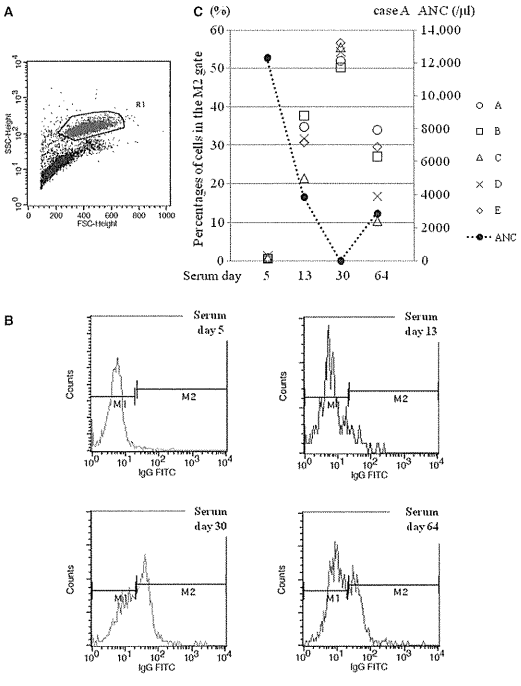

Figure 3 Histogram patterns of fluorescence given by the serum from the neutropenic KS patient. (A) neutrophils were identified by their FSC versus SSC properties and the R1 population collected. (B) the percentage of cells attaining an arbitrary level of fluorescence above the background signal in serum on day 5 (before intravenous immunoglobulin treatment) is indicated as M2. The flow cytometric data in subsequent figures utilized the same M2 gate. The columns show example data that staining of neutrophils separated from case C (shown in Table 1) associated with IgG levels of the serum obtained from patient A at different time points during the course of disease. (C) Percentages of cells in the M2 gate are shown compared with ANC of case A. Data within the graph (case A through E) are listed in Table 1. ANC, absolute neutrophil count.

In primary autoimmune neutropenia, the autoantibody specificity has been defined and the usually recognized human neutrophil antigens (HNAs) are located on glycosylated isoforms of FcγRIIIb (CD16b) [14, 15]. Autoantibody specificity associated with secondary autoimmune neutropenia is often unknown [16] but was recently shown to be associated with pan FcRγIIIb antibodies [17]. In this case, the recognized major HNAs were negative. We tried to evaluate the specificity of the immunoglobulin binding using an immunoblot technique with cell lysates to identity the target antigens. However, we could not identify the specific protein. Little information was found about the target antigens, and further studies are required to determine the mechanism of autoimmune-induced neutropenia in KS.

Granulocyte immunofluorescence test has proven to be the best screening procedure for the detection of neutrophil-specific antibodies [18, 19]. These direct and indirect methods have the advantage of avoiding the non-specific binding of IgG and IgG immune complexes to the neutrophils [20]. Furthermore, flow cytometric analysis of GIFT can be used to detect antibodies of any subclass directly on the patient's neutrophils or indirectly on donor neutrophils after incubation with the patient's serum [21]. This study showed that autoantibodies bound to immature CD13-positive myeloid cells, resulting in myeloid lineage maturation arrest in the bone marrow. In addition, GIFT revealed that autoantibodies to neutrophils were produced and were associated with quantitative variation over time during the clinical course of the patient. Autoimmune neutropenia became increasingly severe as antibodies were directed against not only peripheral neutrophils, but also earlier precursors. Agglutination is the major neutrophil response to anti-neutrophil antibodies, and an activated complement system can cause neutrophil aggregation and adherence to endothelial cells [17]. Phagocytosis of neutrophils that are coated with anti-neutrophil antibodies is another probable mechanism for neutrophil destruction [17]. Furthermore, anti-neutrophil antibodies might have a role in the

myelosuppression by inhibiting the growth of granulocyte/macrophage colony-forming unit, or inhibition of bone marrow granulopoiesis by proinflammatory cytokines [16, 22]. In the light of these considerations, we speculated that newly produced autoantibodies bound to either immature myeloid cells or circulating neutrophils and might have caused severe neutropenia in our patient. D-GIFT was negative in all subjects, even in the patient's leukocytes obtained 89 days after onset when the KS inflammation had completely subsided. However, because of the retrospective analysis, we could not perform D-GIFT using the patient's leukocytes in the middle of the KS inflammation. Given that the antibodies bound to immature CD13-positive myeloid cells, we speculated that the maturational-specific antigens of the autoantibody on the myeloid precursor or neutrophil membrane increased during the acute or subacute phase of KS inflammation, and then gradually decreasing after the KS inflammation had subsided.

We also revealed that the amount of autoantibody produced inversely correlated with the patient's neutrophil counts throughout the patient's hospitalization and outpatient clinic visits. Immune activation is a significant part of the pathogenesis of KS, characterized by an immunoregulatory imbalance that consists of an increased number of activated helper T cells and monocytes, a decreased number of $CD8^+$ suppressor/cytotoxic T cells and marked polyclonal B cell activation [23]. There are also several studies on polyclonal B cell activation, demonstrating an increase in autoantibodies [24, 25]. These polyclonal autoantibodies to foreign antigens might cross-react with self-antigens and, in the case of a normally developed immune systems, this type of immune reaction is self-limiting [21]. Meanwhile, these antibodies may develop as a result of 'molecular mimicry' wherein an epitope on the surface of foreign infectious antigen stimulation. Those produced antibodies are also considered to be polyclonal and are present relatively long period (month or year) [26]. The aetiology of KS remains unknown, although infectious agents are suspected and being discussed even now. Hence, it is conceivable that the possibility of infectious antigens induced these autoimmune phenomena. Various drugs are also thought to be associated with neutropenia [27]. These mechanisms include immune-mediated destruction of granulocytes or granulocytic precursors, dose-dependent inhibition of granulopoiesis and direct toxic effect on myeloid precursors or the marrow microenvironment [28, 29]. In this case, the DLST of PAPM/BP was positive, suggesting that it may be one of the causes of immune-mediated neutropenia. The antibiotics might function as a hapten and recognize antigens on the neutrophil membrane, resulting in the production of neutrophil-specific autoantibody. However, when the drug acts as a hapten, the ANC should also improve within 1–2 weeks after cessation of drug administration [26]. In addition, potential role of IVIG-induced neutropenia also should be considered. IVIG-induced neutrophil apoptosis in KS had been suggested by the rapid occurrence after IVIG administration and was experimentally demonstrated in circulating neutrophils in patients after IVIG administration [7, 30]. The more commonly suggested mechanisms are the presence of anti-neutrophil antibodies in preparing immunoglobulin, and we examined and confirmed the absence of antibodies to neutrophils in the same lots of immunoglobulin used for IVIG treatment. These mechanisms, therefore, did not explain the disease course of the present case. Thus, autoantibodies to immature myeloid cells and neutrophils might be developed as part of a polyclonal activation of B cells and cause transient neutropenia.

In conclusion, an autoantibody to a novel antigen on immature myeloid cells or neutrophils was produced and was revealed as a possible cause of severe neutropenia in a patient with KS. Our findings provide further insight into the potential mechanisms of antibody-induced neutropenia associated with KS.

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Development of Kawasaki syndrome in autoimmune neutropenia after treatment with granulocyte colony-stimulating factor

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Key words autoimmune neutropenia, granulocyte colony-stimulating factor, Kawasaki syndrome.

Kawasaki syndrome (KS) is an acute febrile illness with systemic vasculitis, which may cause coronary artery abnormalities (CAA).[1] Laboratory findings show an increased white blood cell (WBC) count, a shift to the left with segmented neutrophils and increased C-reactive protein (CRP) levels in the acute phase of the disease. Pathological histology also shows infiltrating cells that initially mainly consist of neutrophils, followed in time by macrophages.[2] Primary autoimmune neutropenia occurs frequently in newborns with an incidence of approximately 1/100 000, and is usually diagnosed at the age of 5–15 months.[3,4] Although there is significant neutropenia at the time of disease onset (500–1000 neutrophils/μL), the clinical course tends to resolve by the age of 2 or 3 years in 95% of patients.[4] Patients with autoimmune neutropenia who have severe infections sometimes require not only antibiotics but also additional treatment with corticosteroids, i.v. immunoglobulin (IVIG), and granulocyte colony-stimulating factor (G-CSF).[3]

Here, we describe an 8-month-old boy with autoimmune neutropenia, who developed Kawasaki syndrome soon after being treated with G-CSF. This may provide an interesting viewpoint about potential onset mechanisms of KS.

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Case report

A previously healthy 5-month-old boy had high fever and lymphadenopathy, and was treated successfully with antibiotics. After treatment his lymphadenopathy soon improved but neutropenia with absolute neutrophil counts (ANC) <500/μL was noted (90–400/μL), and the condition lasted for months. He was diagnosed with autoimmune neutropenia because of the presence of anti-neutrophil antibody. At the age of 8 months he had high fever and complained of earache. Laboratory findings were as follows: WBC, 4500/mm^3; ANC, 626/mm^3; CRP, 5.4 mg/dL. He was diagnosed as having acute otitis media, and was admitted to the neighborhood hospital for initial treatment with the antibiotic flomoxef sodium. On the third day of illness he still had high fever, and developed right cervical lymphadenopathy. Computed tomography showed a ring-enhancing lesion, suggestive of a cervical abscess. WBC was 3560/mm^3, ANC, 819/mm^3; and CRP 11.9 mg/dL. The antibiotic was changed from flomoxef sodium to meropenem trihydrate and clindamycin, and G-CSF administration (5 μg/kg per day) was initiated as an additional treatment, but his clinical symptoms (i.e. high fever and lymphadenopathy) did not improve. On the fifth day of illness, 3 days after initiation of G-CSF, he suddenly developed skin rash, peripheral edema, injected lips, and conjunctival injection, and was then diagnosed as having KS. We administered IVIG (2 g/kg) for 1 day and oral aspirin (30 mg/kg) and stopped administration of clindamycin and G-CSF. Although the clinical symptoms partially subsided, he continued to have high fever, and WBC was 8430/mm^3, ANC was 6643/mm^3, and CRP was 22.9 mg/dL. He was then treated with IVIG (2 g/kg) for 1 day additionally on the seventh day, but

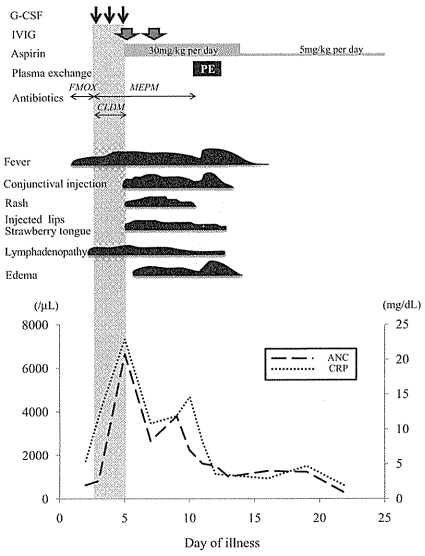

Fig. 1 Clinical course and laboratory data. ANC, absolute neutrophil count; CLDM, clindamycin; CRP, C-reactive protein; FMOX, flomoxef sodium; G-CSF, granulocyte colony-stimulating factor; IVIG, i.v. immunoglobulin; MEPM, meropenem trihydrate.

he had poor clinical resolution of symptoms, and was then referred to Kagoshima University Hospital.

At referral, echocardiography demonstrated mild dilatation of the origin of the left and right coronary arteries; we administered a 2 day course of plasma exchange (PE) with the consent of the parents on the 10th day. During the course of PE therapy, his clinical symptoms gradually subsided, and, similarly, inflammatory markers decreased after PE therapy (total exchange volume, 1625 mL): WBC decreased from 8620 to 4300/mm^3, ANC decreased from 4095 to 860/mm^3, and CRP decreased from 11.9 to 3.5 mg/dL. There were no complications during PE therapy. Aspirin was reduced to 5 mg/kg per day on the 13th day when the serum CRP level decreased. He was discharged on the 25th day. At discharge, echocardiography showed mild dilatation of the left coronary artery and his WBC was 4750/mm^3, and ANC 736/mm^3 (Fig. 1). Sequential follow up with catheter angiography after 4 months from KS onset showed complete regression of the left coronary artery dilatation.

Discussion

We encountered a patient with autoimmune neutropenia who developed KS clinical symptoms rapidly after receiving G-CSF treatment. Furthermore, he needed to undergo PE therapy for resistance to additional IVIG treatment. Functionally activated neutrophils are known to increase in number, and transient infiltration of neutrophils was identified in the early stage of KS.[2] Elevated neutrophil counts are associated with the development of coronary artery lesions, but early neutropenia within 10 days of illness is reported to be associated with CAA formation. The significance of neutrophils in KS has not been fully elucidated.[5] Inflammation associated with KS initially involves elevation of the levels of various cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), and G-CSF may also play an important role in the acute phase of KS.[6,7]

The G-CSF treatment has also been associated with flares in patients with Felty's syndrome or other autoimmune diseases such as systemic lupus erythematosus.[8] Autoimmune neutropenia is likely to have a relatively benign course, but G-CSF treatment is indicated in some children in whom severe infections occur. G-CSF has been identified as a glycoprotein that stimulates the production and functional activation of neutrophils, and modulates the function and activity of matured neutrophils including production of chemokines, phagocytosis and cell surface receptor expression.[9] Activated neutrophils have been reported to induce organ damage by tissue infiltration and release of pro-inflammatory cytokines.[9] G-CSF treatment also induces excessive migration or activation of neutrophils and induces monocyte or macrophage production, which would promote vascular permeability.[9] KS patients, however, who present with only fever and cervical lymphadenopathy at admission have been reported to have an increased risk of additional IVIG treatment and of developing CAA.[10] Therefore it is possible that the present case may have been due to the natural course of the KS itself, but G-CSF treatment may have potentially contributed to the severity of KS inflammation. In light of these considerations, we suggest that G-CSF treatment might be involved in the onset mechanism of KS clinical symptoms.

The present case raises concerns about the potential complication of G-CSF treatment in children with autoimmune neutropenia. Pediatricians should be aware of this risk and ensure careful clinical observation when using G-CSF treatment in children with autoimmune neutropenia complicated with severe or recurrent infections.

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Blepharophimosis-ptosis-epicanthus inversus syndrome

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Key words blepharophimosis, epicanthus inversus, *FOXL2* gene, genetic counseling, ptosis.

Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) (OMIM 110100)[1] is a rare autosomal dominant genetic disease that affects aproximatelly 1 in 50 000 liveborns. It is clinically characterized by a complex eyelid/ocular malformation that includes blepharophimosis, ptosis, epicanthus inversus and telecanthus. It is often associated with premature ovarian failure (POF), which is classified as BPES type I. BPES with normal ovarian function is classified as BPES type II.[2–4]

Mutations in the *FOXL2* gene (forkhead transcription factor gene 2) (OMIM 605597),[1] located at 3q23, have been shown to underly this syndrome. The majority of cases are due to a *de novo* mutation.[5]

Here a case report is provided of a 1-year-old girl with BPES caused by a de novo mutation of the *FOXL2* gene.

Case report

The patient was a Caucasian 1-year-old girl, the only daughter of young and non-consanguineous parents. Both the mother and the maternal grandfather presented a history of bilateral blepharoptosis, entropium, distichiasis and dysmorphic ears. The mother was previously referred for oculoplastic surgery for blepharophimosis correction. The parents had healthy children from their previous marriages. The familial history was negative for cases with POF or infertility (Fig. 1). The patient was born by vaginal delivery, at term, weighing 3500 g (50th–75th centile), measuring 44 cm (<2nd centile), with head circumference of 36 cm (50th–98th centile) and Apgar scores of 8 and 10 at the first and fifth minutes, respectively (Fig. 1). Although the mother's pregnancy was uneventful, the fetal ultrasound identified a right pyelocaliceal dilatation. Renal ultrasound performed soon after birth confirmed this finding. The evaluation made later, through the same exam and through excretory urography, was normal however.

At physical examination, at the age of 1 year and 9 months, the infant presented with a height of 70 cm (50th centile); weight of 8900 g (50th–75th centile) and head circumference of 44.3 cm (2nd–50th centile); supraciliar arch hypoplasia; arched eyebrows; blepharophimosis; ptosis, epicanthus inversus; telecanthus; low and broad nasal root; short columella; long philtrum; high arched palate; carp-shaped mouth; micrognathia; small, low-set and posteriorly displaced ears with overfolded helix; and redundant neck skin (Fig. 2). No heart murmur or sign of cardiorespiratory dysfunction was observed. Ophthalmological assessment did not identify any additional findings. Molecular analysis from a peripheral blood sample through polymerase chain reaction (PCR), sequencing, and multiplex ligation-dependent probe amplification (MLPA) of the *FOXL2* gene showed that the patient was heterozygous for the p.Pro287Fs (c.843-859dup) mutation in the *FOXL2* gene. The molecular evaluation of her mother and grandfather did not show the presence of this mutation, however.

The child presented a normal neuropsychomotor development: she had head support at age 3 months; sat alone at 7 months and started to walk alone at 1 year and 3 months. At 10 months, she was referred for simultaneous corrective surgeries for epicanthus inversus/telecanthus, through the technique of medial canthoplasty using Z-plasty, and for ptosis, through bilateral frontalis suspension. No complications were observed after the surgery.

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特集　川崎病の本態にせまる－古くて新しい研究から－

Ⅲ．治療にせまる（治療法・有用性から本態にせまる）

ステロイドパルス

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要旨　われわれは，免疫グロブリン（IVIG）療法追加不応の川崎病をステロイドパルス（IVMP）で治療してきた．IVIGで加療した川崎病412例中21例にIVMPとリバウンド防止のプレドニゾロン後療法を行ったところ，冠動脈病変（CAL）を2/21例，計3/412例（1%）に認めた．洞徐脈（17例）などの副作用があったが自然軽快した．IVMPは迅速な抗炎症作用がありCAL抑制効果も期待されるため，IVIG不応例や不応予測例には使用する価値があると考える．

Key words　ステロイドパルス，プレドニゾロン後療法，免疫グロブリン不応例

はじめに

ステロイドパルス（intermittent intravenous methylprednisolone，以下IVMPと略す）療法は，ステロイド薬の中でも電解質作用が少ないメチルプレドニゾロンを大量に点滴静注する治療法である．当初，腎移植後の急性拒絶反応に対して開発され，強力な免疫抑制作用を有することからリウマチ性疾患・呼吸器疾患・腎疾患などの重症例や難治例に広く用いられるようになった．

川崎病では，免疫グロブリン（intravenous immunogloburin，以下IVIGと略す）療法が確立する以前の1982年，IVMPで初回治療を行ったKijimaらの報告[1]をもって嚆矢とする．その後，ステロイド薬への旧弊な批判とIVIGの普及に伴い，IVMPは一時使用されなくなった．ところが，1996年のWrightらの論文[2]を契機に，IVIG不応例の治療法として世界的に注目され，わが国でもHashinoら[3]がいち早く導入した．このIVMPの再評価は，川崎病全例に対する初回IVIGとの併用効果を否定したNewburgerらの二重盲検無作為化比較試験[4]以来，トーンダウンしているようにみえる．しかし，重症例では迅速な抗炎症作用が期待できることから，IVMPは依然として有用な治療法であると考える．

本稿では，川崎病に対するIVMPについて，初めにIVIG不応例に対するわれわれの成績を紹介し，次いで全般的な解説を述べる．

当院におけるステロイドパルス療法の成績

1．IVIG初回不応例に対するIVMPとIVIG追加の比較[5]

筆者の所属施設（以下，当院とする）の前身である清瀬小児病院において，2001年1月～2003年5月に，初回IVIG療法終了後も発熱が持続・再燃した22例を対象にIVMPとIVIG追加の非盲検無作為化比較試験を行った（各群11例）．

IVMPによって全例速やかな解熱が得られたが，一部の症例に発熱のリバウンドが生じた（図1）．IVMP群における最高体温はIVIG追加群に比し，投与後2日目では有意に低値であったが（中央値36.9 vs. 37.8℃，$p=0.02$），3日目以降では有意差を認めなかった．結局，両群においてさらに治療を要した例（3例vs. 2例）と冠動脈病変（colonary artery lesion，以下CALと略す）の割合（2例vs. 3例）は同等であった．

副作用に関しては，洞徐脈（9例vs. 2例，$p=0.01$）と高血糖（6例vs. 0例，$p=0.01$）がIVMP群に有意に高率に認められた．IVMP群では体温が急速に降下し，最低体温は有意に低値であった（平均値35.4 vs. 36.1℃，$p=0.002$）．これらの変化はいずれも一過性で自然に回復し，重篤な副作用は両群とも認められなかった．

すなわち，IVMPはIVIG追加に比し，①抗炎症作用は迅速だが時にリバウンドを伴う，②CALの抑制効果は同等の可能性がある，③洞徐脈などの副作用が多い，ことが判明した．

2．IVIG追加不応例に対するIVMPとプレドニゾロン後療法[6]

前述の成績と川崎病に対するステロイド薬の使用は保険適用がない状況をふまえ，IVIG初回不応例はIVIG追加で治療し，IVMPはさらに不応の重症例に限定し，リバウンド防止にプレドニゾロン（PSL）の後療法を行う方針とした．IVMPは30 mg/kg/日を3日間，PSLは1～2 mg/kg/日で開始し2週間で中止（1週間後から3日間ずつ漸減），IVMP療法中はヘパリン併用〔10～20単位/kg/時間活性化部分トロンボプラスチン時間（activated partial thromboplastin time：APTT）が短縮しないように調節〕，IVMP～PSL投与中はファモチジン併用（1 mg/kg/日 分2）を行った．なお川崎病では，低ナトリウム血症の主因が抗利尿ホルモン不適切分泌症候群（syndrome of inappropriate secretion of antidiuretic hormone：SIADH）であること[7]，水分貯留がCALの危険因子になること[8]が示されているので，IVIG投与中は輸液を行わず，その後も輸液量を制限した（維持量の1/2～2/3）．

2003年7月～2008年11月の約5年間の成績では（図2）[6]，10病日未満に当院で初回IVIGを開始した川崎病は412例で，IVIG初回不応74例（18%）にIVIGを追加した．さらに不応の21例（28%，全

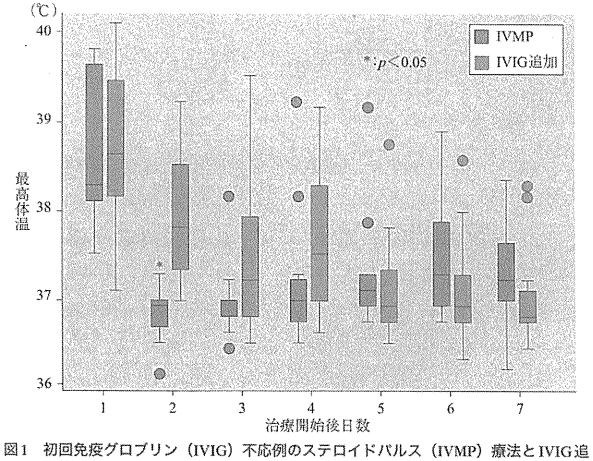

図1　初回免疫グロブリン（IVIG）不応例のステロイドパルス（IVMP）療法とIVIG追加後の体温（文献5）より引用，一部改変）
箱の上端，中央線，下端は順に，75%点，中央値，25%点に相当する．ヒゲは箱の長さの1.5倍以内の値，○印ははずれ値を示す

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412例中の5%）にIVMPを行ったところ，全例速やかに解熱した．その後PSLを2週間経口投与したが，うち7例は発熱の再燃があり3～6週間まで延長した．IVMPの開始は7～12病日で，12例（57%）は8病日以内であった．

CALは，IVIG反応の1例，IVIG追加反応の0例，IVMP反応の2例の計3例〔全412例中の1%，

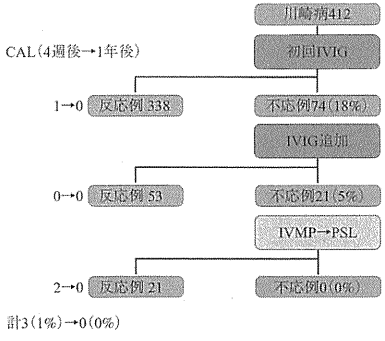

図2　免疫グロブリン（IVIG）追加不応例に対するステロイドパルス（IVMP）療法・プレドニゾロン（PSL）後療法の成績（文献6）より引用，一部改変）
箱の中の数字は症例数（全体に占める割合），左側の数字は発症4週後と1年後の冠動脈病変（CAL）の症例数（合併率）を示す

米国の基準[9]ではおのおの6例，0例，2例の計8例（2%）〕に認めたが，いずれも巨大瘤ではなく1年後に退縮した．すなわち，当院の方針を適切に活用すれば，CALの発生をゼロにするという川崎病急性期治療における究極の目標達成も可能になると考えられる．

IVMPの副作用として，洞徐脈（17例），高血圧（17例），低体温（3例）などを認めたが，PSL後療法中は減少し自然軽快した．治療が必要な重篤な副作用は認めなかった．

川崎病に対するステロイドパルス療法の概論

1．作用機序

ステロイド薬は細胞質内の糖質コルチコイド受容体（細胞質受容体）に結合し，核内で遺伝子の発現を調節して抗炎症作用を示す（ゲノム作用）[10]．IVMPでは細胞質受容体の飽和量を大幅に上回るため，ゲノム作用以外の機序も関与すると予想される．この非ゲノム作用（図3）には，①細胞質受容体と複合体を構成する蛋白質を介する作用，②細胞膜の糖質コルチイド受容体を介する作用，③ステロイド薬の細胞膜への陥入による膜結合蛋白質の機能変化，などがあり，ゲノム作用よりも急速に発現する[10][11]．

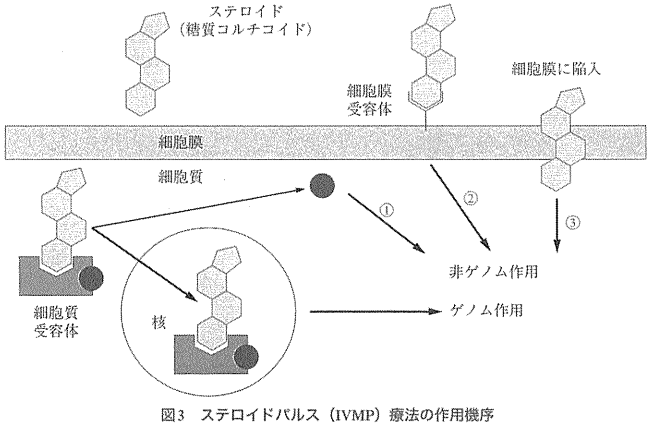

図3　ステロイドパルス（IVMP）療法の作用機序
ステロイドパルス療法はゲノム作用以外にも，①細胞質受容体と複合体を構成する蛋白質，②細胞膜受容体，③細胞膜への陥入，などを介する非ゲノム作用によって作用する

川崎病では，IVMPの抗炎症作用が早期に出現することから，おもに非ゲノム作用によって免疫細胞の働きや炎症性サイトカインを抑制すると推測される．IVIG不応あるいは不応予測例においては，IVMPによって炎症・CALにかかわるサイトカインの分泌〔tumor necrosis factor（TNF）-α，monocyte chemotactic protein（MCP）-1〕[12]や遺伝子の転写量[13]が減少することが報告されている．

2．使用方法

腎疾患や膠原病ではメチルプレドニゾロンを，成人では1～2 g，小児では20～30 mg/kgを2～3時間かけて1日1回 3日間点滴投与する方法が標準的である[11]．IVMPの半減期は3時間と短いが[11]，疾患の性質上，通常はPSLなどが引き続き投与される．

川崎病では，メチルプレドニゾロン30 mg/kgを1日1回，1～3日間とする文献[3)～6)9)13)～17)]が多い．PSLの後療法[6)15)17)]と副作用防止のためのヘパリン・抗潰瘍薬の併用[6)15)]の妥当性に関しては，報告によって相違があり確立していない．

3．適応別の成績

1）初回IVIGとIVMPとの併用

川崎病全例に対して初回IVIGにIVMPを併用する効果は，Newburgerらの二重盲検試験[4]によって否定された．すなわち，IVIG＋IVMP群とIVIG＋プラセボ群の比較では，発熱日数，IVIG追加の割合（12% vs. 16%），CAL合併率（16% vs. 19%），冠動脈径のZスコアなどに有意差を認めなかった．この結果は，ステロイド薬の不要な症例も広く対象にしたためと推察される．

一方，IVIG不応例では初回IVIGとIVMP併用が有用である可能性がある．Newburgerらの試験[4]でも，IVIG追加例に限定した事後解析（post-hoc analysis）では，IVMP併用群のCAL合併率がプラセボ群より有意に低かった（0% vs. 60%，$p=0.002$）．Okadaら[14]は，IVIG不応予測例に対する初回IVIGとIVMPの併用は，IVIG単独に比し解熱が早くCAL合併率も有意に低い（24% vs. 47%，$p=0.03$）ことを報告した．

2）IVIG不応例に対するIVMP

初回IVIG不応例に対する治療としては，IVMPの効果をIVIG追加と比較した場合，解熱効果は早く，CAL合併率は同等（18% vs. 27%，11% vs. 11%，0% vs. 21%）[5)15)16)]で，医療費が安価[16]というメリットを示した複数の研究がある．これらの報告より前にHashinoら[3]は，IVIG追加例（初回2 g/kg後に1 g/kg追加）を対象に同様の成績を報告した（CAL合併率73% vs. 68%）．しかし，いずれの検討でも，非劣性検定によるIVMPとIVIG追加の同等性は証明されていない．

実際には，IVIG追加不応例にIVMPを行っている施設が多く，その一部ではPSL後療法も用いられている[6)15)17)]．米国のガイドライン[9]では，IVMPはIVIG（2 g/kg）を2回行った不応例に限定するべきであると記載されている．IVIG追加不応例をIVMPの適応とすると対象を少数に絞ることができるが，投与が遅れることでCALが発生するおそれも否めない．

3）CAL合併例に対するIVMP

IVIG不応例でCALが生じたため，他施設から当院に紹介される例も少なくない．CAL合併例に対するステロイド薬使用の是非については否定的な見解もあるが，われわれは炎症を終息させるために積極的に投与するほうがよいと考えている．この場合，中等度以上の瘤には抗凝固療法，高血圧があれば降圧薬（プロプラノロールなど）の併用を考慮している．

Adachiら[18]は，IVMPを行ったCAL合併例（巨大瘤を含む）では，IVMPを行わなかった例に比べ退縮率が有意に高いと報告した（100% vs. 46%，$p=0.04$）．川崎病に対するIVMPを初めて報告したKijimaら[1]，CALが高率に改善すると述べている．すなわち，IVMPを行った後に仮にCALを

生じたとしても，長期的な予後は悪くない可能性がある．

4．副作用

IVMPの副作用は[10][11][19]，ステロイド療法全般に共通するものとIVMP特有のものに分けられる．前者には，感染症，高血糖，電解質異常，消化性潰瘍，高血圧，精神障害，大腿骨頭壊死などがあるが，ステロイド薬長期投与に伴う成長障害，副腎機能抑制，骨代謝異常はないか軽微であると考えられる．後者としては，味覚障害，顔面紅潮，けいれん，洞徐脈・房室ブロック・頻拍症などの不整脈などがあげられる．

川崎病におけるIVMPの副作用は，報告によって相違がある．Newburgerらの二重盲検試験では[4]，副作用の出現率はプラセボと有意差はないとされているが，調査項目が十分ではないように思う．合併率に相違はあるが[5][6][15]，洞徐脈・高血圧・低体温などの危険性があるので，IVMP施行時には心電図モニタ・血圧測定などを用いてバイタルサインを注意深く観察するべきである．

最近われわれは，ステロイド薬投与が長期（3週間以上が目安）[20]にわたる例では副腎皮質機能抑制に注目し，内分泌学的検査〔早朝の副腎皮質刺激ホルモン（adrenocorticotropic hormone：ACTH）とコルチゾール測定〕を行っている．コルチゾールが10 μg/dL以下の例では，副腎皮質刺激ホルモン放出ホルモン（corticotropin releasing hormone：CRH）負荷試験やPSLより半減期が短いコートリル®の補充を考慮する．副腎皮質機能抑制下では感染症や炎症が悪化するおそれもあるので，今後，詳しく検討していきたい．

おわりに

川崎病に対するIVMP療法に関し，われわれの成績を含め文献を中心に概観した．ステロイド薬はどの施設でも使用可能，医療経済的に有利，小児科医の経験が豊富といったメリットが大きい．なかでもIVMPは効果が迅速であることから，IVIG不応例におけるIVMPの使用は炎症の終息だけでなくCALの抑制にも有用である可能性がある．

ステロイド薬への旧来の批判は，統計学が未熟な時代の古い報告やステロイド薬使用でCALが悪化したことがあるという経験論に基づいており，否定的なエビデンスは皆無といってよい．後方視的なデータ（たとえば全国調査）を利用して，もともと重症例の多いステロイド薬投与例にCAL合併率が高いとする非難は，明らかに適切ではない．

今後，RAISE study（われわれも2008年12月から参加，別稿の「川崎病におけるプレドニゾロン治療」参照）などによって，川崎病に対するステロイド薬の妥当な使用法も科学的に解明されていくだろう．そして，免疫抑制薬や抗サイトカイン療法はリウマチ性疾患や腎疾患と同様に，ステロイド抵抗例を適応として有効性を検証するべきと考える．

川崎病の本態が感染症によって誘発された免疫異常による血管炎であることは，ほぼコンセンサスが得られているだろう．感染症については，経気道性に持続感染する未知のRNAウイルス[21]など種々の報告があるが，単一の病原体でないかもしれない．むしろ，川崎病に対するステロイド薬の有効性は，免疫異常が病態の主体であることを示唆する．免疫異常については，一般に注目されている全身免疫のほかに，主要症状である皮膚・粘膜の炎症を司る粘膜免疫が関与しているのではないかと推測する[22]．

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ORIGINAL ARTICLE

Steroid pulse therapy for Kawasaki disease unresponsive to additional immunoglobulin therapy

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M Miura, T Tamame, T Naganuma, S Chinen, M Matsuoka, H Ohki. Steroid pulse therapy for Kawasaki disease unresponsive to additional immunoglobulin therapy. Paediatr Child Health 2011;16(8):479-484.

BACKGROUND: The optimal management of Kawasaki disease (KD) unresponsive to intravenous immunoglobulin (IVIG) therapy remains unclear.

OBJECTIVE: To prospectively evaluate the efficacy and safety of intravenous methylprednisolone pulse (IVMP) therapy in KD cases unresponsive to additional IVIG.

METHODS: KD patients who initially received IVIG (2 g/kg/24 h) and acetylsalicylic acid within nine days after disease onset were studied. Patients who did not respond received additional IVIG (2 g/kg/24 h), and those who still did not respond were given IVMP (30 mg/kg/day) for three days, followed by oral prednisolone. The response to treatment, echocardiographic findings and adverse effects were evaluated.

RESULTS: Among 412 KD cases, 74 (18.0%) were treated with additional IVIG; 21 (28.4%) of the latter cases subsequently received IVMP followed by prednisolone. All cases became afebrile soon after IVMP infusion and did not have a high-grade fever during treatment with prednisolone for two to six weeks. Four weeks after disease onset, coronary artery lesions (CAL) were diagnosed according to the Japanese Ministry of Health and Welfare or the American Heart Association criteria in two of the 21 cases treated with IVMP plus prednisolone; among all 412 cases, three (0.7%) and eight (1.9%) had CAL according to each criteria, respectively. All CAL regressed completely one year after disease onset. Adverse effects of IVMP, such as hypothermia and sinus bradycardia, resolved spontaneously.

CONCLUSIONS: In KD patients unresponsive to additional IVIG, IVMP promptly induced defervescence, and subsequent oral prednisolone suppressed recurrence of fever. IVMP followed by prednisolone therapy may prevent CAL, without severe adverse effects.

Key Words: *Coronary artery lesions; Kawasaki disease; Nonresponders to intravenous immunoglobulin; Prednisolone; Steroid pulse*

La thérapie pulsée à base de stéroïdes dans le traitement de la maladie de Kawasaki qui ne répond pas à une thérapie à l'immunoglobuline supplémentaire

HISTORIQUE : On ne connaît pas la prise en charge optimale de la maladie de Kawasaki (MK) qui ne répond pas à une thérapie à l'immunoglobuline intraveineuse (IGIV).

OBJECTIF : Procéder à une évaluation prospective de l'efficacité et de l'innocuité de la thérapie pulsée à la méthylprednisolone par voie intraveineuse (MPIV) dans les cas de MK qui ne répondent pas à une IGIV supplémentaire.

MÉTHODOLOGIE : Les chercheurs ont étudié les patients atteints de la MK qui commencent par recevoir de l'IGIV (2 g/kg/24 h) et de l'acide acétylsalicylique dans les neuf jours suivant l'apparition de la maladie. Les patients qui n'ont pas répondu au traitement ont reçu de l'IGIV supplémentaire (2 g/kg/24 h), et ceux qui ne répondaient toujours pas ont reçu de la MPIV (30 mg/kg/jour) pendant trois jours, suivie de prednisolone par voie orale. Les chercheurs ont évalué la réponse au traitement, les résultats échocardiographiques et les effets indésirables.

RÉSULTATS : Parmi les 412 cas de MK, 74 (18,0 %) ont été traités avec de l'IGIV supplémentaire; 21 (28,4 %) de ces cas ont ensuite reçu de la MPIV suivie de prednisolone. Tous ces cas sont devenus afébriles peu après la perfusion de MPIV et n'ont pas fait de forte fièvre pendant le traitement à la prednisolone de deux à six semaines. Quatre semaines après l'apparition de la maladie, des lésions des artères coronaires (LAC) ont été diagnostiquées selon les critères du ministère de la santé et du bien-être du Japon ou de l'*American Heart Association* dans deux des 21 cas traités à la MPIV et à la prednisolone. Dans les 412 cas, trois (0,7 %) et huit (1,9 %) avaient des LAC selon ces critères, respectivement. Toutes les LAC s'étaient complètement résorbées un an après l'apparition de la maladie. Les effets indésirables de la MPIV, tels que l'hypothermie et la bradycardie des sinus, se sont résolus spontanément.

CONCLUSIONS : Chez les patients atteints de la MK qui ne répondaient pas à de l'IGIV supplémentaire, la MPIV a rapidement induit une défervescence, et l'administration ultérieure de prednisolone par voie orale a supprimé la récurrence de fièvre. Une thérapie à la MPIV suivie de prednisolone peut prévenir les LAC sans causer d'effets indésirables graves.

Kawasaki disease (KD), the most common cause of acquired heart disease in children in developed nations, is an acute systemic vasculitis of childhood, primarily affecting the coronary arteries. Approximately 10% to 20% of patients do not become afebrile after standard therapy with intravenous immunoglobulin (IVIG) and oral acetylsalicylic acid, and are at an increased risk for the development of coronary artery lesions (CAL) (1-6). Such patients generally receive additional treatment with IVIG (1-5), which may prevent CAL if administered promptly (6); however, not all patients respond. Other treatment options, including corticosteroids, cyclosporine, plasma exchange and infliximab, have been reported to be effective in some cases (1-5,7). Although corticosteroids are widely used, the indications and dosage for patients with KD remain

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controversial. Some physicians avoid corticosteroids because of the potential risk of exacerbating CAL, but no study, including the classical work by Kato et al (8), has demonstrated a significant relationship between the use of corticosteroids and CAL.

Intravenous methylprednisolone pulse (IVMP) therapy rapidly resolves fever associated with refractory KD and is, thus, expected to prevent CAL. In small, open-label, randomized controlled studies performed by us (9) and Hashino et al (10), the prevalence of CAL was similar in patients with KD unresponsive to initial IVIG therapy who received IVMP and patients who received additional IVIG. In our study, IVMP induced faster resolution of fever, but caused more adverse effects, such as bradycardia, than did additional IVIG; however, the total antipyretic potency of these treatments was similar because fever recurred after the completion of IVMP therapy (9). In a double-blind, randomized controlled study, Newburger et al (11) reported that the addition of a single dose of IVMP to initial IVIG did not reduce the prevalence of CAL or the total length of the hospital stay; nonetheless, the initial period of hospitalization was shorter for all patients with KD who received IVMP, and the prevalence of CAL was significantly lower in patients unresponsive to IVIG who received IVMP, compared with patients who received placebo. These findings may reflect the prompt and short antipyretic activity of IVMP, and suggest that IVMP may prevent CAL in patients with severe KD.

During the past five years, we treated KD patients who were unresponsive to additional IVIG with IVMP followed by oral prednisolone to prevent recurrent fever. We now report the extremely low prevalence of CAL among the approximately 400 patients with KD managed using this treatment regimen.

METHODS

The present study prospectively evaluated a protocol for the treatment of patients with acute KD. In brief, nonresponders to initial treatment with IVIG received additional IVIG, and patients who still did not respond to additional IVIG were given IVMP followed by oral prednisolone. The subjects were consecutive patients with KD who were given IVIG in the hospital within nine days after disease onset from July 2003 through November 2008. Since December 2008, the hospital has participated in the Randomized controlled trial to Assess Immunoglobulin plus Steroid Efficacy for Kawasaki disease (RAISE) study. Acetylsalicylic acid was given orally (30 mg/kg/day to 50 mg/kg/day) until two or three days after fever cessation, followed by 5 mg/kg/day until no CAL were evident as of eight weeks after the onset of illness. Exclusion criteria were incomplete KD, acetylsalicylic acid therapy alone, initiation of IVIG treatment 10 or more days after the onset of disease, and previous treatment with IVIG or corticosteroids within two weeks before admission.

According to the protocol, an additional dose of IVIG (2 g/kg/24 h) was given to patients who had persistent or recurrent fever 48 h after completion of the initial IVIG infusion. Fever was defined as an axillary body temperature of 37.5°C or higher. Patients in whom fever did not resolve within 24 h after completion of the additional IVIG infusion received IVMP (30 mg/kg/day of methylprednisolone for three days) combined with a continuous infusion of heparin (15 units/kg/h to 20 units/kg/h). Then, oral prednisolone (1 mg/kg/day to 2 mg/kg/day) was given for one week and the dose was tapered over the following week. If fever recurred during this period, the dose of prednisolone was increased and tapered again, thereby extending prednisolone therapy. The subjects also received famotidine orally (0.5 mg/kg/day) during IVMP and prednisolone therapy to prevent gastrointestinal ulcers. The study was approved by the Human Research Ethics Committee of the hospital. Informed consent was obtained from the parents or guardians of the children for all treatments.

Echocardiograms were obtained before the start of the initial IVIG infusion, and two weeks, four weeks and one year after the onset of KD. The internal lumen diameters of the left main coronary artery (LMCA), the left anterior descending coronary artery (LAD), the left circumflex coronary artery and the proximal right coronary artery (RCA) were measured. The presence or absence of CAL was evaluated according to the criteria of the Japanese Ministry of Health and Welfare (JMHW [12]; previously named the Japanese Ministry of Health, Labour and Welfare) and those of the American Heart Association (AHA [3]). The JMHW criteria consider CAL to be present if the luminal diameter is greater than 3 mm in children younger than five years of age, or greater than 4 mm in children five years of age or older; if the internal diameter of a segment measures at least 1.5 times that of an adjacent segment; or if the coronary lumen is clearly irregular (12). The AHA criteria consider CAL to be present if the z score of the internal diameter is at least 2.5 for the LAD or proximal RCA (3). In addition, systolic dysfunction of the left ventricle, defined as a shortening fraction of less than 29%, regurgitation of the mitral and aortic valves, and pericardial effusion were evaluated.

Adverse effects of IVMP followed by oral prednisolone therapy were studied, especially hypothermia, arrhythmias, hypertension, thrombosis, femoral head necrosis, convulsions, secondary infections and gastrointestinal bleeding (9). Hypothermia was defined as an axillary body temperature below 35.0°C; bradycardia as a heart rate below the second percentile of the normal standard (13); and hypertension as a systolic or diastolic blood pressure higher than the 95th percentile of the normal standard (14). As for corticosteroid-related changes in laboratory data, hyperglycemia (fasting blood glucose greater than 6.99 mmol/L) and serum electrolyte imbalance (hyponatremia [lower than 135 mmol/L] and hyperkalemia [greater than 5.5 mmol/L]) were also assessed.

RESULTS

During the study period, 469 cases of KD in 461 Japanese patients, including eight recurrent cases, were admitted to study hospital. The following cases were excluded: incomplete KD (n=35), treated with acetylsalicylic acid alone (n=7), IVIG started 10 days or more after disease onset (n=3), and those previously treated with IVIG therapy before transfer to the hospital (n=12). A total of 412 cases of complete KD in 404 patients, including eight recurrent cases that initially received IVIG and acetylsalicylic acid within nine days after disease onset, fulfilled the inclusion criteria. A total of 227 male and 177 female patients were one to 158 months of age (median 23 months; interquartile range 12 to 45 months) at the onset of KD. The initial IVIG infusion was started two to nine days (median five days; interquartile range four to six days) after disease onset. Underlying diseases were detected in nine patients: ventricular septal defect (n=3), corrected transposition of the arteries (n=1), Down syndrome with ventricular septal defect (n=1), Niikawa-Kuroki syndrome with a double-outlet right ventricle (n=1), Williams syndrome with supra-aortic valvular stenosis (n=1), Sotos syndrome with a bicuspid aortic valve (n=1) and diabetes mellitus (n=1).

Among the subjects, 338 cases in 336 patients, including two recurrent cases, demonstrated defervescence within 72 h

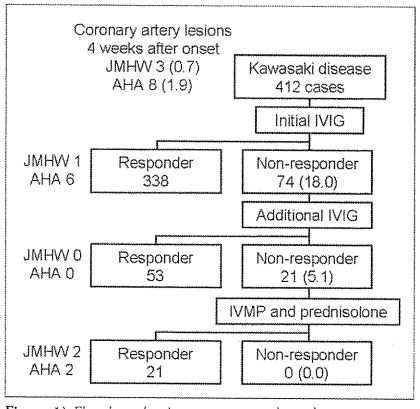

Figure 1) *Flowchart showing treatment results and coronary artery lesions. Numbers in parentheses represent percentages. Numbers beside the boxes indicate the number of cases of coronary artery lesions four weeks after disease onset according to the criteria of the Japanese Ministry of Health and Welfare (JMHW) and the American Heart Association (AHA). IVIG Intravenous immunoglobulin; IVMP Intravenous methylprednisolone pulse*

TABLE 1
Clinical data of cases treated with corticosteroids

Cases, n	21
Age, months, median (IQR)	44 (24–66)
Male patients	11 (55.0)
Illness days at the start of treatments, median, n (IQR)	
IVIG	4 (4–5)
Additional IVIG	7 (7–8)
Intravenous methylprednisolone pulse	8 (8–9)
Days of prednisolone therapy, n (IQR)	15 (13–24)

Cases of coronary artery lesions	4 weeks after onset	1 year after onset
JMHW criteria	2 (9.5)	0 (0)
AHA criteria	2 (9.5)	0 (0)
Cases with adverse effects	**IVMP**	**Prednisolone**
Hypothermia	3 (14.3)	0 (0)
Bradycardia	17 (81.0)	13 (61.9)
Hypertension	17 (81.0)	11 (52.4)
Thrombosis	0 (0)	0 (0)
Femoral head necrosis	0 (0)	0 (0)
Convulsions	0 (0)	0 (0)
Secondary infection	0 (0)	0 (0)
Gastrointestinal bleeding	0 (0)	0 (0)
Hyperglycemia	7 (33.3)	1 (4.8)
Hyponatremia	4 (19.0)	3 (14.3)
Hyperkalemia	0 (0)	1 (4.8)

Data presented as n (%) unless otherwise indicated. AHA American Heart Association; IQR Interquartile range; IVIG Intravenous immunoglobulin; IVMP Intravenous methylprednisolone pulse; JMHW Japanese Ministry of Health and Welfare

after the start of IVIG treatment and remained afebrile (Figure 1). Fever persisted 48 h after the completion of initial IVIG treatment in 53 cases and recurred after defervescence in 21. Consequently, an additional IVIG infusion was used to treat 74 cases (18.0%) in 72 patients, including two recurrent cases. The additional IVIG infusion was started six to 19 days (median seven days) after disease onset and before day nine in 55 cases (74.3%). Despite the second IVIG infusion, 18 cases had persistent fever and three had recurrent fever. These 21 cases (28.4% of the cases treated by additional IVIG; 5.1% of the total cases) in 20 patients, including one recurrent case, were treated by IVMP therapy followed by oral prednisolone. The clinical characteristics of the cases treated with corticosteroids are shown in Table 1. IVMP was started seven to 12 days (median eight days) after disease onset and before day 10 in 17 cases (81.0% of the 21 cases treated by IVMP followed by prednisolone). Body temperature fell rapidly soon after the start of IVMP therapy in all cases and remained below 37.5°C during the two weeks of prednisolone therapy and, subsequently, in 14 cases (66.7%). Because of recurrent low-grade fever during tapering of prednisolone, the dose was adjusted in the remaining seven cases, and the duration of prednisolone therapy was prolonged to three weeks in four cases, four weeks in two cases, and six weeks in one case. None of the subjects required other treatments such as a third course of IVIG, cyclosporine, plasma exchange or infliximab.

Figure 1 and Table 2 show cardiac complications including CAL, ventricular systolic dysfunction, mitral and aortic valvular regurgitation, and pericardial effusion. Before starting the initial IVIG infusion, four cases (1.0%) had CAL according to the JMHW criteria and 15 (3.7%) had CAL according to the AHA criteria among the 410 cases for which echocardiographic studies were available. Four weeks after disease onset, CAL were diagnosed according to the JMHW criteria in one of 338 cases (0.3%) responsive to initial IVIG, zero of 53 responsive (0.0%) to additional IVIG, and two of 21 (9.5%) responsive to corticosteroids; CAL were diagnosed according to the AHA criteria in six (1.8%), zero (0.0%) and two (9.5%) cases, respectively (Figure 1). In total, CAL were present in two of 74 cases (2.7%) unresponsive to initial IVIG and in three of all 412 cases (0.7%), according to the JMHW criteria, compared with two (2.7%) and eight (1.9%), respectively, according to the AHA criteria. The prevalence of CAL eight weeks after disease onset was similar to that four weeks after disease onset.

Among three cases of CAL at four weeks, according to both the AHA and the JMHW criteria, one that responded to initial IVIG had a lesion that was 3.4 mm in diameter (z score = 2.3) in the LMCA and a 3.0 mm lesion (z score = 3.3) in the LAD; one of the two cases that responded to corticosteroids had a 4.9 mm lesion (z score = 7.9) in the RCA and a 3.4 mm lesion (z score = 5.1) in the LAD, and the other case had a 3.4 mm lesion (z score = 3.0) in the LMCA and a 2.5 mm (z score = 2.6) lesion in the LAD. All five cases of CAL according to the AHA criteria, but not the JMHW criteria, responded to initial IVIG; z scores were at least 2.5 for RCA lesions in two cases (2.6 and 2.9) and for LAD lesions in three cases (2.6, 3.0 and 3.5). One year after disease onset, CAL completely regressed in all eight cases; consequently, none of the 384 cases (0.0%), including all 21 cases treated by corticosteroids for which echocardiographic studies were available, had CAL according to the JMHW criteria. Catheter angiography was performed six months after disease onset to evaluate the coronary arteries in two cases responding to corticosteroids, and it also showed regression of

TABLE 2
Echocardiographic findings

	Pre-IVIG (n=410)	Post-IVIG 2 weeks after onset (n=409)	Post-IVIG 4 weeks after onset (n=412)	Post-IVIG 1 year after onset (n=384)
Coronary artery dimensions, mm				
Right coronary artery	1.87±0.37	1.79±0.32	1.74±0.35	1.80±0.31
Left main coronary artery	2.22±0.30	2.12±0.31	2.07±0.31	2.12±0.29
Left anterior descending coronary artery	1.77±0.36	1.69±0.33	1.67±0.30	1.71±0.28
Left circumflex artery*	1.53±0.36	1.48±0.31	1.48±0.33	1.52±0.33
z score of diameters				
Right coronary artery	0.26±0.84	0.04±0.73	−0.08±0.79	−0.05±0.67
Left main coronary artery	−0.01±0.58	−0.24±0.63	−0.36±0.64	−0.45±0.58
Left anterior descending coronary artery	0.40±0.85	0.17±0.85	0.12±0.74	0.04±0.69
Abnormal cases, n (%)				
Coronary artery lesions				
JMHW criteria	4 (1.0)	5 (1.2)	3 (0.7)	0 (0.0)
AHA criteria	15 (3.7)	7 (1.7)	8 (1.9)	2 (0.5)
Ventricular systolic dysfunction	17 (4.1)	2 (0.5)	0 (0.0)	0 (0.0)
Aortic regurgitation	6 (1.5)	6 (1.5)	2 (0.5)	3 (0.8)
Mitral regurgitation†	38 (9.3)	42 (10.3)	23 (5.6)	22 (5.7)
Pericardial effusion	20 (4.9)	10 (2.4)	2 (0.5)	1 (0.3)

*Data presented as mean ± SD unless otherwise indicated. *Diameters of the left circumflex artery were available in 324 cases preintravenous immunoglobulin (IVIG), 343 at two weeks, 305 at four weeks and 301 at one year after onset; †Mitral regurgitation was trivial in 27 cases pre-IVIG, 36 at two weeks, 20 at four weeks and 16 at one year after onset. AHA American Heart Association; JMHW Japanese Ministry of Health and Welfare*

CAL. However, according to the AHA criteria, CAL were newly detected in the LAD in two other cases (0.5%) one year after disease onset: one responsive to initial IVIG had a lesion 2.7 mm in diameter (z score = 2.6) and one responsive to additional IVIG had a 2.7 mm lesion (z score = 2.7). Twenty-two patients (5.7%) had mitral regurgitation, but not CAL, one year after disease onset; the severity was trivial in 16 patients, mild in five and moderate in one.

As for the adverse effects of IVMP, sinus bradycardia in 17 cases (81.0%) and hypertension in 17 (81.0%) were most prominent (Table 1). Hypothermia occurred in three cases (14.3%), hyperglycemia in seven (33.3%) and hyponatremia in four (19.0%). All of these adverse effects improved during the following course of prednisolone and finally resolved with no special treatment. No subject experienced thrombosis, femoral head necrosis, convulsions, secondary infection, gastrointestinal bleeding or severe arrhythmias such as atrial flutter, ventricular tachycardia and arrioventricular block.

DISCUSSION

The results of our study suggest that IVMP therapy followed by oral prednisolone is useful for the prevention of CAL and for defervescence in patients with KD unresponsive to an additional IVIG infusion. Four weeks after disease onset, CAL were present, according to both the AHA and the JMHW criteria, in only two cases (2.7% of 74 cases unresponsive to initial IVIG). The incidences of CAL were, thus, considerably lower than those previously reported for severe cases of KD, ie, 26.7% to 48.6% in cases unresponsive to initial IVIG, and 60.0% to 70.6% in cases unresponsive to additional IVIG (1,2,6,9-11). Furthermore, CAL in our two cases did not have a diameter of at least 5.0 mm or a z score of at least 8.0, and completely regressed within one year after disease onset. We believe that IVMP followed by prednisolone therapy, therefore, prevented CAL in cases unresponsive to additional IVIG, resulting in the extremely low prevalence of CAL in our study group as a whole, ie, 0.7% at four weeks and 0.0% one year after disease onset according to the JMHW criteria, and 1.9% and 0.5%, respectively, according to the AHA criteria. A nationwide survey of KD in Japan (15,16) reported that the prevalence of CAL according to the JMHW criteria was 3.2% to 3.8% four weeks after disease onset; the prevalence of giant coronary aneurysms was 0.25% to 0.35%. In an analysis performed at 27 paediatric hospitals in the United States (7), coronary artery aneurysms were diagnosed in 3.3% of patients with KD, although the criteria were not clearly described. We believe our results are very encouraging because we have almost reached the final goal of treatment for KD (ie, no patients with CAL).

Our indications for corticosteroid therapy are consistent with the AHA statement (3) that the use of corticosteroids should be restricted to patients with KD in whom two infusions of IVIG have been ineffective for alleviating fever and acute inflammation. At present, corticosteroids are not superior to IVIG in terms of experience, the prevention of CAL or possible adverse effects. We, therefore, do not recommend that corticosteroids be administered to all patients with KD as initial treatment (17) or to those not responding to initial IVIG therapy (18), although this policy may change if novel benefits of corticosteroid therapy are established, for example, by the ongoing RAISE study. When our strategy was used for treatment, only 5.1% of subjects received corticosteroid therapy.

Nonetheless, early administration of corticosteroids is essential for patients with severe KD unresponsive to IVIG because prolonged inflammation may induce CAL. Among cases responding to corticosteroids in our study, 81.0% were treated with IVMP therapy before 10 days after disease onset, when CAL become evident pathologically (19) and clinically (6,20). This early onset of IVMP therapy may have played an important role in the low prevalence of CAL. If fever resolves before 10 days after disease onset in patients with severe KD, early treatment for nonresponders to additional IVIG is necessary; the response was, therefore, evaluated 24 h after completion of

the additional IVIG infusion. Corticosteroids are used in various regimens for the treatment of KD; however, we prefer IVMP rather than the conventionally used dosage to resolve fever rapidly because IVMP therapy is started approximately 10 days after disease onset in patients who do not respond to initial or additional infusions of IVIG. We previously reported that IVMP promptly induces defervescence, but fever rebounds in 54.5% of patients within four days after the completion of treatment (9). Indeed, the half-life of pulse methylprednisolone is short (3 h) and the duration of action is only two days (21). In the present study, subsequent administration of oral prednisolone prevented recurrence of fever after IVMP therapy, without the need for any other treatment; seven of 21 cases (33.3%) had low-grade fever during tapering of prednisolone, but all of these cases became and remained afebrile after adjusting the dose of prednisolone. Coincidentally, our strategy is similar to the report by Gong et al (22) with respect to the oral administration of corticosteroids after IVMP therapy given to patients not responding to an additional IVIG infusion.

Corticosteroids may inhibit immune cells and inflammatory cytokines that cannot be completely suppressed by IVIG in patients with KD unresponsive to IVIG. Makata et al (23) showed that IVIG inhibits the activation of macrophages and coronary arterial endothelial cells more strongly than that of T cells in vitro, whereas dexamethasone inhibits the activation of all three cell types. CD8-positive T cells and macrophages infiltrate the tissue of coronary artery aneurysms in patients with fatal KD, and both of these cell types are speculated to play important roles in the pathogenesis of CAL (24). High concentrations of steroids intercalate into the plasma and mitochondrial membranes of immune cells, thereby altering their physicochemical properties and the activities of membrane-associated proteins (25), which are believed to contribute to rapid immunosuppression. Okada et al (26) reported that serum levels of inflammatory cytokines, such as interleukin 2 and interleukin 6, were significantly lower in patients with KD who initially received IVIG combined with prednisolone than in those who received IVIG alone. In our control study of patients with KD unresponsive to initial IVIG (9,27), plasma levels of tumour necrosis factor-alpha and monocyte chemoattractant protein-1 were reduced faster with IVMP therapy than by additional IVIG therapy. Hence, we believe that the mechanisms of corticosteroid therapy complement those of IVIG in refractory KD.

Despite the good outcomes in terms of CAL, our strategy has several shortcomings. An additional infusion of IVIG, an expensive biological product, was used to treat 18.0% of KD cases. The well-known adverse effects of corticosteroids can be induced by IVMP therapy followed by oral prednisolone for two weeks or longer. In our study group, sinus bradycardia, hypertension and hyperglycemia were the most prominent adverse effects, but did not require treatment. There were no severe complications such as thrombosis or gastrointestinal bleeding in any patient, in part because heparin and a histamine type 2 blocker were given prophylactically. We have used heparin with IVMP to prevent potential hypercoagulability and coronary thrombosis as recommended for steroid-resistant nephrotic syndrome in children (28).

The dose of acetylsalicylic acid used to treat acute KD differs from country to country, eg, 30 mg/kg/day to 50 mg/kg/day in Japan (4) and the United Kingdom (5), and 80 mg/kg/day to 100 mg/kg/day in the United States (3). Lower doses may exert lower anti-inflammatory activity than higher doses and, therefore, may have contributed to the rate of nonresponders to IVIG in the present study. However, Japanese physicians prefer lower doses because of a potentially better tolerance (ie, lower risks of liver dysfunction, gastrointestinal ulcers and other side effects). We believe that the dose of acetylsalicylic acid is unimportant because a meta-analysis conducted by Terai and Shulman (29) showed that the prevalence of CAL is strongly dependent on the IVIG dose, but independent of the acetylsalicylic acid dose.

Our study had several limitations. First, some biases may have been present because the study was noncontrolled and performed in a single centre; the effectiveness of IVMP followed by oral prednisolone should, therefore, be verified by a multicentre, controlled study. Second, echocardiographic studies were performed by several people, including paediatric cardiologists and technicians, and one-year follow-up studies were not completed in all 410 patients. Third, the z scores of coronary artery diameters derived from studies in American children (3) were applied to our Japanese subjects, and racial differences may be related to the low incidence of CAL in our study.

CONCLUSION

In cases of KD unresponsive to an additional infusion of IVIG, IVMP therapy induced prompt defervescence and subsequent treatment with oral prednisolone suppressed recurrent fever. This treatment strategy may have decreased the prevalence of CAL. Adverse effects caused by corticosteroid therapy were transient and nonsevere, although sinus bradycardia and hypertension occurred frequently.

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冠動脈後遺症をもつ川崎病

鮎沢　衛*

I 疾患概念

川崎病の治療にガンマグロブリン静注療法（IVIG）が行われ始めたのは，1980年代後半からである。それ以前に発症し，現在成人している川崎病既往者は，20～30%が冠動脈病変の後遺症をもっていると考えられる。全国調査結果[1]に基づくと，IVIGが普及した現在では，発症1か月後の時点で，冠動脈瘤の合併率は約3%に減少したが，患者数が年間1万人以上に増加しているため，毎年新たに約300人の心後遺症患者が発生している。後遺症例の病変分類を図1に示す[1]。

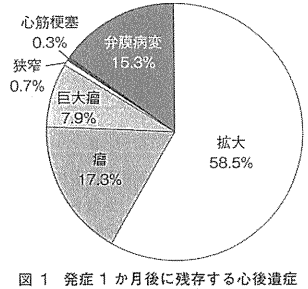

図1　発症1か月後に残存する心後遺症のべ803例の内訳[1]（第20回全国調査：2007～2008発症患者対象）
のべ803例/23337例=3.4%

川崎病の急性期治療が終了し，外来フォローに移った患者の管理は，後遺症としての冠動脈病変の有無と，その程度によって異なるが，2003年以降は，日本循環器学会の川崎病心臓血管後遺症の診断と治療に関するガイドラインによって，基本方針が示されている[2]。

ここでは，冠動脈瘤を合併した患者の管理について注意点を述べる。

II 年齢別健康管理

1．検　査

1）身体所見

川崎病既往者の身体所見は，発病1か月目に四肢末端の所見で，膜様落屑の残存や爪の横溝，頸部リンパ節炎の影響で斜頸の状態がある例以外は，正常である。小児科の外来診療では血圧測定を怠りがちであるが，川崎病の重症後遺症があれば，できるだけ毎回測定し，患者および家族が成長後血圧は重要であるという意識をもつようにすることが重要である。

2）臨床検査

臨床検査では，退院時に炎症所見や血小板数の異常増多，肝機能の異常などを残していた場合は，1～3か月後の時点でフォローするために採血する場合があるが，冠動脈後遺症がある場合には，ワルファリン内服による抗凝固療法のコントロールのためや，あるいは虚血性心疾患としての病状評価をするために，定期的に血液検査を行い投与量の調整を行う。

凝固能検査としては，プロトロンビン時間の国際標準化比（PT-INR）を測定する。筆者は，外傷を起こしやすい幼児期の患者は1.5～2.0，出血性の副作用を理解して注意できる年齢になれば，2.0～2.5を目標にコントロールしている。

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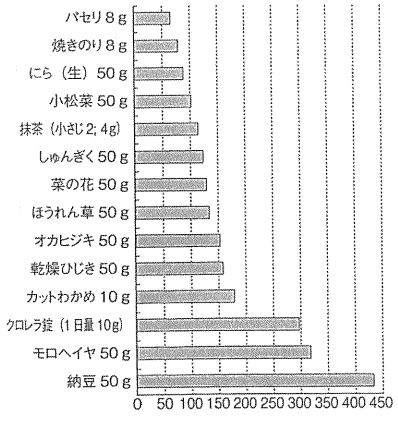

図2　各食品1食分あたりのビタミンK（K_1+K_2）含有量（単位 μg）（文献3をもとに改変）
注：「青汁」は製品により，表示される1杯分のビタミンK含有量に15～180 μgまで幅がある。

食事指導は不可欠で，図2のようなビタミンK含有量の多い食品の制限を指導しておく必要はあるが，あまり厳格にしないで少量は許可して血液検査をみながら投与量でコントロールしていけばよいと考える。

最近，成人の心房細動での抗凝固療法に使用が認可されたトロンビン阻害薬のdabigatranは，凝固能検査によるモニターが不要とされており，小児期からの川崎病冠動脈障害の血栓予防に，その長所が生かされることを期待したい。

3）機能検査

川崎病冠動脈後遺症の評価において，心電図は感度の高い検査法とはいえず，重大な障害が発生するまで変化がないが，何らかの変化が発生した場合には虚血性病変の部位や時間経過について高い診断力をもつ。そのため，安定期にはつい記録を怠りがちになるが，以前の所見からの変化の有無が重要であるため，最低でも半年に1回は記録しておきたい。

川崎病患者の動脈硬化の問題は関心がもたれ，頸動脈の内膜中膜厚（IMT）や硬化指数（AI），末梢動脈の脈波伝導速度（PWV）などは，川崎病冠動脈後遺症者で異常を呈しており，若年成人期に動脈硬化促進性があるのではないかという研究が報告されている[2]。一般的注意点として，冠動脈障害を残した小児には，肥満，高脂血症，喫煙などの危険因子を生涯にわたって避けるように，より強調して指導する必要がある。

4）画像検査

冠動脈および心筋血流に関する正確な画像検査情報は，川崎病冠動脈障害の評価に不可欠である。

心エコーは最も汎用される画像診断であり，発症後も5年目まではくり返し外来で実施されるが，中学生以上になると，十分な画像は得られにくくなるので，発症後1年までに病変の部位や程度を明確にしておく必要がある。

病変が疑われる例には，現在でも発症3か月ごろまでを目安として，一度は冠動脈造影を行っておきたいが，生後6か月未満の例などでは，疑われる病変の程度により，10 kgをこえるころまで待ってから行うこともやむをえない。

冠動脈障害をもった児に，競争的運動を許可するかどうかには，運動負荷または薬物負荷心筋SPECTによって正確に虚血の有無が評価されることが必要である。負荷時に虚血がなければ，管理指導区分はE可または禁でよいが，症状，所見が出現する場合は，D禁区分よりも厳しい制限が必要である。

最近，多列式のCT（図3）が急速に普及してきており，乳幼児での解像度がさらに向上すれば，冠動脈病変が疑われた場合のスクリーニングとして，有益であると思われる。施設によっては，以前に年長児以上のフォローのために行っていた，3～5年に1回の冠動脈造影は，ほとんどCTで代用することが増えていると思われる。ただし，結果が他の臨床所見，とくに核医学的検査における血流分布と矛盾して，重症の狭窄性病変や虚血が疑われる場合には血管造影所見を確認しなければならない。今後は，CTにおける被曝線量の低減が重要な課題である。

2．臨床評価

小児期に，心筋虚血の程度を臨床的に診察所見のみから判断することは困難である。冠動脈後遺

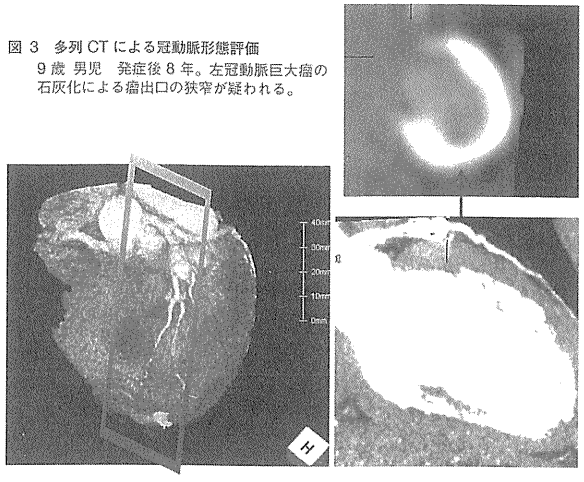

図 3　多列 CT による冠動脈形態評価
9 歳 男児　発症後 8 年。左冠動脈巨大瘤の石灰化による瘤出口の狭窄が疑われる。

症の影響を正しく把握するには，上記に述べた各種検査の意義を熟知することと，これまでの川崎病後遺症例での経験に基づいてどのような病態が起こっているかを予測する必要がある。

現在すでに，成人に達した後遺症患者が 70～80%を占めてきており，徐々に成人循環器科への引き継ぎを必要としている。

3．予想される課題（身体的，社会的，その他）に対する指導

1）急性期カードの活用

川崎病の後遺症患者は，多くは無症状で成人期に達し，発病が乳幼児期であったため，患者が自分の病状を知らないことが多い。保護者も時間とともに正確な内容を忘れるため，患者が進学，就職して転居した場合などに別な病院を受診したとき，既往歴が正しく把握されないことが多い。そのため，急性期 1 か月間の病状を記録した，川崎病急性期カード（図 4）を記入し，大切に保管するように指導しておくと有益である。

2）予防接種の可否

退院後に多くの患者から予防接種の可否について尋ねられる。インフルエンザ，DPT などの不活化ワクチンや，BCG，ポリオワクチンは，ガンマグロブリン投与（IVIG）の影響はないと考えられており，これらのうち必要なものは接種を開始してよい。麻疹・風疹（MR），水痘，おたふくかぜなどの生ワクチンは，総量 2 g/kg までの IVIG を施行した患者では 6 か月以降，IVIG の追加投与例は 9 か月目以降が適当と考えられている[5]。

3）ワルファリン副作用に対する工夫

幼児期はしばしば転倒，衝突などするため，ワルファリンのコントロール下では頭部の打撲で大きな皮下血腫や帽状腱膜下血腫などを合併することがある。頭部保護用の帽子やインナーキャップなども，医療介護用品として各種そろえられてきているので，年齢，性別に合わせて，機能，デザインなどを選ぶよう勧めるとよい。

また，学校生活をする年齢層では，冠動脈障害に伴い心筋虚血がある場合には，運動制限が必要になるが，とくにワルファリンによる抗凝固療法中の場合は，出血性副作用を防ぐために，格闘技やラグビーなどの contact sports は止めるよう指導する。

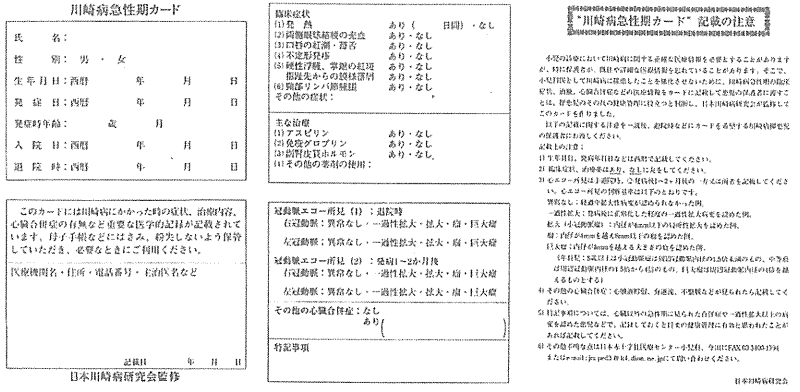

図 4　川崎病急性期カード

表　年齢別健康管理スケジュール

		発症 1 か月（1 歳）	発症 3～6 か月	発症 1 年後（2 歳）	発症 5 年後（小学校入学）	発症 10 年後（中学校入学）	発症 15 年後（16 歳）
内服薬	アスピリン内服	●	●	●	●	●	●
	ワルファリン内服	●	●（1～2 か月ごと）	●	●	●	●
血液検査	心筋虚血の評価（TnI，hs-CRP，BNP）			●（退縮，内膜肥厚開始後）	●	●	●
	凝固系（PT-INR）	●	●	●	●	●	
	急性期炎症反応，血小板数，肝機能	●	▲				
非侵襲的画像および機能検査	胸部 X 線	●		●	●（側面；石灰化の有無）	●（側面；石灰化の有無）	●（側面；石灰化の有無）
	心電図	●	（●）	●	●（運動負荷）	●（運動負荷）	●（運動負荷・24 時間）
	心エコー	●	●	●	●	▼	
冠動脈 MDCT 核医学検査 TI 心筋 SPECT 同時検査				●（施設により）	●	●（症例毎に適応判断）	
冠動脈造影			●		上記で予層あるとき・手術適応判定時		
不活化ワクチン（含ポリオ），BCG 許可			2～3 か月以後●				
生ワクチン（上記以外）許可			6 か月以後●				
外来指導		急性期カード記載と携行指導			学校生活管理指導（年 1 回）		
			ワルファリン食事指導	出血副作用予防具の紹介		本人へ病状説明，ドロップアウト防止	（女子）妊娠と薬剤の関係

▲は発症 1 か月時に異常を残している場合
▼は明瞭に冠動脈が抽出できる場合

4）女性患者の妊娠と出産について

成人期までワルファリンを服用することになる場合，女性の患者であれば，適切な時期に，ワルファリンの胎児への影響について話し，妊娠，出産は主治医，産科医と十分相談するよう理解させる必要がある[5]。先天性心疾患での経験では高校生でも妊娠の報告があり，最初に説明する時期としては，家族の了解を得たうえで，高校入学後がよいと考える。

Key Points

❶ 退院後の生活指導は，合併症の有無と程度によって内容が異なる。

❷ 成人期にドロップアウトしないように，中学卒業までに病状の説明を本人に十分しておく。

❸ 非侵襲的検査が普及しつつあり，検査が多様化しているので，各法の特性を十分把握する。

Ⅲ 年齢別健康管理スケジュール

表に示した。

Ⅳ 結 論

川崎病心後遺症患者は，成人の比率が年々増大しており，20歳以上が70～80%を占めてきている。生活制限の必要性は虚血の有無で決定されるので，経過と各検査結果を成人期までの数十年間のスパンで評価していくことが重要である。

進学や就職に伴う転居などにより，ドロップアウトすることを防止し，今後，成人内科医に協力を求めることが望まれる。

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Estrogen-like activity and dual roles in cell signaling of an *Agaricus blazei* Murrill mycelia-dikaryon extract

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ABSTRACT

Agaricus blazei (*A. blazei*) Murrill mycelia-dikaryon has attracted the attention of scientists and clinicians worldwide owing to its potential for the treatment of cancer. However, little is known about its effect on other pathologies. This study sought to extend the potential medical usefulness of *A. blazei* for preventing vascular damage and to unravel its mechanism of action. The *A. blazei* extract showed estrogen-like activity in both gene expression profiling and a luciferase assay. Indeed, the extract inhibited oxidized low-density lipoprotein-stimulated activation of Erk1/2, Akt and p38 in HUVECs and macrophage-derived TIB-67 cells. Moreover, the extract enhanced transcription of the glutathione peroxidase 3 (*GPX3*), α-synuclein (*SNCA*) and endothelial nitrogen-oxide synthase (*eNOS*) genes. Furthermore, atherosclerotic lesions in rabbits were reduced by intake of *A. blazei* powder. Therefore, *A. blazei* may be useful for preventing atherosclerosis via dual roles in cell signaling, suppression of macrophage development and the recovery of endothelial cells from vascular damage.

Introduction

Agaricus blazei (*A. blazei*) Murrill mycelia-dikaryon, a mushroom native to Brazil where it is known as the sun mushroom, has recently attracted the attention of scientists and clinicians worldwide for its potential adjuvant role in the treatment of cancer (Firenzuoli et al. 2008). Experiments *in vitro* and *in vivo* using various chemicals including polysaccharides isolated from mycelia and the fruiting body of *A. blazei* have shown anti-tumor activity potentially via activation of the host immune response (Kawagishi et al. 1989; Itoh et al. 1994; Kobayashi and Masumoto 2010; Oliveira Lima et al. 2011). Additionally, *A. blazei* components have been reported to affect the activity of natural killer cells and mitogen-induced lymphoproliferative activity in spleen cells of Ehrlich tumor-bearing mice (Kaneno et al. 2004), to have anti-mutagenic potential in Chinese hamster lung-derived V79 cells (Guterrez et al. 2004), and rat livers exposed to carcinogenesis (Barbisan et al. 2003), and to show chemoprevention of preneoplastic foci in rat livers (Pinheiro et al. 2003). Recently, agaritine purified from *A. blazei* was shown to exert direct anti-tumor activity against leukemic tumor cells *in vitro* (Endo et al. 2010). However, there have been few reports of comprehensive gene expression analyses for bioactive components in *A. blazei*, and little is known about their mechanism of action and the effect on other pathologies. During our early clinical study, we obtained preliminary data suggesting that the extract of *A. blazei* has positive effects on atherosclerotic symptoms, which prompted us to the molecular study of the extract.

Coronary disease is less common among premenopausal women than men but its risk is low in men with cirrhosis-related hyperestrogenemia and increases for women after menopause (Kalin and Zumoff 1990; Baker et al. 2003). The most likely explanation for this is that estrogen is anti-atherogenic: estrogens alter

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the lipoprotein profile, increasing high-density lipoprotein (HDL), decreasing low-density lipoprotein (LDL) and inhibiting oxidation of LDL, and act on the vascular endothelium and vascular smooth muscle to prevent monocyte infiltration and promote vasodilation (Nathan and Chaudhuri 1997; White 2002). Arterial vasodilation caused by estrogen likely occurs through a rapid estrogen-signaling pathway (Mendelsohn 2002, 2009). These include nuclear receptor-dependent and independent signaling pathways (reviewed in Zhao et al. 2010; Mendelsohn and Karas 2010).

Phytoestrogens, including those from soybeans, may prevent atherosclerosis by inhibiting the infiltration and proliferation of monocytes at atherosclerotic lesions (Raines and Ross 1995), reducing LDL concentrations (Clarkson 2002), and inhibiting production of pro-inflammatory cytokines, cell adhesion proteins and nitric oxide (Cassidy et al. 2003). Accumulative clinical data have shown that phytoestrogens are generally considered safe and cause no obvious side effects (Deng 2009). However, the benefits of phytoestrogens based on their atheroprotective effects as well as the advantages of their clinical use over synthetic estrogens need further characterization at the molecular level. Notably, differences in the signaling initiated with phytoestrogen from that initiated with estrogen, such as variations in receptors, modulation in estrogen signaling and cross talk in signaling, require elucidation.

Here, we report that the *A. blazei* extract has dual roles in cell signaling, suppression of macrophage development and the recovery of endothelial cells from vascular damage. The effects of the extract may partly be due to its estrogen-like activity, which has not been reported before. The potential molecular mechanisms of the extract's effect are the enhanced expression of genes related to reducing levels of reactive oxygen species, and activation of the oxidized LDL (OxLDL)-induced down-regulation of these genes, suggesting that these genes play roles in protecting endothelial cells from oxidized damage caused by the *A. blazei* extract.

Materials and methods

Materials

Estrogen (17β-estradiol or E_2) was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). *A. blazei* powder (*A. blazei* mycelia-dikaryon (strain my26)) was prepared by the JMCU Center Corporation. The aqueous extract of *A. blazei* mycelia-dikaryon was prepared as reported previously with minor modifications (Talorete et al. 2002). Briefly, an extract was obtained from 2.5 g of the *A. blazei* powder boiled in 22.5 ml of Milli-Q water for 10 min. The supernatant was recovered by centrifugation at 3300 × *g* for 3 min and sterilized by filtration through 0.22 μm filters (Millipore; Billerica, MA). OxLDL was prepared according to Itabe et al. (1996).

DNA microarray assay

A focused oligonucleotide-DNA (oligo-DNA) microarray was manufactured by Invitrogen (Tokyo, Japan) by mechanical spotting of oligo-DNA on a glass slide for 203 genes including a set of 172 estrogen-responsive genes as described previously (Terasaka et al. 2004). For microarray assay, MCF-7 cells were cultured in phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days and treated with 10 nM of E_2, 2 μg/ml of the *A. blazei* extract, or the vehicle (DMSO, control) for 72 h. Total RNA was then isolated using a RNeasy Plus Mini kit (QIAGEN), and antisense RNA (aRNA) was prepared from 5 μg of total RNA using a SuperScript RNA Amplification kit (Invitrogen). Then, cDNA was synthesized and labeled with Cy3 (GE Healthcare) using a SuperScript Indirect cDNA Labeling kit (Invitrogen). Hybridization was performed as described previously (Terasaka et al. 2006). The slides were then scanned using FLA-8000 (Fujifilm). The assay was performed three times using independent cell cultures.

Image analysis was performed using ArrayGauge software (Fujifilm) according to the manufacturer's instructions and the data was further analyzed with Microsoft Excel software. The microarray data are available in the Gene Expression Omnibus database in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) with Accession No. GSE30193. For data processing, the signal intensity for Cy3 was averaged among duplicated spots and the ratio of the mean signal intensity for the indicated chemical-treated sample to that for the control sample was then calculated for each gene. The ratios of signal intensity for all genes were normalized against the mean ratio for the 28 control genes. Normalized ratios were $\log_2$-transformed and used for correlation analyses and *t*-tests. The control genes were selected from housekeeping genes and the genes that did not change their expression levels after the treatment with estrogen in MCF-7 cells (Terasaka et al. 2004). The UniGene names for the 150 selected genes were obtained from the Entrez database (http://www.ncbi.nlm.nih.gov/); these 150 genes were selected as highly reproducible estrogen-responsive genes from the original 172 genes (Terasaka et al. 2006).

Reporter gene assay

The reporter plasmid pG-ERα-AB containing the promoter region of the human estrogen receptor gene α (ERα) was used to examine the estrogenic activity (Inoue et al. 2002). The plasmid DNA (2.0 μg/10^5 cells) was transfected into MCF-7 cells with Lipofectin (Gibco-BRL). After incubation of the cells in RPMI 1640 medium (phenol red-free) with 10% DCC-FBS for 24 h, the *A. blazei* extract (0.01, 0.1, 1, 2, 4 or 8 μg/ml), E_2 (10 nM), or the *A. blazei* extract + 1 μM ICI 182,780, was added to the medium, and the cells were incubated for another 24 h. The extracts of the cells were assayed using a luciferase assay kit (Promega, Madison, WI). Luciferase activity was examined with four independent transfections for each treatment and data were analyzed with a *t*-test.

Real-time quantitative RT-PCR

Real-time reverse transcription (RT)-polymerase chain reaction (PCR) was performed using Two-step qRT-PCR with a SYBR Green kit (Invitrogen, Carlsbad, CA). PCR was performed three times for each gene. The reaction consisted of denaturation at 95 °C for 3 min, followed by 50 cycles of 95 °C for 5 *s* and 60 °C for 30 sec using a LightCycler (Roche; Mannheim, Germany). β-*Actin* (GenBank Accession No.: NM_001101) mRNA was used as an internal control for normalizing the mRNA amount in cells after different treatments. The primers used for PCR were as follows: 5′-CGCAGGTCAAGAGCAAGG-3′ and 5′-GCGCATCTTGGCCTTGTC-3′ for *CEBPB*; 5′-CTCTACAAAACAACCAAGATG-3′ and 5′-TGACTGCCATCCTTTGTATC-3′ for *RBBP8*; 5′-AGCCTCGACCTGACCTGT-3′ and 5′-TGGTGAGCAGCAGAAAGAG-3′ for *IER3*; 5′-GTCTTGTCATTCCAGGTGC-3′ and 5′-CACAAGACCCTGCTAAGAC-3′ for *DHCR24*; 5′-CCTCACTAGCCTCTACTCCCTGT-3′ and 5′-ACTGAGGTGACTTGAGTGTTGG-3′ for *ARHGDIA*; 5′-ATGGATTTGAGAGGAAAGAGAGG-3′ and 5′-AGCACCCTCCTAAGGTTACTCAC-3′ for *IGFBP5*; 5′-CAAGAGATGGCCACGGCTGCT-3′ and 5′-TCCTTCTGCATCCTGTCGGCA-3′ for *β-actin*; 5′-CCTTAGCCTGAATGCACTAA-3′ and 5′-AGAGATCAACCTCCCTGAGA-3′ for *GPX3*; 5′-ACATATGCCTAAGCATGGGC-3′ and 5′-GGTGTTATCCAATGGGATGC-3′ for *SNCA*; 5′-AGGGCTCTGCTGGACAC-3′ and 5′-GGCTGGGAGGCTCTAAAG-3′ for *eNOS*; and 5′-ACCGAGAGGCTGAGACTA-3′ and 5′-AGCAGCAGGCACAGAAGG-3′ for *MCP-1*.

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Western blotting

HUVECs (Cambrex; East Rutherford, NJ) were cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% (v/v) dextran-coated charcoal-treated FBS (DCC-FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The macrophage cell line TIB-67 was obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS at 37 °C under 5% CO_2. Cells were serum-starved in phenol red-free RPMI 1640 medium for 16 h and then treated with the *A. blazei* extract (10 μg/ml), 10 nM E_2, or vehicle (0.1% DMSO, v/v) in the presence or absence of ICI 182,780 (ICI). For the inhibition assay, HUVECs and TIB-67 cells were treated with the *A. blazei* extract (10 μg/ml), LDL (50 μg/ml), OxLDL (50 μg/ml), or vehicle for 6 h. For the inhibition of OxLDL signaling, the *A. blazei* extract (10 μg/ml) or anti-LOX-1 (5 μg/ml) was added 1 h before OxLDL. Total protein was extracted with SDS buffer and sonicated on ice for 30 sec. After incubation at 95 °C for 5 min with loading buffer, the protein sample (20 μg) was resolved by SDS–PAGE on a 5–20% gradient gel, then electrotransferred onto nitrocellulose membranes (Millipore, Billerica, MA) using a semi-dry transfer cell (Bio-Rad, Hercules, CA) at 1 mA/cm^2 for 1 h. Phospho-Erk1/2 (P-Erk1/2), phospho-Akt (P-Akt) and phospho-p38 (P-p38) were analyzed by probing membranes that had been preblocked in Tris-buffered saline containing 0.1% Tween-20 and 5% BSA (TBST–BSA). Membranes were probed with antibodies against Erk1/2, P-Erk1/2, Akt, P-Akt, p38, P-p38, eNOS, β-actin (Cell Signaling Technologies, Ipswich, MA), GPX3 or Lox-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a sufficient dilution (1:200–1:1000) in TBST–BSA overnight at 4 °C. Complexes of rabbit or mouse antibodies-antigens were detected with horseradish peroxidase-coupled goat antibodies against rabbit or mouse IgG (Cell Signaling Technologies) after 1:3000-dilution with TBST–BSA, and then visualized using the ECL-plus Western Blotting Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL).

Animal experiments

Sixteen male Japanese inbred white rabbits (Clea Japan; Tokyo, Japan) weighing about 2.5 kg were used in this study. They were randomly divided into two groups: I, *A. blazei* powder (−) group ($n = 8$); II, *A. blazei* powder (+) group ($n = 6$); and non-treated control rabbits ($n = 2$). At first, the aorta of animals in groups I and II was injured using a balloon-tip catheter (Sanborn et al. 1982). Then, all animals were housed individually and given free access to food (100 g/day) and water as follows. Group I and group II rabbits were fed with a high-cholesterol diet [0.5% cholesterol added to regular CLEA Rabbit Diet CR-3 (Clea Japan; Tokyo, Japan)] for 12 weeks. Then, for the 23 subsequent weeks, rabbits in group II were fed with 0.01% *A. blazei* powder (3.3 mg/kg: the same ratio as for human intake) in CR-3, while those in group I were fed with CR-3 alone as a regular diet. Non-treated control rabbits ($n = 2$) were fed with regular CR-3 for the whole 35 weeks. At the end of this period, the weights of both group I and group II rabbits were about 3.5 kg. At the end of the 12th and 35th weeks, the tunica intimal thickness of descending ventral aorta was measured using ultrasonic echo. Rabbits were anesthetized with Nembutal (50 mg/kg) and were sacrificed after 35 weeks. All rabbit experiments were approved by the Animal Ethics Committees of Tokyo Women's Medical University.

Histological and immunohistochemical analyses

Histological sections of the coronary artery and descending aorta from all animals were stained with hematoxylin–eosin (HE), Masson's trichrome, van Gieson, and Victoria blue using conventional methods. Immunochemical analysis was also performed on all animals using rabbit anti-macrophage 11 (RAM11), α-SMA for smooth muscular actin.

Results

Estrogen-like activity of A. blazei extract

Estrogen-like activity of the *A. blazei* extract has never been examined. However, it is known that a number of useful compounds, phytoestrogens for example, have estrogen-like activity and the activity plays an important role in their actions (see Introduction). Here, we started our study of the effects of the extract by examining the similarities as well as the differences between the extract and estrogen (17β-estradiol or E_2).

We first examined the effect of the *A. blazei* extract on cell growth using breast cancer MCF-7 cells and found that the extract inhibited cell growth in a dose-dependent manner, 50% at 4 μg/ml and 80% at 6 and 8 μg/ml (data not shown). This finding was not surprising because such inhibitory activity had been reported (Grube et al. 2001). We assumed that any potential estrogen-like activity would be masked by this growth-suppressive effect. We therefore examined whether the *A. blazei* extract shows activity similar to estrogen at the gene expression level using a customized DNA microarray containing a set of 150 estrogen-responsive genes (Fig. 1A). Similar expression profiles were obtained between cells treated with 10 nM E_2 and those treated with 2 μg/ml of the *A. blazei* extract (correlation coefficient or $R = 0.84$), suggesting the presence of estrogen-like activity in the extract. The 150 genes were further categorized into six groups with specific functions (enzymes, signaling, proliferation, transcription, transport and others) and used for a correlation analysis (Fig. 1B). Significant correlations were observed between the *A. blazei* extract and E_2 for the groups enzymes, signaling, transcription, transport and others, while a significant but less correlation was observed for the group proliferation (Fig. 1B).

We then examined the estrogen-like activity by conducting a reporter gene assay using the promoter (ProAB) of the human estrogen receptor α (ERα) gene, which responds to estrogenic compounds and xenoestrogens (Inoue et al. 2002). We transfected a reporter plasmid containing a ProAB/luciferase fusion gene into MCF-7 cells and examined the estrogen-like activity of the extract at various concentrations (Fig. 1C). The *A. blazei* extract exhibited luciferase activity greater than the control at 0.1–4 μg/ml, with a maximal level at 2 μg/ml, which was 70% of that with 10 nM E_2. Note that the luciferase activity was blocked by treatment with an estrogen antagonist, ICI 182,780.

Despite the overall similarity in the response of genes, the effect of the extract on gene expression is apparently different from that of E_2. To see this difference, we performed ANOVA- and *t*-tests for the estrogen-responsive genes used for the DNA microarray assay. Six genes (*CEBPB*, *RBBP8*, *IER3*, *DHCR24*, *ARHGDIA* and *IGFBP5*) exhibited significantly ($p < 0.05$) different responses between treatments while most of the other genes showed similar responses (data not shown). To confirm these differences, we carried out *real-time* quantitative RT-PCR (Fig. 2). All selected genes except *DHCR24* exhibited statistically significant differences in expression, suggesting that E_2 and the *A. blazei* extract share similar activities that are yet distinguishable at the gene expression level.

Blocking of OxLDL-induced slow activation of Erk1/2, Akt and p38 with the A. blazei extract

We examined if there is any effect of the *A. blazei* extract on the level of OxLDL, because one of the clinical implications that we obtained was the improvement in the OxLDL levels of

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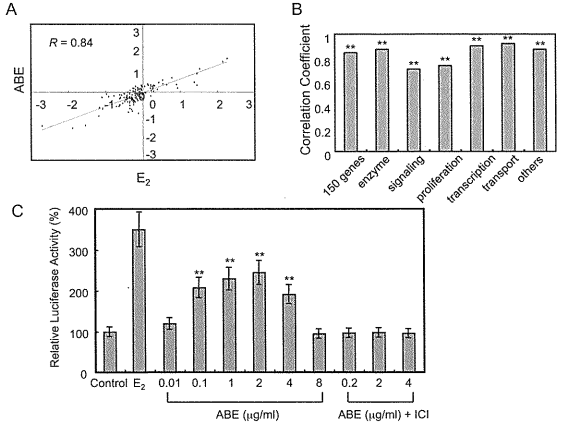

Fig. 1. Identification of estrogen-like activity of the *A. blazei* extract. (A) Correlation between the expression profiles of estrogen-responsive genes in MCF-7 cells treated with the *A. blazei* extract (ABE). The average of the results of triplicate gene expression profiling after treatment with 2 μg/ml of the *A. blazei* extract was compared with the average of triplicate profiles obtained with 10 nM E_2. The axes show $\log_2$-transformed ratios of signal intensities with and without the *A. blazei* extract or E_2. Correlation coefficients (*R* values) were calculated based on a linear regression of the two profiles. (B) Evaluation of estrogenicity of the *A. blazei* extract based on quantitative profiling of functional groups. The bars indicate the correlation coefficients for a total of 150 genes or for the genes categorized into six groups (enzymes, signaling, proliferation, transcription, transport, and others). $^{**}p < 0.01$. (C) Estrogen-responsive luciferase-reporter gene assay with the *A. blazei* extract. MCF-7 cells were lipofected with pG-ERα-AB vector DNA and treated with the vehicle as a control, 10 nM E_2, and the *A. blazei* extract with or without ICI 182,780 (ICI) at the indicated concentrations. The luciferase activity was assayed 24 h after the treatment. The values represent the mean ± SD of the data from four independent experiments. $^{**}p < 0.01$.

patients. The dysfunction of endothelial cells and accumulation of OxLDL in macrophages play important roles in the development of atherosclerotic plaques (Faxon et al. 2004). We used human umbilical vein endothelial cells (HUVECs) and macrophage-derived TIB-67 cells to examine the cell signaling induced by OxLDL and the *A. blazei* extract (Fig. 3). The activation of Erk1/2, Akt, and p38 mitogen-activated protein kinase (MAPK) by OxLDL in both HUVECs and TIB-67 cells suggests the involvement of these molecules in endothelial dysfunction, the proliferation of macrophages, and the accumulation of foam cells, and has thus been targeted as a potential treatment for atherosclerosis (Senokuchi et al. 2004; Patten et al. 2004; Yano et al. 2007; Chen et al. 2007). Here, we found that the OxLDL-induced slow activation (compared with the rapid estrogen-signaling pathway, see Introduction and Mendelsohn and Karas 2010) of Erk1/2, Akt, and p38 were inhibited by the *A. blazei* extract (Fig. 3A and B, lane 7) in a manner identical to that observed for LOX-1 using an antibody against the OxLDL receptor (Fig. 3A and B, lane 5) both in HUVECs and in TIB-67 cells, suggesting that the *A. blazei* extract could also be used to treat atherosclerosis by targeting the response to OxLDL.

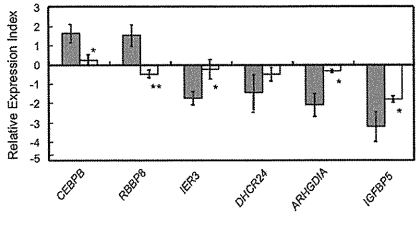

Fig. 2. *Real-time* RT-PCR analysis for the genes showing expressional differences between the *A. blazei* extract (2 μg/ml) and E_2 (10 nM). Six genes (*CEBPB*, *RBBP8*, *IER3*, *DHCR24*, *ARHGDIA* and *IGFBP5*) were analyzed by *real-time* RT-PCR. The bars show the mean ± standard deviation of $\log_2$-tansformed ratios in response to E_2 (gray bars) or the *A. blazei* extract (white bars). $^{*}p < 0.05$, $^{**}p < 0.01$ between the *A. blazei* extract and E_2.

Gene expression induced with the A. blazei extract

We further investigated the contribution of the *A. blazei* extract to the prevention of endothelial dysfunction at the level of gene expression, by examining atherosclerosis-related genes (Fig. 4). The extract enhanced the expression of two genes related to the reduction of a reactive oxygen species (ROS): the glutathione peroxidase 3 (*GPX3*) gene and α-synuclein (*SNCA*) gene (Fig. 4A). Since estrogen up-regulates *GPX3* and *SNCA* expression in an ER-dependent manner (O'Lone et al. 2007), we hypothesized that an antioxidative effect could be induced by the estrogen-like activity of the *A. blazei* extract as seen in Fig. 1 and contribute to suppression of the plasma OxLDL level. The extract also enhanced the expression of the endothelial nitrogen-oxide (NO) synthase (*eNOS*) gene and activated OxLDL-mediated down-regulation of *eNOS* expression (Fig. 4A). Note that NO suppresses the development of arteriosclerosis (see Discussion). The extract alone down-regulated the expression of monocyte chemotactic protein-1 (*MCP-1*) gene, a key regulator for the initiation and development of atherosclerotic lesions (Harrington 2000), and inhibited the expression of *MCP-1* in response to OxLDL, which supports the results obtained with *eNOS*. The effects of the *A. blazei* extract on the expression of *GPX3* and *eNOS* were also confirmed at the protein level (Fig. 4B).

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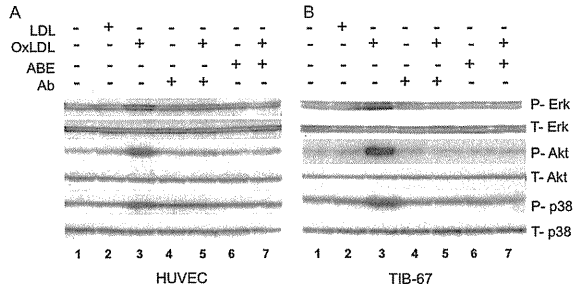

Fig. 3. Blocking of OxLDL-induced activation of Erk1/2, Akt and p38 with the *A. blazei* extract. HUVECs (A) and TIB-67 cells (B) were treated with the *A. blazei* extract (10 μg/ml), LDL (50 μg/ml), OxLDL (50 μg/ml), or vehicle for 6 h. For inhibition of OxLDL signaling, the *A. blazei* extract (10 μg/ml) or an antibody against LOX-1 (Ab: 5 μg/ml) was added 1 h before OxLDL. Protein was extracted with SDS sample buffer and analyzed by Western blotting.

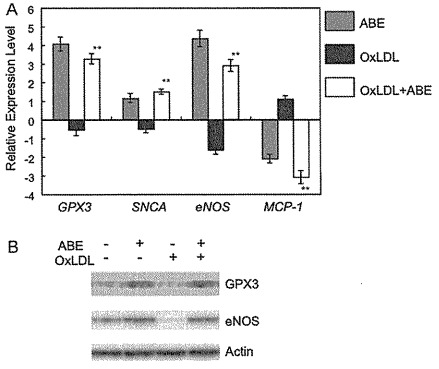

Fig. 4. Expressional analysis of *GPX3*, *SNCA*, *eNOS* and *MCP-1*. (A) *Real-time* RT-PCR of mRNA in HUVECs. HUVECs were treated with the *A. blazei* extract (10 μg/ml), OxLDL (50 μg/ml), the *A. blazei* extract (10 μg/ml), OxLDL (50 μg/ml), or vehicle for 24 h. Then, total RNA and protein were collected. The expression level of each gene in a $\log_2$-transformed value was normalized against that of β-actin and expressed relative to that in the control cells. The data represent the averages of triplicate experiments. $^{**}p < 0.01$ between OxLDL with and without the *A. blazei* extract. (B) Western blotting of GPX3 and eNOS proteins in HUVECs. Western blotting of β-actin was carried out to check the amount of loaded protein.

Immunohistochemical analysis of the effect of A. blazei powder on coronary vessels

We examined the effect of *A. blazei* powder on atherosclerosis using two groups (I and II) of hyperlipidemic inbred Japanese white rabbits. Both groups, I ($n = 8$) and II ($n = 6$), were fed a high cholesterol diet (0.5% cholesterol) for 12 weeks. Then, group II rabbits were fed with a regular CR-3 diet plus 0.01% *A. blazei* powder, while group I rabbits were fed with the regular diet only for 23 subsequent weeks. Non-treated control rabbits ($n = 2$) were fed with a regular diet for the whole 35 weeks. During the 23-week period following the high cholesterol diet, four rabbits (50%) in group I died of myocardiac infarction (as demonstrated in the post-mortem), while no rabbits died prematurely in group II. There were no differences observed between group I and II rabbits in the brain, hypophysis, skeletal muscle, kidney, ureters, aorta, pancreas, testes, colon, lungs, liver, spleen, thymus, stomach, small intestines, and adrenal gland; only the coronary arteries showed detectable differences in histological sections (see below).

The intimal thickness of the descending aorta was also analyzed by echocardiography. Data showed similar increases in intimal thickness for all rabbits in groups I and II during the 12 weeks of high-fat diet (I: 0.63 ± 0.17 μm and II: 0.67 ± 0.10 μm) that returned to normal levels 23 weeks after suspension of the high fat diet (I: 0.38 ± 0.04 μm and II: 0.41 ± 0.08 μm). Controls showed consistent intimal thickness throughout the treatment (0.40 ± 0 μm). Although these findings suggest that *A. blazei* powder does not reverse the intimal thickening of the descending aorta, histological data showed marked differences from the untreated animals (Fig. 5).

Intimal proliferation with foamy macrophage infiltration was found in the coronary arteries of the atria and both ventricles in group I rabbits, particularly in the ventricular septum, indicating the presence of intimal atherosclerotic lesions (Fig. 5A). Prominent proliferation of foamy macrophages, detected by immunostaining with RAM11 (Fig. 5B), and smooth muscle cells, immunostained against α-smooth muscle actin (Fig. 5C), was observed in the coronary lesions of group I rabbits. Group II rabbits (Fig. 5D–F) showed minimal lesions in the coronary artery that were not apparently different from those in control rabbits (Fig. 5G–I). These results document that atherosclerosis are present in the coronary arteries of group I rabbits, whereas no sclerotic lesions were detected in group II rabbits or controls.

Discussion

Estrogen has cardioprotective activity in blood vessels (Mendelsohn and Karas 1994; Farhat et al. 1996). Phytoestrogens, compounds from plants having estrogen-like activity, can have similar effects (Tham et al. 1998; Cassidy et al. 1999). We also clinically observed estrogen-like effects of *A. blazei* powder: in individuals orally administered the powder, menstruation restarted following menopause or hair began to grow (data not shown), which prompted us to the study of estrogen-like activity in *A. blazei*. The results of the DNA microarray analysis and luciferase assay indicate the presence of estrogen-like activity in the *A. blazei* extract. Furthermore, a part of the signaling could be mediated by the estrogen receptor, because the activity was inhibited by treatment with ICI 182,780 (Fig. 1C). It is necessary to elucidate

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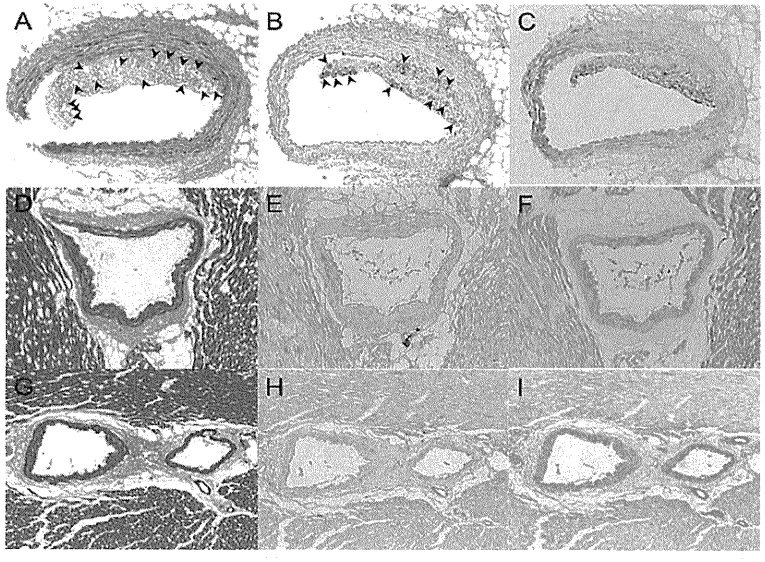

Fig. 5. Immunohistochemical analysis of coronary vessels. Atherosclerotic lesions in the septal coronary vessel were examined for *A. blazei* powder (−) group I rabbits (A–C), and lesions in the left ventricle coronary vessels of *A. blazei* powder (+) group II rabbits (D–F) and controls (G–I) were also examined. Each sample was examined by Masson's trichrome staining (A, D and G), or by immunostaining with RAM11 (B, E and H) or a monoclonal antibody against α-smooth muscle actin (C, F and I). Infiltration of foamy macrophages in coronary lesions (A) and their proliferation in coronary lesions of rabbits fed with cholesterol for 12 weeks (B) are shown by wedges. Magnification: ×100.

the molecular identity responsible for the estrogen-like activity of *A. blazei* and its mechanism of action in a future study.

The production of NO by eNOS contributes to many endothelial functions and is important for maintaining the balance between vasodilation and vasoconstriction, thrombosis and anticoagulation, and modulation of inflammation (Ross 1995; Grube et al. 2001). Furthermore, activation of eNOS causes vasodilation in both muscular conduit vessels and resistance arterioles (Lieberman et al. 1996; Ludmer et al. 1996). Decreased bioavailability of NO is indicated in patients with atherosclerosis (Lieberman et al. 1996; Ludmer et al. 1996). Moreover, the effect of estrogen on cardiovascular diseases is related to the activation of eNOS via Akt and Erk1/2 cascades (Klinge et al. 2005; Hisamoto et al. 2001). Therefore, the estrogen-like activity of the *A. blazei* extract, which effectively blocks OxLDL-induced activation of Akt and Erk1/2 (Fig. 3), possibly prevents vascular damage, enhances recovery from such damage, and contributes to balanced vascular function through an up-regulation of eNOS expression (Fig. 4A and B).

The dysfunction of endothelial cells and accumulation of OxLDL in macrophages play important roles in the development of atherosclerotic plaques (Faxon et al. 2004). Furthermore, the production of OxLDL is related to the reduction of ROS. Since estrogen up-regulates the expressions of *GPX3* and *SNCA* (O'Lone et al. 2007), which have functions of reducing ROS, they could also play a role in the reduction of ROS in the progress of atherosclerosis. Their expressions were up-regulated by the addition of the *A. blazei* extract (Fig. 4A), suggesting that the extract has a positive effect in reducing ROS produced by the stimulation of OxLDL.

Based on our findings, we propose a model for the effects of the *A. blazei* extract on human vascular endothelial cells (Fig. 6). In this model, we suggest that the suppression of the OxLDL-induced activation of Akt, Erk1/2 and p38 is due to estrogen response element (ERE) for estrogen but triggered here by the *A. blazei* extract, and

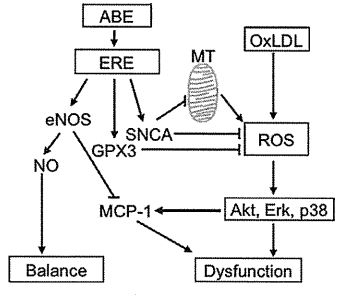

Fig. 6. A proposed model for the effect of the *A. blazei* extract on vascular endothelial cells. The extract enhances the expression of the *eNOS*, *GPX3*, and *SNCA* genes through estrogen response element (ERE) responsive pathway. GPX3 and SNCA proteins contribute to a reduction in reactive oxygen species (ROS) generated in mitochondria or in response to OxLDL. eNOS protein down-regulates *MCP-1* expression and increases production of NO, which is an important signal mediator for balancing vascular function.

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is subsequently followed by the activation of eNOS. The activated eNOS contributes to the balance of endothelial functions, while suppression of ROS is crucial to the dysfunction of endothelial cells as discussed above.

Taken together, our findings suggest that some components of *A. blazei* mycelia-dikaryon are responsible for the prevention of and recovery from endothelial vascular damage. These effects seem to be mediated by ERE signaling pathways that regulate eNOS and inhibit OxLDL. It is clear that further studies are needed to clarify the intracellular mechanism responsible for the effects reported here. The striking *in vivo* and *in vitro* effects of the *A. blazei* mycelia-dikaryon extract may ultimately prove therapeutically useful for the prevention of cardiac vascular disease.

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