinapelline N-oxide	Mesaconitine
Rate of wound diameter reduction, mm/day	days 1-3	0.37±0.05	0.69±0.07*	0.44±0.06	0.36±0.06	0.43±0.05	0.40±0.03
	days 3-9	0.69±0.05	0.57±0.05	0.68±0.10	0.70±0.06	0.70±0.08	0.64±0.06
	days 9-16	0.72±0.04	0.75±0.02	0.73±0.12	0.71±0.06	0.68±0.60	0.85±0.05
Reduction of the mean diameter of the wound, %	days 1-3	10.14±1.37	19.39±1.81*	12.90±1.85	10.34±1.54	11.78±1.34	10.94±0.98
	days 3-9	42.08±2.58	39.24±2.35	45.29±7.91	45.82±3.96	43.85±4.92	38.37±3.31
	days 9-16	89.72±4.72	100	100	100	89.93±5.53	97.8±2.2

thin and crawled onto the wound like a wedge; numerous capillaries of vertical direction were detected in the granulation tissue. Applications of songorine, napelline, or hypaconitine significantly reduced the leukocytic infiltration of the wound edges, derma, and sublying tissues on day 3 of the experiment. In addition, applications of these substances increased the counts of fibroblasts in the granulation layer, forming, among other things, cell cords of appreciable size by day 5 of the experiment (Fig. 1).

The study of the mechanisms of wound-healing effects of alkaloids selected for further studies (songo-

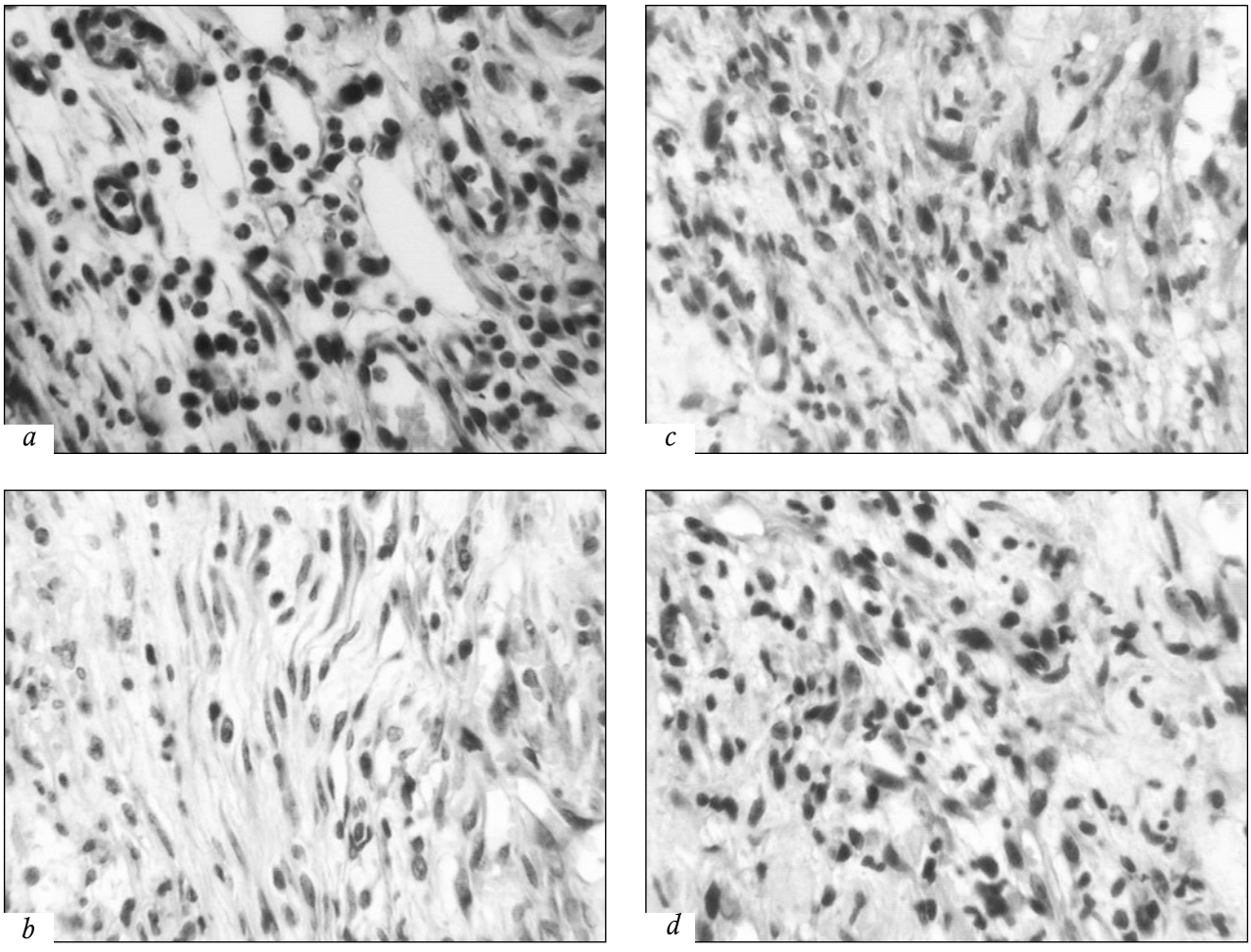

Fig. 1. Effects of Baikal aconite diterpene alkaloids on skin wound granulation tissue in male outbred mice on day 5 after wound creation (hematoxylin and eosin staining, ×400). *a*) control; *b*) songorine; *c*) napelline; *d*) hypaconitine.

rine, napelline, and hypaconitine) showed their pronounced effects on the resident precursor cells. Application of Baikal aconite extracts was associated with an appreciable increase in CFU-F count in the wound surface (Fig. 2); this parameter peaked on day 3 and constituted 970, 448, and 422% of the control for songorine, napelline, and hypaconitine, respectively.

Similar changes were recorded in studies of the intensity of precursor elements differentiation. A higher maturation index was recorded in all cases, the maximum in mice receiving songorine applications (up to 179% of control on day 3). Importantly, the alkaloids exhibited a direct stimulatory effect on the mesenchymal precursors. All of them significantly stimulated fibroblast colony formation after addition to culture medium (Fig. 2).

On the other hand, the important role of tissue microenvironment cells in determination of the proliferative status of stem cells and triggering of their differentiation programs is a known fact. The status of progenitor cell pool largely depends on the secretion of humoral regulation factors by the stromal elements [1,3,4]. The study of the production of colony-stimulating substances by the cells collected from the surface layers of damaged tissues have shown an appreciable increase of conditioned media CSA under the effects of alkaloids (Fig. 2). The most significant changes were observed in mice treated with songorine. The studied parameter increased to 245 and 284% of the control on days 3 and 5, respectively.

The results confirmed high regenerative activity of songorine, napelline, and hypaconitine. Their therapeutic activities are based on stimulation of the functions of resident mesenchymal precursors (containing, in addition to stromal precursors, multipotent – true stem cells [4,11]) due to the direct effects of these alkaloids on the precursors and higher production of growth factors by the skin stromal cells. Songorine exhibited the highest specific activity due to more intense stimulation of progenitor cell differentiation as a result of maximum stimulation of the secretory function of the microenvironment cells. These specific mechanisms of this alka-

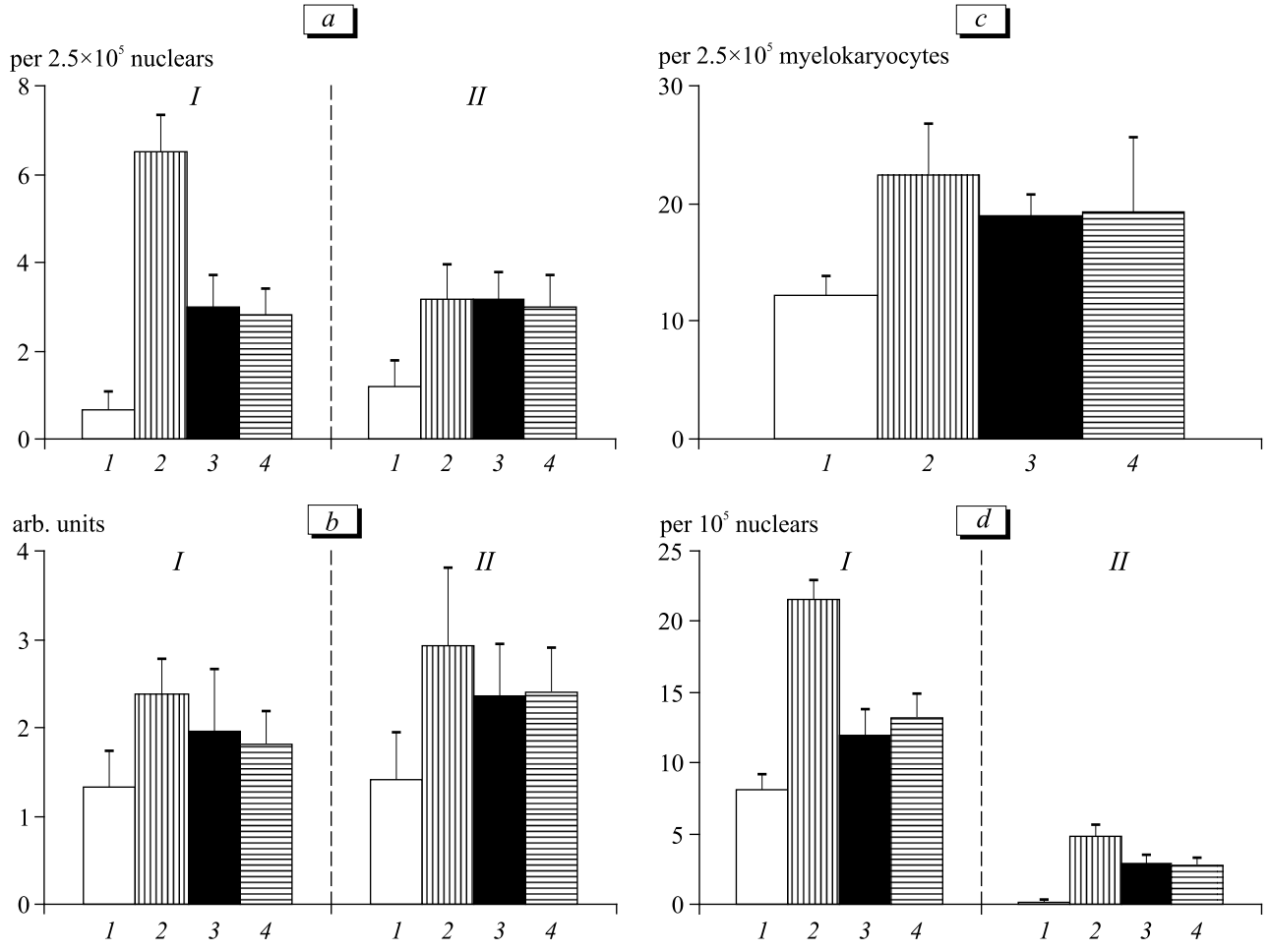

Fig. 2. Effects of Baikal aconite alkaloids on the levels of mesenchymal precursor cells (CFU-F) in wound surface in outbred mice (*a*), these cells' differentiation index (*b*), CFU-F growth from bone marrow cells (*c*), and CSA levels in the wound surface microenvironment cell conditioned media (*d*). I: day 3; II: day 5. *1*) control; *2*) songorine; *3*) napelline; *4*) hypaconitine. Confidence intervals at p=0.05.

loid's effects indicate its good prospects as a candidate drug for regenerative medicine [8-10].

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