The effects of Kampo herbal medicines (Scutellariae Radix, Carthami Flos, Linderae Radix) on the atherosclerosis mouse model introduced with heat shock protein (Hsp) 60 and high cholesterol diet

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Abstract

In the process of the formation of atherosclerosis, many immune factors, such as cytokines and chemokines are involved. On the other hand, heat shock proteins (Hsps) work as a Chaperon and are considered to have an effect that protects the cell from protein damage by restoration of the degenerated proteins. We were successful in establishing an atherosclerosis mouse model in C57BL/6NJc mice immunized with Hsp60 and simultaneously treated with a high cholesterol diet (HCD). At this time, using this model, we verified the effects of Kampo herbal medicines, Scutellariae Radix (SR), Carthami Flos (CF) and Linderae Radix (LR), on the pathological atherosclerositic change in the aorta, change in body weight, and alteration of serum cytokine levels. At first, compared with the control group, the reduction in the body weight of the groups that was administered with SR and CF were suppressed significantly ($p<0.05$). On the other hand, the production of IFN-γ of the groups that were administered with SR and CF were suppressed significantly, but the LR group only showed a tendency of suppression. The lipid deposits that we observed have a tendency to increase the volume and area gradually from the aortic valve to the root of ascending aorta. The deposits were observed in each mouse of the control group and SR group, but only 20 to 60% of the mice in the remaining groups (CF group and LR group) exhibited lipid deposition. Consequently we found that the herbal medicines reduced the adjuvant function of Hsp60, and simultaneously reduced the progression of the atheroscleosis.

Key words Hsps, Hsp60, Hsp70, atherosclerosis, Kampo herbal medicines, model mouse, anti-Hsp antibody, Scutellariae Radix, Carthami Flos, Linderae Radix.

Abbreviations CF, Carthami Flos; Hsps, heat shock proteins; HCD, high cholesterol diet; LR, Linderae Radix; SR, Scutellariae Radix.

Introduction

Atherosclerosis develops easily in medium and large-sized arteries, and the best-known mechanism of the onset of atherosclerosis is the response-to- injury hypothesis by R. Ross, 1973.[1,2)]

On the other hand, heat shock proteins (Hsps, or Chaperonins[3)]) are a group of proteins that are not only introduced and released from cells by many stimuli, but also exist within the cell without any stimuli. Hsps work as Chaperons that inhibit the over-expression of heteroproteins produced by external microorganisms, or the production of toxins, and the degeneration and aggression of proteins in the host cells while the dysbolism is caused by injury that was introduced by infection.[4)] Furthermore hsps are also considered to protect the cell from protein injury by restoring degenerated proteins.[5)]

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Hsps are generally determined by their molecular weight and are classified into four groups: Hsp90s family; Hsp70s; Hsp60s, and low molecular weight family. Of these, Hsp60 and Hsp70 play important roles in maintaining the immune system.[5]

For the purpose of studying the process of the formation of atherosclerosis, numerous animal models were designed, such as apolipoprotein deficient mice, ApoE knockout mice, LDL receptor knockout mice and transient rats. However, these models are relatively expensive.[6,7]

From the early 1990's, investigations into the relationship between Hsps and atherosclerosis have been done, since Berberian and his group first reported in 1990 the localization of Hsp70 in atherosclerosis specimens of human arteries.[8] The first reported animal model for Hsp induced atherosclerosis is Xu *et al.* They succeeded in inducing atherosclerosis by immunizing rabbits with Hsp65 under normal serum cholesterol levels.[9]

With reference to related research, our group at first investigated the Hsp60 and anti-Hsp60 antibody levels of serum from human type II diabetes mellitus cases treated with several formulations of Kampo herbal medicines. We found that anti-Hsp60 antibody levels were significantly decreased in the cases treated with the formulation "Hachimi-jio-gan (Ba-wei-di-huang-wan)", and the general conditions of these cases improved remarkably.[10]

Subsequently, in 2000, we were successful in establishing an atherosclerosis mouse model in C57BL/6NJc mice immunized with Hsp60 and simultaneously treated with a high cholesterol diet.[11]

At this time, using this model, we verified the effect of the Kampo herbal medicine on the pathological atherosclerositic change in the aorta, change of the body weight, and alteration of serum cytokine levels.

Materials and Methods

Mice : Seven-week-old female C57BL/6Njcl mice were purchased from Japan Coria Co.Ltd, Tokyo, Japan, and were provided with commercial pellets (CE2 Clea Japan) and tap water ad libitum until the start of the treatment.

Preparation of herbal medicines : Scutellariae Radix (SR, Ougon, Huang-qin), Carthami Flos (CF, Kouka, Hong-hua) and Linderae Radix (LR, Uyaku, Wu-yao) were obtained from Uchida Co. (Tokyo, Japan), and 50g of a crude herb was boiled with 1000 ml of distilled water until the volume was reduced to 500 ml. The supernatant fluid was filtered and lyophilized. Each herbal extract was given as drinking water. The concentration of the extract was adjusted to 1g/kg body weight/day of the Kampo herb weight.

The animals (total number: 20) were divided into 4 groups: the control group: 4; the SR group: 5; the CF group: 5 and the LR group: 6.

The control group was given water only. The herbal medicines were administered to other groups consecutively for 12 weeks.

High cholesterol diet (HCD) : High cholesterol diet purchased from The Oriental Enzyme Industry, Co.Ltd. Tokyo, Japan, was prepared according to the method described by Nishina *et al.*[12] and was composed of 15 % cocoa butter, 50 % sucrose, 20 % casein, 1 % corn oil, 5.07 %cellulose, 5 % AIN-76 mineral mix, 1 % chlorine chloride, 0.3 % DL-methlonime, 0.13 % DL-atocopherol, 0.5 % sodium cholate and 1 % cholesterol.

Immunizations : The recombinant heat shock protein (Hsp)60 was purchased from Stress Gen Biotechnologies Corp. (Victoria, BC, Canada), and was used as an antigen. (1μg/mouse). Freund's incomplete adjuvant was obtained from DIFCO Laboratories (Detroit, MI). One week, 4 weeks and 7 weeks after the administration of the herbal medicine extracts, the mice were immunized with 1μg of Hsp60 emulsified in 25 μl of physiological saline and 25 μl of incomplete Freund's adjuvant (FIA) by injection subcutaneously, excluding the intact group.

Measurement of anti- Hsp60 antibody production in serum : At 12 weeks, after the sacrifice of the animals, the blood was collected from the trunk, allowed to clot for 1 hour and centrifuged at 1000 mg for 15 min at 4 ℃. Serum was stored at -20℃ for assay. Anti-Hsp IgG2a antibody in serum was measured using the ELISA method. The 96-well plates were coated with 4 μg/ml Hsp60 in bicarbonate buffer, pH 9.6, at 4 ℃. Wells were blocked with 1 % BSA (Fraction V, Calbiochem) for 2 hours at room temperature. Diluted sera were applied to the wells and incubated for 2 hours at 37 ℃. Bound antibodies were directed by incubation with HRP-conjugated anti-IgG2a (Pharmingen, San Diego, CA) for

1 hour at 37℃. The reaction was developed with 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS, Sigma) for 0.5 hour and optical density (OD) 405 nm was read using a plate reader (BioRad, Hercules, CA).

Cytokine production : At autopsy, the spleen was immediately removed and pressed with slide glass in PBS (-). The cell suspension was passed through a #200 metal sieve and washed three times with PBS (-). Spleen cells were resuspended at 3×10^6 cells/ ml in RPMI1 640 medium containing 10 % fetal calf serum (FCS, Bioscience) and stimulated with 1 μg/ml anti-CD3 antibody or 5 μg/ml LPS. After incubation for 36 hours, the supernatant was collected and stored at 20℃. The content of IFN- γ , IL-4 and IL-12 were evaluated using ELISA kits (Biosource International, Camarillo, USA).

Pathological analysis of the aorta (aortic valve and ascending aorta) : For quantitative assessment of the atherosclerotic lesions, we used a modification of the method of Paigen *et al.*[13] In brief, the heart and the upper portion of the aorta were removed from the animals and the peripheral fat was carefully separated. After fixation in 10 % buffered formalin, the upper section of the heart was embedded in OTC compound and frozen. The frozen sections were discarded until the 3 valve cusps of the aorta appeared. The presence of the 3 valve cusps of the aorta was determined by examining the sections microscopically with toluidine blue stain. Once the appropriate section was located, sectioning continued along the root of the ascending aorta away from the heart until the valve cusps were no longer visible. The 4 μm sections were fixed on gel-coated microscope slides, stained with Oil red O, and counterstained with hematoxylin. The evaluation of the pathologic changes in the aorta was performed by the pathologist Prof. Y. Mori, without preliminary knowledge to the identity of the sample. This pathological evaluation method of atheroscoerosis is showed in table I.

Table I The pathological evaluation method of atherosclerosis

1. The range of lipid deposition:
The proportion of sub-epithelium lipid deposition area to the circumference of the cross section of the aorta.
2. The deposition score is determined as below:
Grade 0 : No deposition was found. Grade 1 : Slightly deposited. Grade 2 : Moderately deposited (without hypertrophy of the endothelium). Grade 3 : Highly deposited (the hypertrophy of the endothelium is remarkable).
3. The index:
Range of the lipid deposition × deposition score/10.
4. The incidence ratio:
The number of individuals that exhibited the lipid deposition/total number of the group.

Statistics analysis : Data were analyzed by Student's t-test to determine significance.

Results

Alteration of body weight

There was a remarkable reduction in body weight in the control group from week 7. The reduction ratios were 95.9% at week 8, 85.7% at week 10, and 76.37% at week 11, compared to the original weight. Finally at week 12, the reduction levelled at 75%. In contrast, the reduction in body weight among the groups administered with SR (the reduction ratio was 114.33% at week 12, compared to the original weight) and CF (117.22% at the same time) were significantly suppressed ($p<0.05$). The LR group showed no significant change. (93.06% at the same time) (Fig. 1)

Cytokines

The results of cytokine production in the control group were IFN- γ : 113 ± 91.7pg/ml; IL-4 : 38.2 ± 9.6

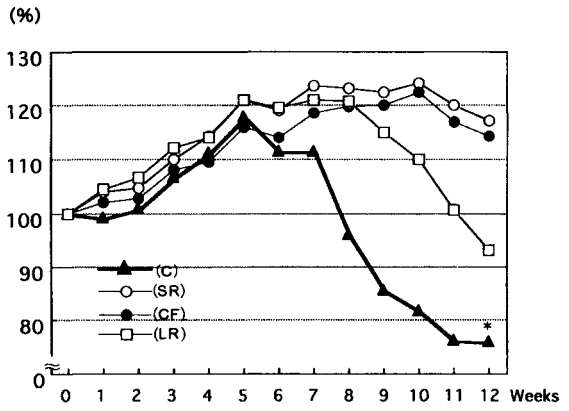

Fig. 1 The Alternation of Body Weight of the Mice.
Mean±S.E.
The reduction ratios of the control group (C) were 95.9% at week 8,85.7% at week 10, and 76.37% at week 11, 75.0% at week 12 compared to the original weight. In contrast, the reduction in body weight among the groups administered SR and CF were suppressed significantly. (the reduction ratio of SR was 114% and CF group was 117% at week 12, *$p<0.05$) The LR group showed no significant change. (93.1% at the same time)

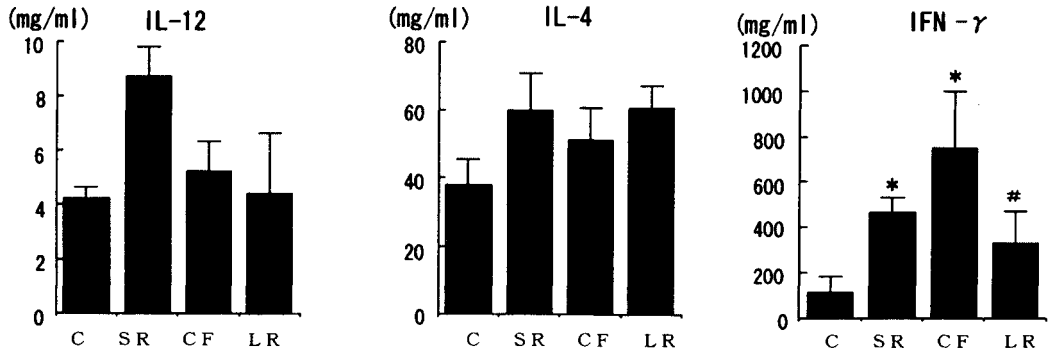

Fig. 2 The Cytokine Productions.
Mean±S.D.
The cytokine production in the control group (C) were IFN-γ : 113±91.7pg/ml; IL-4:38.2±9.6pg/ml; IL-12 : 4.2±0.6pg/ml; In contrast, the SR group were : INF-γ : 464±92.2.pg/ml; IL-4:60.3±10.6 pg/ml; IL-12 : 8.7±1.1 pg/ml; and the CF group are : INF-γ :751.2±251.3pg/ml; IL-4 : 51.5±9.0pg/ml; IL-12 : 5.25±1.1pg/ml; The LR group were:IFN-γ : 329.5±146.3pg/ml; IL-4 : 60.6±6.5pg/ml; IL-12 : 4.4±2.2pg/ml; The production of INF-γ in the SR and CF groups were significantly suppressed (*p<0.05) but the LR group only showed a tendency of suppression (#). No changes were confirmed regarding the production of IL-4 and IL-12.

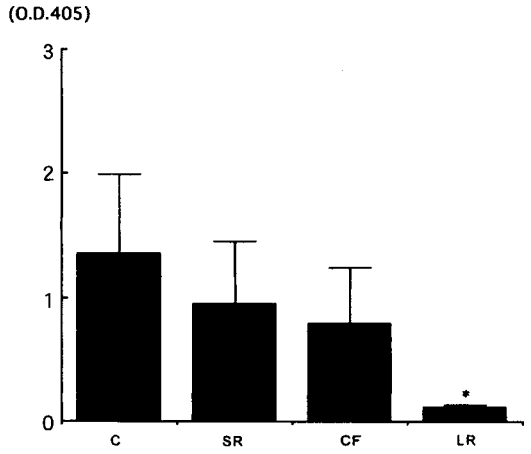

Fig. 3 The Production of Anti-Hsp60 Antibody
Compared with the control group (C), anti-Hsp60 antibody production : 1.51 O.D. 450nm, the SR group was 0.99 O.D. 450nm, the CF group was 0.79 O.D. 450nm and the LR group was 0.11 O.D. 450nm. The significant reduction of the titer of the anti-hsp60 antibody was identified in the LR group compared with the control group. (*p<0.05) The other two groups showed a tendency for reduction but not significantly.

Table II Pathological evaluation of atherosclerosis

	Aorta Valve				Root of Ascend Aorta			
	Range	Score	Index	Ratio	Range	Score	Index	Ratio
Cintrol	33.3%	1~2	4.7	100%	13.3%	0~1	1.3	100%
SR	38.0%	1~3	7.0	100%	20.0%	0~1	2.4	60%
CF	22.0%	0~2	4.9	75%	8.3%	0~1	0.8	25%
LR	26.0%	0~2	4.2	60%	10.0%	0~1	1.2	60%

Mean;
The lipid depositions were found in each group to a different degree. It shows a tendency to increase in volume and area gradually from the aortic valve to the root of ascending aorta. The deposits were observed in each mouse of the control group and SR group, but only 20 to 60% of the mice in the remaining groups exhibited lipid deposition.

pg/ml; IL-12 : 4.2±0.6pg/ml; In contrast, the SR group were : IFN-γ : 464±92.2pg/ml; IL-4 : 60.3±10.6pg/ml ; IL-12:8.7±1.1 pg/ml; and the CF group are : IFN-γ : 751.2±251.3pg/ml; IL-4 : 51.5±9.0pg/ml; IL-12 : 5.25±1.1pg/ml; The LR group were : IFN-γ : 329.5±146.3pg/ml; IL-4 : 60.6±6.5pg/ml; IL-12 : 4.4±2.2pg/ml; The production of IFN-γ in the SR and CF groups with were significantly suppressed, but the LR group only showed a tendency of suppression. No changes were confirmed regarding the production of IL-4 and IL-12. (Fig.2)

Anti-Hsp60 antibody

Compared with the control group (anti-Hsp60 antibody production: 1.513 O.D.450nm), the SR group was 0.99 O.D.450nm, the CF group was 0.79 O.D.450nm and the LR group was 0.11 O.D.450nm. The significant reduction of titer of the anti-Hsp60 antibody was identified in the group administered with LR compared with the control group. The other two groups showed a tendency for reduction, but not significantly. (Fig.3)

Pathological evaluation of atherosclerosis (Table II) and the pathological findings of the aorta (Fig.4)

The lipid depositions (fatty streak) on the endothelium and sub-endothelium of the root of the aorta were found in each group to a different degree. At the aortic valve, the results of lipid deposition area were: control group : 33.3%; SR group : 38.0%; CF group : 22.0%; LR group : 26.0%; The results of the deposition score were: control group : 1 to 2; SR group : 1 to 3; CF : 0 to 2; LR

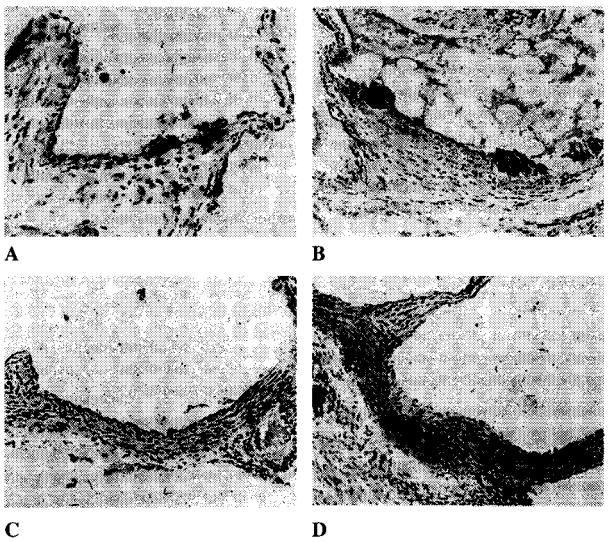

Fig. 4 Pathological findings of the aorta. (x200)
The lipid depositions were stained with oil red O.
A: Control;
B: SR
C: CF
D: LR

group : 0 to 2; The results of the index of atherosclerosis were: control group : 4.7; SR group : 7.0; CF group: 4.9; LR group : 4.2; The results of the incidence ratio were: control group : 100%; SR group : 100%; CF group : 75%; LR group: 60%;

The results from the valuation of the upper valve portion were: the lipid deposition area: the control group: 13.3%; SR group : 20.0%; CF group : 8.3%; LR group: 10.0%; The results of the deposition score were : control group : 0 to 1; SR group : 0 to 1; CF: 0 to 1; LR group: 0 to 1; The results of the index of atherosclerosis were: control group : 1.3; SR group : 2.4; CF group : 0.8; LR group: 1.2; The results of the incidence ratio were : control group : 100%; SR group : 60%; CF group : 25%; LR group : 60%;

The lipid deposits that we observed had a tendency to increase in volume and area gradually from the aortic valve to the root of ascending aorta. The deposits was observed in each mouse of the control group and SR group, but only 20 to 60% of the mice in the remaining groups exhibited lipid deposition.

Discussion

Atherosclerosis is the primary cause of ischemic heart disease and stroke. From the pathogenic point of view, possible causes of atheresclerosis include elevated and modified LDL; free radicals caused by cigarette smoking, hypertension, diabetes mellitus, aging, elevated plasma homocysteine concentration; infectious microorganisms such as Clamydia penemoniae or herpes virus or Helicobacters pyloris, or any combinations of these or other factors.[14]

The principle of the response-to-injury hypothesis about the formation of the atherosclerosis is that it is at the point on the artery wall where the endothelium injured. The platelets are aggregate and release several factors that introduce the proliferation of the vascular smooth muscle cell. This is more important that the dysfunction of the endothelium that is induced by the invasion of macrophages and T-lymphocytes in the wall of the aorta.[2]

In the process of the formation of atherosclerosis, many immune factors, such as cytokines and chemokines are involved, especially M-CSF, TNF-α, and INF-γ are induced when monocytes are derived to macrophages. Among these, PDGF, M-CSF, TNF-α, and IL-12 promote the differentiation of macrophages and work as accelerators of atherosclerosis.[15]

On the other hand, the Hsps of Clamydia pnuemoniae and humans are both recognized at the atherosclerosis (atheroma) and have the same molecular structure, and show the same function of activating the endothelium and macrophages. Although it has been reported in the past that hosts and invaded cells have their own Hsps, it has recently been reported that the Hsps from different species exhibit high conservation. Hsps are found in all organisms from E. coli to human beings and their cognates show highly homologous sequences between different species. Another feature of Hsps is that they have high immunogenesis, although there is a very strong homology in amino acid aligment.[16]

So the recent point of view is that the existing knowledge of immunology fails to explain many aspects of disease with the usual self-non-self theory.[3] According to Janeway's hypothesis of Pathogen Associated Molecular Patterns (PAMPs), in addition to the general immune system of recognizing self-non-self by T and B cells, common structure molecules of bacteria, parasites, and fungi are recognized by innate immune cells such as macrophages and dendritic cells. For example : the lipopolysacchride of gram negative bacteria, the lipoara-

binomannan of mycobacteria, and double stranded RNA of viruses- are all PAMPs .[17] Hsps are considered to be a kind of PAMPs.[3]

From another point of view, Matzinger recommended a new model of immune systems: the danger model hypothesis. This idea proposes that, instead of the major function of the immune system being to discriminate between self and non-self, its function is actually to recognize danger, especially certain danger signals that emanate from the tissues of the body, rather than from the pathogens. In this model, it is self Hsp that is being recognized, not from the pathogen. It would seem that Hsps play a central role in the initiation of the host immune responses including atherosclerosis.[19]

These new points of view using the Hsp-anti-Hsp theories may lead to a new therapy or treatment for disease.

We had already investigated the effects of traditional Kampo herbal medicine on the production of Hsps. First of all, we examined the effects of 231 extra herb medicines on the expression of Hsp70 on IMR-32 cells under thermal stress and found that Scutellariae Radix (SR), Linderae Radix (LR), and Carthami Flos (CF) caused an enhancement by 2 times on the activity of the Hsp70 expression.[20]

Secondly, we checked the effect of 234 extra herbal medicines on the induction of Hsp70 on the human active T-lymphocyte, two herbs: SR and CF showed a remarkable enhancement on the activity of the Hsp70 expression.[21]

In our investigation, the significant reduction of body weight of the mice of the control group fed with HCD induced hyperlipidermia, and additionally immunizing with Hsp60, which works as an adjuvant, is considered to exacerbate the status. This is because the anti-Hsp60-antibody works on the cell membrane to induce an antigen-antibody reaction, a rapid progression of atherosclerosis by macrophages, severe ischemia, or so called microangiopathy due to an embolism, and the dysfunction of the main vital organs of the host.

Although the pathogenic investigation confirmed that the aorta of each group of mice had the depositions of lipids to some extent, the depositions in the mice treated with Linderae Radix and Carthami Flos were suppressed to some extent. We concluded that this effect was due to the herbal medicines, especially Linderae Radix, reducing the adjuvant function of the Hsps, and simultaneously reducing the progression of the atheroscleosis. Those with a reduced function exhibited a suppression in the titer of the anti-Hsp60-antibody with Linderae Radix.

The complete effect of cytokines on the progession of atherosclerosis has not yet been detected. However, it has been reported that the Th1 group of cytokines, such as INF-γ which was measured in this study, is the M-CSF inducing factor which is found at the pre-inflammation stage of atherosclerosis as the accelerator of the monocyte/macrophage differentiation and cloning, and an inhibitor of vascular smooth muscle proliferation. This inhibition of vascular muscle accelerates the break down of plaque by decrement of the extra-cellular matrix.[22]

On the other hand, the Th2 group of cytokines, such as IL-4 and IL-12, are considered to be the anti-inflammatory factors, those have been reported to inhibit the formation of atherosclerosis. However, the IL-4 is also considered to be a main cytokine at the immune construction of the atherosclerosis.[15]

The herbal medicines we investigated showed different effects on the active immune system, such as: LR and SR enhancing the production of cytokines. SR significantly enhanced Th1 group production and showed a tendency to accelerate the Th2 group. CF mainly accelerated the Th1 group. On the other hand, LR showed a tendency to accelerate the Th2 group cytokine IL-4 only. The mechanism of herbal medicine on (acceleration or inhibition) the formation of atherosclerosis is activation of the transfer factor of heat shock, activation of the translation, and activation of the protein synthesis.

It was reported that CF reduces the serum concentration of cholesterol because of linoleic acid component. This function will influence the hypothesis of formation of atherosclerosis but it could not interpret that why other herb medicines, especially LR, have the same effect without containing linoleic acid.

Although the new mechanism of herbal medicine with Hsp-anti-Hsp systems is almost unknown, a clue for the development of a new therapy may be found by clarifying the substances that adjust Hsps expression.

和文抄録

動脈硬化の形成過程では免疫関連因子，各種サイトカインやケイモカインが関与している。一方，ストレス蛋白（Heat Shock Proteins. Hsps, Chaperonins）はシャペロンという機序を通して，外来微生物が産生する異種蛋白の発見，毒素の産生，或いは感染による宿主細胞内代謝障害の際における蛋白質の変性や凝集を抑制し，さらには変性蛋白質を修復することにより，蛋白の損傷から細胞を保護する機能を担っていると考えられる。我々はすでにC57BL/6NJcマウスを用いて，Hsp60で免疫すると同時に高脂肪症食を与えることによって，動脈硬化症を誘導することに成功した。今回，このモデルマウスに，黄芩，紅花，烏薬，を投与し，動脈硬化の病理学的変化，体重，血清サイトカインへの影響などを検討した。その結果，対照群に対して，黄芩と紅花を投与した群は有意に体重減少の抑制を認めた。($p<0.05$) また，これらの群におけるIFN-γも有意の変化が見られた。さらに，心臓弁膜から大動脈起始部にかけて見られた対照群その他の脂質の沈着も紅花及び烏薬群では20～60％しか見られなかった。従って，生薬によるHsp60のアジューバンド効果への抑制と同時に，動脈硬化への抑制効果を確認した。

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