

Inhibitory effect and mechanism of action of sanggenon C on human polymorphonuclear leukocyte adhesion to human synovial cells¹

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KEY WORDS sanggenon C; neutrophils; synovial membrane; adhesions; NF-kappa B

ABSTRACT

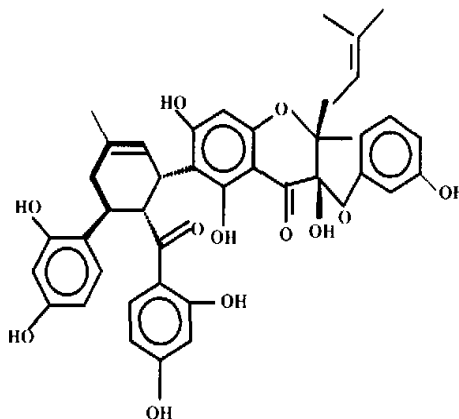
AIM: To examine the effect of sanggenon C on human polymorphonuclear leukocyte (PMN) adhesion to human synovial cell (HSC), and explore its mechanism.

METHODS: Adhesion of PMN to HSC was measured by MTT colorimetry. Cell-ELISA and RT-PCR methods were used to examine the expression of adhesion molecules ICAM-1 and VCAM-1. Activation of nuclear factor-kappa B (NF- κ B) was measured by electrophoretic mobility shift assays (EMSA) method. **RESULTS:** Sanggenon C effectively inhibited TNF- α (50 kU·L⁻¹ for 12 h) and IL-1 β (50 kU·L⁻¹ for 12 h) induced adhesion of PMN to HSC (IC₅₀ 27.29 nmol·L⁻¹ and 54.45 nmol·L⁻¹, respectively) in a concentration-dependent manner. Adhesion molecule VCAM-1 surface protein and mRNA expression induced by TNF- α 50 kU·L⁻¹ were significantly inhibited by sanggenon C, nevertheless, for ICAM-1 only surface protein expression being inhibited. The activation of NF- κ B was also extensively inhibited by sanggenon C. **CONCLUSION:** Sanggenon C inhibited TNF- α -stimulated PMN-HSC adhesion and expression of VCAM-1 by suppressing the activation of NF- κ B.

INTRODUCTION

Rheumatoid arthritis (RA) is currently considered an autoimmune disease in which a pathologic immune response attacks synovial cells, cartilage, and bone resulting in joint destruction and permanent disability.

The pathogenesis of RA was not known clearly. Recently, it is found that adhesion molecules, such as ICAM-1 and VCAM-1^[1,2], are highly expressed in synovial cells in the presence of proinflammatory cytokines, such as TNF- α and IL-1 β ^[3,4], while the expression level in resting synovium is quite low^[5]. Furthermore, there are multiform inflammatory cytokines and mononuclear cells and granulocytes filtrated in the arthrosis cavity in RA patient^[6]. The infiltration of polymorphonuclear leukocyte (PMN) and macrophages to the arthrosis cavities of RA patients was associated with characteristic lesion for RA, and these infiltrated cells presented some counter-receptor of HSC adhesion molecules. Moreover, the activation of adhesion molecule will induce the release of matrix metalloproteinase, as a result of which arthrosis cartilage is destroyed^[7]. Based on these facts, we hypothesize that granulocytes can adhere to synovial cell and the adhesion process plays a crucial role in the pathogenesis of RA. Indeed, this adhesion phenomenon was observed in our previous experiment^[8].



Structure of sanggenon C

Sanggenon C is a Diels-Alder type adducts

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compound, isolated from the root bark of *Morus Cathayana*. In the present study, we explored the inhibitory effect and mechanism of sanggenon C on adhesion of PMN to HSC.

MATERIALS AND METHODS

Chemicals RT-PCR primer sequence was designed using Primer Premier Software 3.0 and synthesized by Shanghai Sangon Co. Sanggenon C (purity > 90 % assayed by HPLC) was provided by Professor MAO Lin (Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences).

Isolation of human polymorphonuclear leukocyte Human blood was obtained from male healthy volunteers. Peripheral blood samples of the human were drawn from the median cubital vein, and PMN was isolated as described previously⁽⁹⁾.

Human synovial cell (HSC) culture Synovial membrane tissue was obtained aseptically from nonarthritic joint at the time of joint replacement surgery. Synovial membrane tissue was isolated and synovial cells were cultured as described previously^(8,10).

Adhesion assay of PMN and HSC Assays were performed as described previously⁽⁸⁾.

Enzyme immunoassay for the adhesion molecule of HSC Cell-ELISA system was developed according to the method of Tessier to measure CD54/or CD106⁽¹¹⁾. Briefly, human synovial cells were seeded to 96-well plates, with 3×10^3 cells/well, and grown to confluence. The cells were treated with sanggenon C for 30 min before TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$, Biotinge-Tech Co, China) was added, and then incubated for 12 h. The supernatants were removed and the cells were fixed with $100 \mu\text{L}$ /well acetone for 10 min at room temperature. The plates were gently washed in PBS for thrice and incubated with monoclonal antibody against CD54/or CD106 (Pharmingen Co, USA) at a concentration of 1:500 at 37°C for 1 h. The supernatants were removed, and the plates were gently washed with 0.05 % Tween-20-PBS for thrice. A $50 \mu\text{L}$ solution of goat anti-mouse IgG-HRP (SABC, China) was added to the wells, and the plates were incubated for a further 30 min at 37°C . The plates were washed thrice in 0.05 % Tween-20-PBS, and the solution of substrate (phosphate buffer containing 2 % OPD, Sigma Co) was added. The plates were incubated at 37°C for 20 min,

thereafter, $50 \mu\text{L}$ of H_2SO_4 9 mol/L was added to stop the reaction. The optical density at 490 nm was read on Bio-Rad reader.

RT-PCR analysis of adhesion molecule mRNA expression Total RNA was isolated from HSC with TRIzol according to the manufacturer's instructions. RT-PCR was conducted using Access RT-PCR System (Promega, USA). The sequence of primers are as follows; ICAM-1 sense 5'-GGC AGT CAA CAG CTA AAA CC-3'; antisense 5'-AGT GCG GCA CGA GAA ATT-3', 269 bp; VCAM-1 Sense 5'-CTG CAA GGT TCT AGC GTG TA-3', antisense 5'-GGA AGG GCT GAC CAA GAC G-3', 292 bp; GAPDH sense 5'-GAG GGG CCA TCC ACA GTC TT C-3'; antisense 5'-CAT CAC CTC TTC CAG GAG CG-3', 357 bp. ICAM-1, VCAM-1, and GAPDH were transcribed and extended, respectively. The final products were detected with 2 % agarose gel in TBE buffer in the presence of ethidium bromide, and the bands were visualized under UV light and photographed.

Nuclear extract preparation and electrophoretic mobility shift assays The experiments were performed as described previously⁽¹²⁾. Oligonucleotides used for the gel shift analysis were as follows: NF- κB (5'-AGTTGAGGGGACTTCCCAAGGC-3', 3'-TCAACTCCCCTGAAAGGGTCCG-5'), (Pharmacia Co, USA) mut-NF- κB (5'-AGTTGA-GGC-GACTTCCCAAGGC-3', 3'-TCAACTCCGCTGAAAG-GGTCCG-5', gifted by professor Xin-Sheng SONG, University of Virginia, USA).

Statistical analysis Data were presented as $\bar{x} \pm s$. Differences were analyzed for significance by the two-tailed *t*-test.

RESULTS

Activation of PMN adhesion to HSC by TNF- α and IL-1 β Adhesion of PMN to HSC was stimulated significantly by TNF- α in a concentration-dependent manner at concentrations of $1 - 50 \text{ kU} \cdot \text{L}^{-1}$ for 12 h. As to IL-1 β , this stimulating effect was observed only at $50 \text{ kU} \cdot \text{L}^{-1}$ (Fig 1).

Inhibition of sanggenon C on the adhesion of PMN to HSC stimulated by TNF- α and IL-1 β Adhesion of PMN to HSC induced by TNF- α and IL-1 β ($50 \text{ kU} \cdot \text{L}^{-1}$) for 12 h was remarkably inhibited by sanggenon C in a concentration-dependent manner at concentrations of $0.01 - 10 \mu\text{mol} \cdot \text{L}^{-1}$, with IC_{50} of 27.29 and $54.45 \text{ nmol} \cdot \text{L}^{-1}$, respectively (Tab 1).

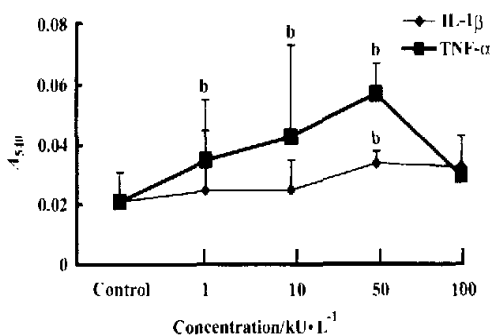


Fig 1. Effects of IL-1 β and TNF- α on PMN adhesion to HSC. Treated with stimulants of different concentrations for 12 h. $n = 3$. $\bar{x} \pm s$. ^b $P < 0.05$ vs control.

Effects of sanggenon C on ICAM-1 and VCAM-1 surface protein expression in HSC

ICAM-1 and VCAM-1 surface protein expression in HSC induced by TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$) for 12 h was significantly inhibited by $10 \mu\text{mol} \cdot \text{L}^{-1}$, the inhibitory rates of which were 38.8 % and 29.6 %, respectively (Tab 2).

Effects of sanggenon C on ICAM-1 and VCAM-1 mRNA expression in HSC

Expression of VCAM-1 mRNA was significantly inhibited by sanggenon C at concentrations of $1 - 10 \mu\text{mol} \cdot \text{L}^{-1}$ in HSC stimulated by TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$) for 6 h (Fig 2). But for ICAM-1, no significant effect was observed.

Effects of sanggenon C on the activation of NF- κ B in HSC The activation of NF- κ B was markedly inhibited by sanggenon C at concentrations of $1 - 10 \mu\text{mol} \cdot \text{L}^{-1}$ in HSC stimulated by TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$) for 1 h (Fig 3).

DISCUSSION

Our study suggested that sanggenon C, which was

screened from more than thirty compounds extracted from natural products, was an effective inhibitory agent. It can significantly inhibit the adhesion of PMN to RSC, the chemotaxis and respiration burst of rat PMN *in vitro*, and carrageenin-induced paw edema of mice *in vivo* (data not shown). Our novel data indicated that the adhesion of PMN to HSC induced by TNF- α and IL-1 β was also significantly inhibited by sanggenon C.

For exploring the inhibitory mechanism of sanggenon C on PMN adhesion to HSC, we examined the surface protein and messenger RNA expression of ICAM-1 and VCAM-1. The results indicated that the surface protein expression of ICAM-1 and VCAM-1 was significantly inhibited by sanggenon C, the VCAM-1, but not ICAM-1, mRNA expression was also inhibited by this compound. The results were same as the effect of

Tab 1. Inhibition of sanggenon C on PMN adhesion to HSC stimulated by TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$) and IL-1 β ($50 \text{ kU} \cdot \text{L}^{-1}$) for 12 h. $n = 6$. $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^e $P < 0.05$, ^f $P < 0.01$ vs TNF- α . ⁱ $P < 0.01$ vs IL-1 β .

Concentrations/ $\mu\text{mol} \cdot \text{L}^{-1}$	A ₅₄₀	Inhibition rate/%	IC ₅₀ / $\text{nmol} \cdot \text{L}^{-1}$
Control	0.024 ± 0.012		
TNF- α	0.050 ± 0.015 ^c		
Sanggenon C	0.001	29.4	27.29
	0.01	53.6	(0.13 - 57.86)
	0.1	59.0	
	1.0	63.7	
	10.0	66.4	
Control	0.029 ± 0.014		
IL-1 β	0.096 ± 0.019 ^c		
Sanggenon C	0.01	42.1	54.45
	0.10	48.5	(0.42 - 70.45)
	1.0	69.0	
	10.0	89.4	

Tab 2. Inhibition of sanggenon C on surface protein expression of ICAM-1 and VCAM-1 of HSC induced by TNF- α ($50 \text{ kU} \cdot \text{L}^{-1}$) by Cell-ELISA method. $n = 6$. $\bar{x} \pm s$. ^c $P < 0.01$ vs control. ^e $P < 0.05$, ^f $P < 0.01$ vs TNF- α .

Concentrations $/\mu\text{mol} \cdot \text{L}^{-1}$	ICAM-1		VCAM-1	
	A ₄₉₀	Inhibition rate/%	A ₄₉₀	Inhibition rate/%
Control	0.029 ± 0.016		0.06 ± 0.04	
TNF- α	0.20 ± 0.05 ^c		0.12 ± 0.04 ^c	
Sanggenon C	0.01	10.2	0.102 ± 0.022	14.3
	0.1	35.2	0.10 ± 0.03	18.7
	1.0	32.9	0.10 ± 0.04 ^c	20.4
	10.0	38.8	0.084 ± 0.028 ^f	29.6

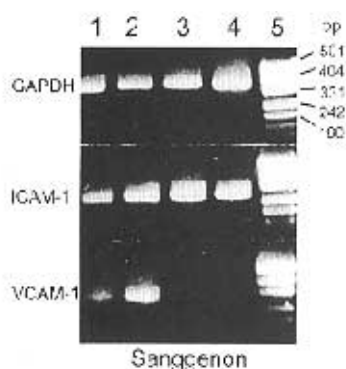


Fig 2. The inhibition of sanggenon C on ICAM-1 and VCAM-1 mRNA expression in cultured HSC stimulated by TNF- α 50 $\text{kU}\cdot\text{L}^{-1}$ for 6 h. GAPDH, ICAM-1, and VCAM-1 levels in cultured HSC were detected with the method of RT-PCR. The samples were loaded on a 2% agarose gel. Lane 1: control; Lane 2: TNF- α ; Lane 3: TNF- α + sanggenon C 1 $\mu\text{mol}\cdot\text{L}^{-1}$; Lane 4: TNF- α + sanggenon C 10 $\mu\text{mol}\cdot\text{L}^{-1}$; Lane 5: pUC19DNA/MspI DNA Marker.

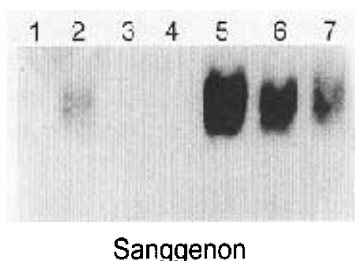


Fig 3. The inhibition of sanggenon C on NF- κ B activity in cultured HSC stimulated with TNF- α 50 $\text{kU}\cdot\text{L}^{-1}$ for 1 h. Lane 1: probe alone; Lane 2: control; Lane 3: NF- κ B + Nuclear extracts; Lane 4: mut + Nuclear extracts; Lane 5: TNF- α alone; Lane 6: TNF- α + sanggenon C 1 $\mu\text{mol}\cdot\text{L}^{-1}$; Lane 7: TNF- α + sanggenon C 10 $\mu\text{mol}\cdot\text{L}^{-1}$. In lanes 3 a 100-fold molar excess of unlabeled specific oligonucleotide was added to the binding reactions.

steroid anti-inflammatory drug, hydrocortisone, on the expression of ICAM-1 and VCAM-1 in HSC^[13], but significantly differed from non-steroid anti-inflammatory drug, meloxicam (data not shown). Above results suggested that both sanggenon C and hydrocortisone might have same anti-adhesion mechanism. It is likely that the inhibition of adhesion is mediated at least in part by attenuation of VCAM-1 and ICAM-1 expression.

As the 5'-regulatory region of VCAM-1^[14] has

binding site of NF- κ B, and suppressing activation of NF- κ B is one of the mechanism that hydrocortisone inhibits expression of adhesion molecule, we examined the inhibition of sanggenon C on NF- κ B activation in HSC. The results indicated that the activation of NF- κ B induced by TNF- α was significantly suppressed by sanggenon C at 1 ~ 10 $\mu\text{mol}\cdot\text{L}^{-1}$, the potency was same as hydrocortisone^[13], but was more potent than that of meloxicam (data not shown) at the same concentrations. These results suggested that inhibition of sanggenon C and hydrocortisone on the NF- κ B activation related to the suppression of VCAM-1 expression.

In current experiments, we also observed that surface protein but not mRNA expression of ICAM-1 was suppressed by sanggenon C, suggesting that sanggenon C may participate in the process of post-transcription of ICAM-1, the mechanism of which need to be elucidated.

In conclusion, our results suggest that suppressing the activation of NF- κ B, attenuating the surface protein and mRNA expression of VCAM-1, or suppressing the surface expression of ICAM-1 may be the inhibitory mechanism of sanggenon C on adhesion of PMN to HSC.

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Sanggenon C 对人多形核白细胞和滑膜细胞粘附的抑制作用及其机制¹

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关键词 sanggenon C; 中性白细胞; 滑膜; 粘附; NF- κ B

目的: 观察化合物 Sanggenon C 对人外周血多形核白细胞(PMN)与人滑膜细胞(HSC)粘附的抑制作用, 并探讨其作用机制。 **方法:** MTT 比色法研究 PMN 与 HSC 粘附, Cell-ELISA 及 RT-PCR 法研究 HSC 粘附分子 ICAM-1 和 VCAM-1 表达, EMSA 研究核转录因子 NF- κ B 的活化。 **结果:** Sanggenon C 在 0.01-10 $\mu\text{mol}\cdot\text{L}^{-1}$ 范围内均可显著抑制 TNF- α 50 $\text{kU}\cdot\text{L}^{-1}$ 与 IL-1 β 诱导的 HSC 与 PMN 粘附, 其 IC₅₀ 分别为 27.29 $\text{nmol}\cdot\text{L}^{-1}$ 和 54.45 $\text{nmol}\cdot\text{L}^{-1}$; Sanggenon C 可显著抑制 HSC 表面 ICAM-1 和 VCAM-1 蛋白表达, 同时也显著抑制 VCAM-1 mRNA 表达, 但对 ICAM-1 mRNA 表达无显著影响; Sanggenon C 在 1-10 $\mu\text{mol}\cdot\text{L}^{-1}$ 浓度下也可显著抑制 TNF- α 对 NF- κ B 的活化。 **结论:** Sanggenon C 是一个有效的人 PMN 与 HSC 粘附抑制剂, 其作用机制可能是通过抑制 NF- κ B 的活化, 进而抑制 HSC 表面 VCAM-1 的表达或抑制 ICAM-1 转录后调控过程而实现的。

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