Study of the Accelerating Effect of Shikonin and Alkannin on the Proliferation of Granulation Tissue in Rats

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The present study was carried out to compare the accelerating effect of shikonin and alkannin and to elucidate the expression of CD antigen and histological changes on the proliferation of granulation tissue in rats. Shikonin and alkannin produced a dose-dependent acceleration of the cotton pellet-induced granuloma formation and this accelerating potency of both compounds on the proliferation of granulation tissue was about the same 5 and 10 d after implantation of the cotton pellet. Also, both compounds increased the ratio of CD11b + cells in the granulation tissue 5 and 10 d after implantation of the cotton pellet. Both compounds increased the expression of CD11b + cells with granulocytes such as macrophages and histiocytes, and then accelerated the proliferation of fibroblasts and collagen fiber. On the other hand, neither compound increased the ratio of CD3 + cells in the granulation tissue after 5 and 10 d. These results suggest that shikonin and alkannin accelerate the proliferation of granulation tissue induced by the cotton pellet and this accelerating effect may be attributed to an increase in the expression of CD11b + cells, and the acceleration of the proliferation of fibroblasts and collagen fiber in the granulation tissue.

Key words shikonin; alkannin; granulation tissue proliferation; CD antigen expression; histological change

We previously reported that the other soluble extracts of “Koushikōn”, the root of Lithospermum erythrorhizon Sch. et Zucc., and “Nanshikōn”, the root of Macrotomia eu-chroma Pauls., produced an accelerating effect on the proliferation of granulation tissue induced by cotton pellet in rats, and that the accelerating potency induced by both extracts was about the same, although the nature of the constituents and the ratio of the optically active isomers of the constituents in the ether extracts differed. 1 – 3) We showed that shikonin, alkannin and acetylshikonin, isolated from ether extracts, accelerated the proliferation of granulation tissue in rats and the accelerating potency of these compounds was about the same. From these results, it was suggested that the absolute configuration of the hydroxy group in the side-chain and the presence of an acetyl group on the hydroxy group of the side-chain of shikonin or alkannin may not be important for the accelerating effect. 4)

Although many pharmacological studies of Koushikōn and Nanshikon and their constituents have been published, there have been very few reports about the expression of CD change and histological studies of the accelerating effect on granuloma formation induced by ether extracts of these materials and their constituents. 5 – 12)

The present study was carried out to compare the expression of CD antigen and histological changes in the accelerating effects on the proliferation of granulation tissue induced by shikonin and alkannin, typical constituents of Koushikōn and Nanshikon, respectively.

MATERIALS AND METHODS

Isolation of Shikonin and Alkannin Optically pure shikonin (R-configuration, R-type) and alkannin (S-configuration, S-type) were separated from commercial shikonin (Funakoshi Co., Ltd.) by HPLC under the conditions described below. An optically active column (Chiralcel OJ, 4.6 mm×250 mm, Daicel Chemical Industries, Ltd.) and a mobile phase of n-hexane:2-propanol:acetic acid=95:5: 0.3 were used. The flow rate was 1.0 ml/min at a column temperature of 40 °C and the column eluate was monitored at 273 nm. The retention times of shikonin and alkannin were 13.5 and 15.8 min, respectively. Both compounds were recrystallized from ether, and the chemical structures were confirmed by circular dichroism (CD), secondary ion mass spectrometry (SIMS) and 1H-NMR spectral data. The chemical structures of shikonin and alkannin are shown in Fig. 1.

Cotton Pellet-Induced Granuloma Formation Test Shikonin and alkannin were dissolved in 1% Tween 80 and each solution as topically applied to a cotton pellet. Male Wistar rats weighing about 250 g were used and were anesthetized with ether. After being sterilized in 2% acrilin, the back skin of the rats was opened and a sterile cotton pellet about 20±1.0 mg, to which 0.1 ml test solution had been topically applied, was implanted subcutaneously in the shoulder region. Control rats were treated similarly, except that they had a cotton pellet implanted to which 0.1 ml 1% Tween 80 solution had been applied.

The cotton pellets and granulaoma tissue were removed 5 and 10 d after implantation of the cotton pellet, respectively. The pellets were dried in an incubator at 60 °C for about 24 h.

Fig. 1. Chemical Structures of Shikonin and Alkannin

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until a constant weight was obtained. The incremental change in dry weight was a measure of granuloma formation.

The results are expressed as the percentage increase in dry weight due to granuloma formation (%), compared with the initial cotton pellet weight.

**Flow Cytometric Analysis of Lymphocyte Antigen Expression** Analysis of lymphocyte antigen expression in the granulation tissue was carried out using flow cytometric analysis 5 and 10 d after implantation of the cotton pellet. After 5 and 10 d, the cotton pellets with granulation tissue were removed, and the pellets were minced in phosphate-buffered saline (PBS).

The cell suspension was passed through a 300-mesh filter to remove the cotton pellet fragments. Following centrifugation, the cells were hemolyzed with ammonium chloride in Tris–HCl buffer (pH 7.65). The washed cells were incubated with different fluorescent-labelled antibody, R-PE conjugated anti-CD3 antibody (Pharmingen) and FITC conjugated anti-CD11b antibody (Pharmingen) for 30 min at room temperature. The stained cells were analyzed by FACScan (Becton Dickinson). The ratio of CD3⁺ to CD11b⁺ cells as expressed as a percentage of the total white blood cells.

**Histological Examination** After 5 and 10 d, the implanted cotton pellets were removed with the skin and granulation tissue and fixed in 10% formalin. The skin was dehydrated with ethanol, embedded in paraffin, cut into sections 4 μm thick and stained with hematoxylin and eosin.

**Statistical Analysis** Data are expressed as the mean value±standard error (%). The statistical significance of differences between the groups was assessed by Duncan’s multiple range test.

### RESULTS

**Accelerating Effect of Shikonin and Alkannin on Cotton Pellet-Induced Granuloma Formation** Shikonin and alkannin produced an acceleration of cotton pellet-induced granuloma formation, but this was not significantly different from the control 5 d after implantation of the cotton pellet.

On the other hand, both compounds produced a dose-dependent acceleration of the cotton pellet-induced granuloma formation 10 d after implantation of the cotton pellet. Although, the potency of shikonin appeared somewhat stronger than that of alkannin, the difference was not significant.

Shikonin and alkannin, in the doses used in this experiment, were found to be free from any apparent toxic effects, especially in the cotton pellet experiment; neither shikonin nor alkannin inhibited weight gain in rats. These results are shown in Tables 1 and 2.

**Expression of CD3⁺ and CD11b⁺ Cells Induced by Shikonin and Alkannin** The flow cytometric analysis of the changes induced by shikonin and alkannin in the infiltrating cells in the granulation tissue around the cotton pellet are shown in Fig. 2. The bulk of the infiltrating cells were CD11b⁺ cells and only a small number of CD3⁺ cells were found in the granulation tissue. CD11b⁺ cells induced by shikonin and alkannin appeared in the granulation tissue 10 d after implantation of the cotton pellet.

On the other hand, no CD3⁺ cell induction by shikonin and alkannin was observed in the granulation tissue 5 and 10 d after implantation of the cotton pellet.

**Histological Examination in Skin** Shikonin and alkannin produced an increase in the subcutaneous tissue thickness around the granulation 5 and 10 d after implantation of the cotton pellet. These results are shown in Figs. 3 and 4.

The increase in the subcutaneous tissue thickness was followed by an increase in the number of infiltrating cells, such as macrophages, lymphocytes and histiocytes induced by shikonin and alkannin in the granulation tissue 5 d after implantation of the cotton pellet. In addition, the extracellular matrix appeared to have an increased number of reticular fibers and many new blood vessels filled with red blood cells were observed (Fig. 3, c and e). At 10 d after implantation of the cotton pellet, the number of infiltrating cells decreased, and then fibroblasts, and the synthesis of collagen and re-epithelization were observed in the granulation tissue around the cotton pellet (Fig. 4, d and f).

The acceleration of the increase in the subcutaneous tissue thickness induced by alkannin was somewhat greater than that of shikonin, although the difference was not significant.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (%)</th>
<th>No. of animals</th>
<th>Weight gain (%)</th>
<th>Increase in dry weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.3±0.7</td>
<td>139.3±10.6</td>
<td></td>
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<tr>
<td>Shikonin</td>
<td>0.3</td>
<td>3.2±0.9</td>
<td>167.3±19.4</td>
<td></td>
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<tr>
<td>Alkannin</td>
<td>0.3</td>
<td>4.2±0.8</td>
<td>163.7±20.6</td>
<td></td>
</tr>
</tbody>
</table>

* a) Not significantly different from the control.  b) Not significantly different from alkannin.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Dose (%)</th>
<th>No. of animals</th>
<th>Weight gain (%)</th>
<th>Increase in dry weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>17.2±2.6</td>
<td>145.5±4.4</td>
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<tr>
<td>Shikonin</td>
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<td>180.6±16.2</td>
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<tr>
<td>Alkannin</td>
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<td>14.7±1.3</td>
<td>221.6±13.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>15.2±1.8</td>
<td>169.6±14.8</td>
<td></td>
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<tr>
<td></td>
<td>0.3</td>
<td>15.7±1.7</td>
<td>209.9±19.1</td>
<td></td>
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</tbody>
</table>

* a) Not significantly different from the control.  b) Significantly different from the control at p<0.01.  c, d) Not significantly different from the low and high dose of alkannin, respectively.
Fig. 2. Expression of CD3⁺ and CD11b⁺ Cells in Granulation Tissue 5 and 10 Days after Implantation of the Cotton Pellet Treated with Shikonin and Alkannin in Rats

a) Not significantly different from alkannin.  b) Significantly different from the control at p<0.05.

Fig. 3. Histological Observation of Skin 5 Days after Implantation of the Cotton Pellet Treated with Shikonin (c, d) and Alkannin (e, f) in Rats

Magnification: a, c and e, 40×; b, d and f, 400×.
Fig. 4. Histological Observation of Skin 10 Days after Implantation of the Cotton Pellet Treated with Shikonin (c, d) and Alkannin (e, f) in Rats
Magnification: a, c and e, 40×; b, d and f, 400×.

DISCUSSION

As far as the naphthoquinone derivatives and their optically active isomers obtained from Koushikou and Nanshikon are concerned, Tukada et al. reported that Koushikou contained a major quantity of the R-type (shikonin-type) with a smaller amount of the S-type (alkannin-type), whereas Nanshikon contained a major amount of the S-type with a minor quantity of the R-type. They also showed that shikonin and alkannin were typical constituents of Koushikou and Nanshikon, respectively.\(^{[3]}\)

It has been reported that shikonin and alkannin produced a dose-dependent acceleration of cotton pellet-induced granuloma formation and their potency was about the same, although the former was somewhat stronger; also, the absolute configuration of the hydroxy group in the side-chain of shikonin or alkannin may not be important in producing this effect.\(^{[4]}\)

In the present study, it was found that shikonin and alkannin showed a dose-dependent accelerating effect on cotton pellet-induced granuloma formation after 10 d, and both compounds tended to accelerate granuloma formation 5 d after implantation of the cotton pellet. The accelerating potency of shikonin tended to be somewhat stronger than that of alkannin, although the difference was not significant.

It has been reported that shikonin and alkannin exhibit bactericidal and antitumor activity and an accelerating effect on the proliferation of granulation tissue, the potency of these effects, induced by both compounds, being about the same.\(^{7–10}\)

From these results, it is suggested that the potency of the accelerating effect on the proliferation of granuloma tissue induced by shikonin and alkannin is about the same, and that their absolute configurations may not be important as far as producing this effect is concerned.

The proliferation of granulation tissue is known to play an important role in wound healing which involves three overlapping stages, that is, inflammation, formation of granulation tissue, and matrix formation.\(^{[14]}\) The injured tissue releases various inflammatory cells, and the migration of monocytes and their conversion at the injury site is critical for the initiation of tissue repair. Macrophages release biologically active substances such as cytokines and growth factors, and then, these substances facilitate the recruitment of additional inflammatory cells, augment macrophage-mediated tissue debridement, and initiate granulation tissue formation.\(^{[5]}\) New blood vessels are formed in the granulation tissue to supply nutrients and oxygen to the injury site. Thus, it is considered that the proliferation of fibroblasts, macrophages, and angiogenesis are important factors in promoting the proliferation of granulation tissue.

In this study we observed the expression of CD antigen and histological changes associated with shikonin and alkannin on the proliferation of granulation tissue induced by cotton pellets in rats. Results of flow cytometric analysis of lymphocyte antigen expression showed that the number of CD11b\(^+\) cells in the granulation tissue were increased by shikonin and alkannin 10 d after implantation of the cotton
pellet. The CD11b$^+$ cells are specifically the membrane antigen of leukocytes, and the number of CD11b$^+$ cells increase following inflammatory stimulation. The CD11b$^+$ cells are involved in cell adhesion, phagocytosis, chemotaxis and express macrophages, monocytes, and granulocytes. These results suggest that shikonin and alkannin increase the number of macrophages, monocytes, and granulocytes in the granulation tissue around the cotton pellet.

Histological observations of skin around the cotton pellets in rats showed that shikonin and alkannin accelerated the increase in the subcutaneous tissue thickness, followed by acceleration of the proliferation of granulation tissue. Many infiltrating cells, such as macrophages, lymphocytes and histiocytes, new blood vessels to promote metabolism, were observed in the subcutaneous skin tissue. Proliferation of fibroblasts and the synthesis of collagen were also observed.

These results suggest that shikonin and alkannin accelerate the proliferation of granulation tissue and the accelerating effect induced by both compounds may be attributed to an increase in infiltrating cells such as macrophages, lymphocytes and histiocytes, and acceleration of the proliferation of fibroblasts and collagen.

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REFERENCES