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Inflammation Research

Cytokine induction by a linear 1,3-glucan, curdlan-oligo, in mouse leukocytes *in vitro*

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Abstract. *Objective:* Curdlan, an extracellular bacterial polysaccharide, is a linear β-1,3-glucan. Previously, we developed Curdlan-oligo (CRDO). We investigated its effect on the production of cytokines in leukocytes from mice, and compared its activity with that of SCG, a 6-branched 1,3-β-glucan.
Methods: Splenocytes from DBA/2 mice were cultured with CRDO or SCG (0, 1, 10 or 100 μg/ml) *in vitro*, and then the supernatants were collected to measure cytokines. Bone marrow-derived dendritic cells (BMDCs) were cultured with CRDO (0, 1, 10 or 100 ng/ml) *in vitro*, and then the supernatant was collected to measure cytokines.
Results: SCG stimulated splenocytes in DBA/2 mice to produce GM-CSF, IFN-γ and TNF-α. CRDO induced production of GM-CSF and IFN-γ, but not TNF-α. The amounts of GM-CSF and IFN-γ were small compared with those produced in response to SCG. The effect of SCG on TNF-α production was partially inhibited by CRDO. In bone marrow-derived dendritic cells, CRDO induced production of TNF-α and IL-6.
Conclusion: Taken together, these results suggest that CRDO stimulated mouse leukocytes to induce the production of cytokines, and the mechanism of the effect of CRDO on leukocytes is different from that of SCG.

Key words: Glucan – Curdlan-oligo – TNF-α, IFN-γ

Introduction

Glucans are natural polysaccharides found in a wide variety of plants, algae, bacteria, fungi and yeast sources. Some β-glucans are well-known biological response modifiers. We and others have demonstrated that the immunomodulating activity of β-glucans is mainly related to their effects on immune effecter cells, such as macrophages, mononuclear cells, and neutrophils involved in innate immunity, resulting in the production of cytokines [1, 2]. The body's defense against microbial attack and against spontaneously arising malignant tumor cells comprises a dynamic orchestrated interplay of innate and acquired immune responses, and the effectors of innate immunity can initiate these systems. These findings indicated that β-glucan is an important player in both host defense against fungi and cancer immunotherapy.

SCG is a major 6-branched 1,3-β-D-glucan in *Sparassis crispa Fr.* showing antitumor activity [3, 4]. SCG enhanced the hematopoietic response in cyclophosphamide-treated leukopenic mice [5, 6]. SCG stimulated leukocytes to produce cytokines in preparations of human peripheral blood mononuclear cells [7], splenocytes [8] and bone marrow-derived dendritic cells (BMDCs) [9] from mice. These results demonstrated that SCG could enhance immune responses *in vivo* and *in vitro*. The study of glucans has been confounded by the presence of endotoxin in glucan preparations. No endotoxin was detected in SCG with endospecy (<30 pg/mg) [10]. So SCG is also used as a purified soluble β-glucan in investigations on cellular receptors and molecular mechanisms [11].

Curdlan from *Alcaligenes faecalis var. myxogenes*, has unique rheological and thermal gelling properties, with applications in the food industry and other sectors. Curdlan is structurally the simplest of the β-1,3-D-glucans with no glycosyl side chains [12]. Curdlan is slightly soluble in water when its molecular weight is relatively high (ca. >8000) and insoluble in alcohols and most organic solvents, but dissolves in dilute basic (0.25 M NaOH), dimethylsulphoxide (DMSO), formic acid and aprotic reagents such as N-methylmorpholino-N-oxide and lithium chloride in dimethylacetamide [13]. Curdlan's conspicuously unusual rheological properties among natural and synthetic polymers underlie its

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use as a biothickening and gelling agent in foods. Its safety has been assessed in studies with animals and tests *in vitro* and it is approved for use in Korea, Taiwan and Japan as an inert dietary fiber and is registered in the United States as a food additive [14]. Curdlan, like other β-1,3-glucans, has medical and pharmacological potential. The reported immunomodulating and pharmacological responses include antitumorigenicity, anti-infective activities against bacterial, fungal, viral and protozoal agents, anti-inflammatory activity, wound repair, protection against radiation, and anti-coagulant activity [13]. The effectiveness of curdlan in eliciting these responses depends on chemical structure, molecular mass, and conformation [1]. Previously, we have successfully developed Curdlan-oligo (CRDO) which is soluble in water (Japanese patent No. 10-194977). CRDO could induce proliferation of T cells and B cells in the presence of ConA or LPS (Japanese patent No. 10-194977). These results suggested that CRDO has immuonomodulating activities.

We have found that there are strain differences in the reactivity of mice to β-glucan, and that DBA/1 and DBA/2 mice are highly sensitive to β-glucan *in vitro* [4, 8] and in vivo [15]. SCG induced leukocytes from DBA/1 and DBA/2 mice to produce large amounts of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-12p70 (IL-12p70), and granulocyte macrophage – colony stimulating factor (GM-CSF) *in vitro*, and GM-CSF is one of the key factors in the reactivity to β-glucan [16, 17]. The expression level of the β-glucan receptor, dectin-1, on BMDCs in DBA/2 mice was highest in DBA/2 among C57BL/6, BALB/c, C3H/HeN and C3H/HeJ mice [9]. The finding suggests DBA/2 mice to be a useful model for studying β-glucan. We also reported that the pattern of the response of the leukocytes differed between soluble glucan and particulate glucan [4]. In this study, we investigated the activity of CRDO in leukocytes from DBA/2 mice, and compared it with that of SCG.

Materials and methods

Animals

DBA/2 male mice between 6 and 7 weeks of age were purchased from Japan SLC, Shizuoka. The experimental protocol was approved by the Committee for Animal Care and Use of Tokyo University of Pharmacy and Life Science. Mice were maintained under specific pathogen-free (SPF) conditions, at 23 plus-minus 1 degree, with a constant humidity of 55 plus-minus 5 %, under a cycle of 12 h of light and 12 h of dark, and had free access to food and tap water according to the Guidelines for Experimental Animal Care issued by the Prime Minister's Office of Japan.

Preparation of CRDO

To prepare CRDO, a suspension of Curdlan (30 g) derived from *Alcaligenes faecalis var. myxogenes* in a 85 % formic acid solution (3 L) was heated at 90 °C for 20 min. After cooling until room temperature, the resulting solution was concentrated *in vacuo* and neutralized by 5N NaOH. The reaction mixture was centrifuged to collect the soluble fraction, soluble fraction were boiled for 120 min to remove formyl groups, then neutralized by 2N NaOH. The resulting solution was dialyzed against distilled water, and then lyophilized (Japanese patent No. 10-194977). The CRDO solution was prepared by dissolving the lypholized powder in saline and autoclaving. The average molecular weight of CRDO was 340 ~ 4000 when estimated by HPLC.

Preparation of SCG

Fruit bodies of *Sparassis crispa* were cultured by Minahealth Co. (Saitama, Japan). SCG was prepared as previously described [5]. Briefly, air-dried and powdered S. crispa was extracted with cold alkali (10 % NaOH/5 % urea, 4 degrees, 2 days). The extract dissolved in 8 M urea was applied to a DEAE Sephadex A25 (Cl-) column equilibrated with 8 M urea, and the pass-through fraction was collected and extensively dialyzed against tap and distilled water, and then lyophilized (elemental analysis C : H : N = 40.06 : 6.77 : 0.08). The SCG solution was prepared by dissolving the lypholized powder in 0.5 N NaOH, followed by immediate dialysis against saline for 3 days. The dialyzed fraction was then autoclaved and frozen until used.

Materials

Tween 20 was purchased from Wako Pure Chemical Co. (Osaka, Japan). Hank's balanced salt solution (HBSS) was purchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Gentamycin sulfate, RPMI 1640 medium, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Sanko Junyaku Co., Ltd. (Tokyo, Japan). Recombinant mouse GM-CSF and recombinant mouse IL-4 were from BD Biosciences (USA).

Preparation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow was removed from mice. Bone marrow cells obtained by flushing femoral shafts were suspended in HBSS containing 50 μg/ml gentamycin sulfate. After centrifugation, the single cell suspension was treated with an ACK-lysing buffer (8.29 g/l NH_4Cl, 1 g/l $KHCO_3$, 37.2 mg/l EDTA/2Na) to lyse red blood cells. After further centrifugation, the cells were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamycin sulfate containing 5 % heat-inactivated FCS, 10 ng/ml recombinant mouse GM-CSF, and 5 ng/ml recombinant mouse IL-4 and cultured in 24-well flat-bottomed plates at 1×10^6 cells per well in 1 ml of culture medium at 37 degrees in a humidified 5 % CO_2: 95 % air atmosphere. Nonadherent and loosely adherent cells were removed by pipetting on day 2 and replated with fresh cytokine-containing media in the plate. CRDO was added on day 5. On day 7, culture supernatant was collected.

Preparation of splenocytes

Splenocytes were prepared as previously described [8]. Briefly, the spleen was teased apart in RPMI 1640 medium, and after centrifugation, the single cell suspension was treated with ACK-lysing buffer (8.29 g/l NH_4Cl, 1 g/l $KHCO_2$, 37.2 mg/l EDTA/2Na) to lyse the red blood cells. After further centrifugation, splenocytes were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamycin sulfate and 10 % FCS. Cells were cultured in 24-well flat-bottomed plates, and stimulated with CRDO or SCG.

CellTiter 96 (R) AQ_{ueous} One Solution Cell Proliferation Assay

Splenocytes were cultured in 96-well flat-bottomed plates, and stimulated with CRDO or SCG. After 24h incubation, CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, USA) was added to each well. After 1, 2, 3 and 4 h at 37 degrees in a humidified 5 % CO_2: 95 % air atmosphere, the absorbance at 490 nm was recorded.

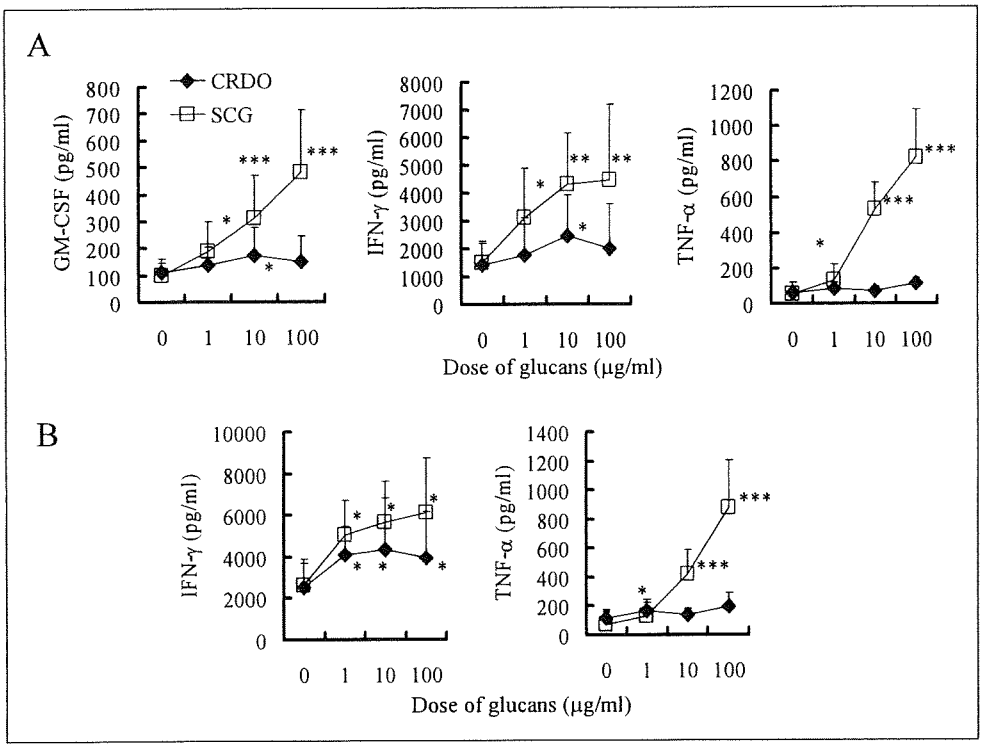

Fig. 1. Dose of CRDO and SCG for cytokine production in splenocytes from DBA/2 mice. Splenocytes were isolated from DBA/2 mice.
Cell suspensions were adjusted to 5×10^6 cells/ml in 10% FCS/RPMI medium. A: Cells were incubated for 48 h with CRDO or SCG (0, 1, 10, or 100 μg/ml). The supernatant was then collected, and the concentrations of IFN-γ, TNF-α and GM-CSF were determined with an ELISA. B: Cells were incubated for 48 h with CRDO or SCG (0, 1, 10, or 100 μg/ml) in the presence of rmGM-CSF (1 ng/ml). The concentrations of IFN-γ and TNF-α in the supernatant were determined with an ELISA. The data represent the mean of six samples, and were reproducible in at least three independent experiments. Significant differences from the control, *P<0.05, **P<0.01, ***P<0.001.

Measurement of cytokines

Concentrations of cytokines were measured using ELISA. All monoclonal antibodies and corresponding recombinant cytokines used were purchased from BD Biosciences. The sensitivity of the GM-CSF and IFN-γ ELISAs were 31.25 pg/ml. The sensitivity of TNF-α and IL-6 ELISAs were 62.5 pg/ml.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The significance of differences between means was measured using Student's t-test. For all statistical calculations, a p-value <0.05 was considered significant.

Results

Comparison of the ability of CRDO and SCG to induce cytokine production in splenocytes

First we examined the induction by CRDO in splenocytes from DBA/2 mice. The cells were cultured with CRDO or SCG (0, 1, 10 or 100 μg/ml) *in vitro* for 48 h, and then the supernatant was collected. The concentrations of GM-CSF, IFN-γ and TNF-α were measured. As shown in Fig. 1A, SCG stimulated splenocytes to produce GM-CSF, IFN-γ and TNF-α in a dose-dependent manner. CRDO induced production of GM-CSF and IFN-γ, but not TNF-α. The levels of GM-CSF and IFN-γ were low compared with those produced in response to SCG.

Our previous studies demonstrated that the production of IFN-γ and TNF-α induced by β-glucan depended on the amount of GM-CSF in the culture medium of the splenocytes. To better evaluate the ability of CRDO to induce production of IFN-γ and TNF-α, a sufficient amount of rmGM-CSF was added to the medium. Splenocytes from DBA/2 mice were cultured with CRDO or SCG (0, 1, 10 or 100 μg/ml) *in vitro* for 48 h in the presence of rmGM-CSF (1 ng/ml), and the supernatant was collected. The concentrations of IFN-γ and TNF-α were measured. As shown in Fig. 1B, CRDO induced production of IFN-γ, but still not TNF-α, and the levels of GM-CSF and IFN-γ were low compared with those produced in response to SCG. These results indicated that CRDO induced the production of cytokines in splenocytes, and the pattern of the induction would be different from that by SCG.

Effect of CRDO on cytokine production induced by SCG in splenocytes

Next we tested the effect of CRDO on the cytokine production by SCG. Splenocytes were incubated with CRDO (0, 1, 10 or 100 μg/ml) for 1 h and then exposed to SCG (100 μg/ml) or saline. After 48 h of incubation, the supernatant was collected, and the concentrations of GM-CSF, IFN-γ and TNF-α in it were measured. As shown in Fig. 2A, the production of TNF-α was inhibited by CRDO in a dose-dependent manner. On the other hand, CRDO had no effect on the production of GM-CSF and IFN-γ induced by SCG. These results indicated that the inhibitory effect of CRDO is not dependent on the presence of GM-CSF. Further, we tested the effect of CRDO on the induction by SCG of TNF-α after preincubation with a sufficient amount of GM-CSF. Splenocytes were incubated with rmGM-CSF (1 ng/ml) for 1 h. After incubation, cells were stimulated with CRDO (0, 1, 10, or 100 μg/ml) for 1 h, and then exposed to SCG (100 μg/ml) or saline. After 48 h of

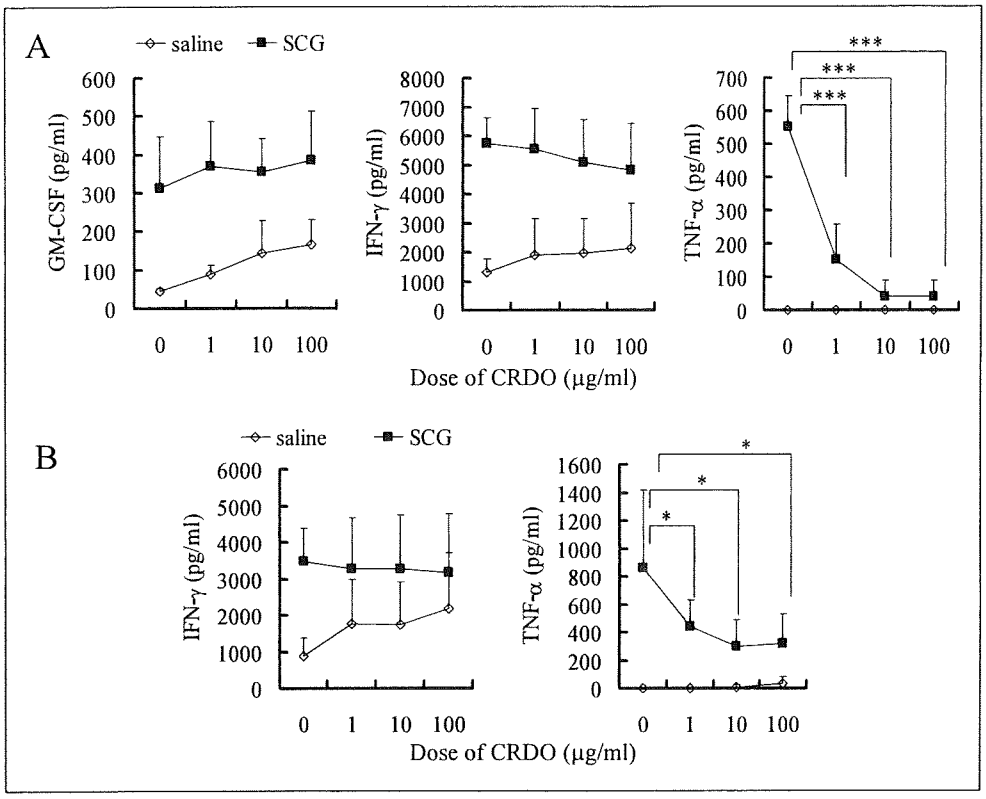

Fig. 2. Effect of CRDO on cytokine production induced by SCG in splenocytes from DBA/2 mice.
Splenocytes were isolated from DBA/2 mice. Cell suspensions were adjusted to 5×10^6cells/ml in 10% FCS/RPMI medium. A: Splenocytes were incubated with CRDO (0, 1, 10 or 100μg/ml) for 1h and then exposed to SCG (100μg/ml) or saline. After 48h of incubation, the supernatant was collected, and the concentrations of GM-CSF, IFN-γ and TNF-α were determined with an ELISA. B: Splenocytes were incubated with rmGM-CSF (1ng/ml) for 1h. After incubation, cells were stimulated with CRDO (0, 1, 10, or 100μg/ml) for 1h, and then exposed to SCG (100μg/ml) or saline. After 48h of incubation, the supernatant was collected, and the concentrations of IFN-γ and TNF-α were determined with an ELISA. The data represent the mean of six samples, and were reproducible in at least three independent experiments. Significant differences from the control, *P<0.05, ***P<0.001.

incubation, the supernatant was collected, and the concentrations of IFN-γ and TNF-α in it were measured. As shown in Fig. 2B, the production of TNF-α was partially inhibited by CRDO. On the other hand, CRDO had no effect on the production of IFN-γ induced by SCG. We tested the effect of CRDO on cell viability in splenocytes using CellTiter 96 (R) AQ_{ueous} One Solution Cell Proliferation Assay (Fig. 3). Regardless of presence of GM-CSF, CRDO showed no effect on cell viability in this culture condition. Our previous study indicated that the production of each cytokine induced by SCG could be mediated though different receptors on splenocytes [17]. The result that CRDO partially competed with SCG to induce TNF-α production suggested that CRDO could associate with the receptor for the induction by SCG.

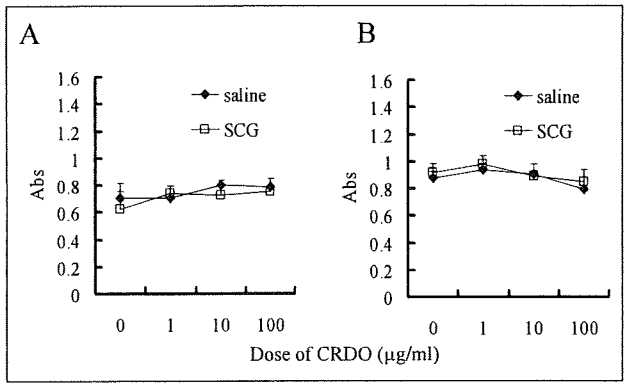

Fig. 3. Effect of CRDO on cell viability.
A: Splenocytes were incubated with CRDO (0, 1, 10 or 100μg/ml) for 1h and then exposed to SCG (100μg/ml) or saline. B: Splenocytes were incubated with rmGM-CSF (1ng/ml) for 1h. After incubation, cells were stimulated with CRDO (0, 1, 10, or 100μg/ml) for 1h, and then exposed to SCG (100μg/ml) or saline. After 24h incubation, CellTiter 96 AQ_{ueous} One Solution Reagent was added to each well. After 4h incubation, the absorbance at 490nm was recorded.

Cytokine induction of CRDO in dendritic cells

Previously, we reported that SCG induced cytokine production, particularly TNF-α and IL-6, in dendritic cells (DCs) from DBA/2 mice [9]. Next, to examine the effect of CRDO on DCs from DBA/2 mice, we tested CRDO induction of TNF-α and IL-6 induction of CRDO from BMDCs. The BMDCs were cultured with CRDO (0, 1, 10 or 100ng/ml) *in vitro* for 48h, and then the supernatant was collected. The concentrations of IL-6 and TNF-α were measured. As shown in Fig. 4, production of both IL-6 and TNF-α was induced by CRDO. These results demonstrated that CRDO induced the production of cytokines in BMDCs.

Discussion

The structural features of β-1,3-glucans, including the primary structure, solubility, degree of branching, conformation and molecular weight, could be dependent on both the source and methods of isolation. All these factors play an important role in various kinds of glucan-associated biological activities [1]. The difference in β-1,3-glucan structure occurs primarily

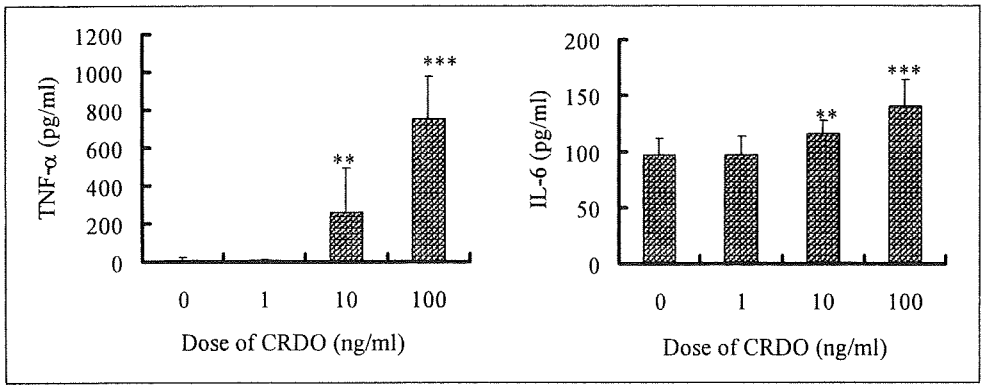

Fig. 4. Cytokine production induced by CRDO in BMDCs from DBA/2 mice.
BMDCs from DBA/2 mice were cultured with CRDO (0, 1, 10, or 100 ng/ml) *in vitro* for 48 h. The concentrations of TNF-α and IL-6 in the supernatant were then determined with an ELISA. The data represent the mean of four samples, and were reproducible in two independent experiments. Significant difference from the control, $^{**}P<0.01$, $^{***}P<0.001$.

in the side chain. Curdlan is structurally the simplest of the β-1,3-D-glucans with no glycosyl side chains [12]. Sonifilan, which has been used clinically to treat cancer in Japan, and SCG have a 1,6-glucose side chain on every third glucose unit along the main chain [18, 19]. CSBG and OX-CA, which are derived from *Candida albicans*, have long side chains of irregular length [20]. Solubility plays an important role in the induction by glucans in DBA/2 mice [4]. OX-CA, from *C. albicans*, is used as a particle β-glucan in investigations of cellular receptors and molecular mechanisms, whereas SCG is a soluble β-glucan [11]. OX-CA, as well as SCG, stimulates splenocytes of DBA/2 mice to produce cytokines. Interestingly, the production of cytokines was also induced by OX-CA, but not by SCG, in splenocytes from C57BL/6 mice. These findings suggested that not all the pathways by which OX-CA induces the production of cytokines in the spleen would be simply mediated through GM-CSF, for example the pathways triggered by SCG, and that the pattern of association of the β-glucan receptor with other receptors could differ between a soluble β-glucan, e.g. SCG, and a particle β-glucan, e.g. OX-CA. In this study, CRDO induced production of GM-CSF and IFN-γ, but not TNF-α, whereas SCG induced production of TNF-α, as well as GM-CSF and IFN-γ (Fig. 1). These results demonstrated that a difference exists in the pattern of induction between SCG and CRDO. Previously, we reported that there are different mechanisms for how the production of individual cytokines by SCG in DBA/2 mice [17]. SCG induced GM-CSF and IFN-γ production through cell-cell contact mediated by the association of $CD4^+$ T cell-expressed LFA-1 and APC-expressed ICAM-1 molecules. On the other hand, SCG directly induced adherent cells to produce TNF-α. These findings suggested that CRDO could activate the pathway related to the production of IFN-γ, but not TNF-α. The fact that even with a sufficient amount of GM-CSF, CRDO could not induce production of TNF-α suggested that the affinity of CRDO for the receptor associated with TNF-α induction is lower than that for the receptor associated with IFN-γ induction.

The β-glucan receptor dectin-1 plays a crucial role in the detection of β-glucan and live pathogenic fungi by macrophages and dendritic cells [11]. The SCG-induced production of cytokines in wild-type dendritic cells was completely abolished in cells homozygous for dectin-1-knockout [11]. BMDCs from DBA/2 mice, which are sensitive to β-glucan, highly expressed dectin-1 [9]. A recent report demonstrated that dectin-1-Syk-CARD9 signaling induced DC maturation and the secretion of proinflammatory cytokines, and that signaling through Syk and CARD9 can couple innate to adaptive immunity independently of Toll-like receptor signals [21]. Dectin-1 interacted with 1,3-β-glucans with an affinity that varied widely. Structural analysis demonstrated that the glucan backbone's chain length and the 1,6-β side chain's branching pattern strongly influenced dectin-1's binding affinity. Palma et al. [22] assigned oligosaccharide ligands for dectin-1 using neoglycolipid-based oligosaccharide microarray technology, and demonstrated that dectin-1 bound exclusively to 1,3-linked glucose oligomers, the minimum length required for the binding being a 10- or 11-mer. Adams et al. [23] demonstrated that dectin-1 did not interact with linear 1, 3-β-glucan oligosaccharides composed of less than seven glucose subunits, and that its affinity for synthetic glucans increased as the number of glucose subunits in the polymer backbone increased. These findings indicated that the branched glucan oligosaccharides were recognized by dectin-1 with higher affinity than the comparable linear glucan, and the binding affinity also increased with polymer length. Tada et al. [24] reported that barley β-glucan, a linear mixed-linkage β-glucan composed of 1,3- and 1,4-β-glucopyranose residues but without a the continuous 1,3-linkage, was recognized by dectin-1. These findings implied that CRDO, used in the present study, interacts with dectin-1 with less affinity than SCG, which is a high molecular weight 6-branched 1,3-β-D-glucan. They suggested that such a difference in affinity for dectin-1 influences the ability of these β-glucans to induce the production of cytokines.

As mentioned above, the effectiveness of curdlan and other β-glucans in eliciting biological responses depends on their chemical structure, molecular mass and conformation. Structure/activity relationships of β-1,3-glucans show that the intactness of the triple helical structure is of importance in receptor binding [25]. However, Takaoka et al. [26] reported that the activity of curdlan is dramatically enhanced by pretreatment with sodium hydroxide or dimethyl sulfoxide, which disrupts multiple-stranded helices of β-1,3-glucans, or with zymolyase, which releases β-1,3-glucans with a single helical structure from the glucan-networks assembled by multiple-stranded helices. Other studies suggest that the curdlan single helix is more potent than the triple helix as an anti-tumour agent [27]. The carboxymethyl ether and the sulphate and phosphate esters of curdlan with increased

solubility in water, show enhanced biological activity and, moreover, a water-soluble aminated curdlan derivative has tumorigenic properties [13]. Curdlan sulphate has anti-HIV activity [28] and inhibitory effects on the development of malarial parasites *in vitro* [29]. A previous report demonstrated that CRDO induced proliferation of T cells and B cells in the presence of ConA or LPS (Japanese patent No. 10-194977). In the present study, CRDO activated splenocytes and BM-DCs to induce the production of cytokines in mice.

Taken together, these results suggested that CRDO activated leukocytes from mice, the pattern of its effect being different from that of 6-branched β-1,3-glucans. These immunomodulating activities of CRDO may be used to develop functional foods.

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ちてく・あらかると

サイトカインハンティング
—先頭を駆け抜けた日本人研究者達—

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プロローグ

第74回日本インターフェロン・サイトカイン学会(JSICR)学術集会(2009年6月26～27日)で,サイトカインハンティング:先頭を駆け抜けた日本人研究者達—名誉会員に聴く—を企画した.10名の名誉会員にお願いしたところ,外国出張と重なった谷口唯継,長田重一先生を除き,新井賢一,小島保彦,笠倉新平,川出由巳,岸本忠三,高津聖志,平野俊夫,本庶 祐先生(以降敬称略)の豪華メンバーが一堂に揃った.先立つこと,2003年秋,JSICR Newsletter編集長となった私は,目玉企画を考えていた.この号には川出から—インターフェロン(IFN)の歴史から学ぶ:長野・小島の「ウイルス抑制因子」はIFNか—という非常に挑戦的な原稿が寄せられた.その後,これに対する小島,他の反論が寄せられ,議論が盛り上がっていく.また,今だったら語れる,記録に残しておきたいと多くの方が書いてくださり,編集委員会では「サイトカインハンティング:先頭を駆け抜けた日本人研究者達」の企画を,展開していくことになる.

サイトカイン発見の夜明け(1954～1960年)

「感染性のない,遠心上清に残る免疫原性を有する粒子の大きさはどのくらいでしょうか.Seitz EKは通りませんか」と北大 山田守英教授が質問,発表者である小島保彦は「35,000回60分の遠心で沈殿しない.SeitzをpH6.4～6.6の20%正常マウス血清で前処理すると,発痘阻止因子は通過するようである」.1954年10月に開催された第2回日本ウイルス学会総会での1コマである(通常のウイルスはSeitz濾過器を通過するが,ワクシニアウイルスは大きなウイルスでこれを通過しない.したがってこの事実は,ウイルス本体ではなく,液性部分に活性があることを強く示唆していた).小島・長野は同様の報告を1954年9月の日仏生物学会で発表,同年にC. R. Soc. Biol.(仏生物学会誌)に「紫外線不活化ワクシニアウイルスの免疫力」として掲載した.この論文をIFNの発見と評価している人がいる一方,その論文のタイトルと,考察のあいまいさゆえに異議を唱える人もいる.ただ,前述のように第2回日本ウイルス学会総会の場では,液性因子に議論が集中したことは,疑いがない.抗体ではない液性因子がウイルスの増殖の抑制に関与する,サイトカイン研究の夜明けには違いなかった.

免疫を動かす液性因子の存在(1960～1979年)

笠倉は一方向性MLR(リンパ球混合反応)を開発する過程で,アロ抗原による第一のシグナルの他にリンパ球の増殖を誘導する可溶性因子が必要であることを明らかにした(1965).これはBF(blastogenic factor)と名づけられたが,IL-2の発見と言ってもよかろう.さらに笠倉はBFが非特異的に免疫反応を惹起することを報告するが,当時(1970年ごろ)免疫学の世界では,免疫とは抗原特異的を前提としていたことから,非特異的リンパ球増殖因子が実際に免疫応答に関与するという考えはなかなか受け入れられなかったらしい.

一方,同時期,高津は抗体産生を刺激する抗ハプテン抗体産生増強因子(1972～1973年)を発表したが,抗原非特異的な因子についての学会の反応は冷たかったようである.ところが,1973年に高津がちょうど留学したジョンズホプキンス大学の石坂公成研究室では,岸本が抗体産生を促進する因子の研究結果に興奮してい

たという．日本の非常識は米国の常識である，この落差に愕然としたと，高津は言う．

とにもかくにも，1970年代には免疫・発生・分化に関わる数多くの液性因子の発見が相次ぎ，報告された時期である．

同時期IFNの実用化に向けた動きが急速に進む．1970年代には科学技術庁・厚生省の研究班でその精製と臨床応用の競争がなされていた．ただ，IFN実用化の動きが，免疫の生理活性物質と必ずしも連動していたわけではないらしい．IFNは抗ウイルス薬であり，抗癌薬であり，免疫とはかけ離れた存在であったようだ．

分子としてサイトカインをとらえる：タンパク質の精製，遺伝子の単離，遺伝子組換え標品の作製（1979年～）

今でこそ一般的な分子生物学的技法を駆使すればさほど困難なものではない機能性微量タンパク質の同定は，1970年代後半～1980年代初めのころ困難を極めた．1979～1980年にかけて，谷口・長田によりIFN-βおよびαのクローニングがなされる．そして1980年にはIFN-α，β，γとして命名された．1970年からIFNの精製に取りかかった川出は言う．これは明らかに液性因子という漠としたものから，サイトカインが明確な分子として認識されたことを物語っていると．

最初にIFN遺伝子のクローニングに成功した谷口は1.5×10^9個の細胞から250 μgのmRNAを得，そしてcDNAを得たと言う．関係者に聞いたところでは，150平方cmのフラスコで800本ぐらいの量の細胞を培養したと言う．当時とすれば大変な細胞量であった．また，高津は抗ハプテン抗体産生増強因子の精製のために，B151K12細胞の培養上清を約1トン集めたと言う．そこからタンパク質を精製，mAbを得ていた．mAbができれば，タンパク質の精製が進み，機能の特定が可能となる．一方でmRNAを分画，逆転写酵素で二本鎖cDNA作製，大腸菌でcDNAライブラリーを作製，ハイブリダイゼーションでmRNAを取り，各分画mRNAをアフリカツメガエル卵母細胞でタンパク質に翻訳，どの分画に目的のものが含まれるかを検定するという過程を繰り返していた．1983年にはIL-2（谷口・羽室），1986年IL-4（本庶），IL-5（本庶・高津），IL-6（平野・谷口・岸本）と次々とクローニングがなされる．一方，DNAX研究所（新井ら）では，IL-3，IL-3R，GM-CSFと次々とサイトカインやその関連遺伝子がクローニングされていく．新井曰く，DNAXには世界中からはみ出し者が集まった，だからできたと．DNAX研究所ではいち早くP. Berg研究室で開発されたcDNAのクローニングと動物細胞での発現の技術（Okayama-Berg法）を用いている．これ以降，クローニングに必要な細胞量は1/100以下にはなったのではなかろうか．このようにクローニング技術もどんどんと進化していく．

1980年代には谷口・長田・新井・本庶といった分子生物学者がサイトカインハンティングで活躍する．免疫学出身の研究者と相互に連携しまた競争することで，次々とクローニングが進む．そして対象はサイトカインからそのレセプターへと広がっていく．

この時代，細胞を対象とした研究から，遺伝子を明らかにする時代に移り，乗り遅れた人間は退場を余儀なくされた．岸本は言う．自分は旧い人間であることを自覚していたので，分子生物学者のもとへ若手を積極的に習いに行かせたと．そのとき本庶の下へ行ったのが審良である．また，臨床を経験していることを生かせと，IL-6受容体を探索，ついにはその抗体を治療に結びつけた．それができたのは自分達が発見したのがIL-6という多機能サイトカインであったからと言う．

名誉会員の先生方が異口同音に言われたことがある．強い意志を持ってやること．既成概念にとらわれない見方，そしてデータを大切にすること．最後にはヒューマンネットワーク，求めれば，必ず応えてくれる人がいると．

そして今

1990年代になるとサイトカインハンティングの様相はさらに大きく変わる．シグナル配列トラップ法は，特にケモカインの発見に大きな力を発揮した．さらに1995年になると，データベースからホモロジー検索により新規ケモカインの存在が明らかにされるようになる．これ以後，存在が示唆，遺伝子の単離，機能解明と，今までとはまったく逆のコースを辿るようになっている．今後の方向性は？ 明らかに旧い研究者である私には？ である．ただ，1980年代の状況を今西錦司が

"Cloning and Sequence Syndrome" と言ったと言う．今や生物を知らないバイオ研究者が増えていることを何と考えるべきか．細胞を見つめ，組織を見つめ，個体を見ることも大切よ，としか言えない．

こうしてサイトカインハンティングの歴史を辿ってみると，サイトカインハンティングの歴史はバイオの発展の歴史でもある．サイトカインハンティングを通じて，色々なバイオ技術が発展してきたと言ってもよいだろう．その先頭を駆け抜けた研究者達は，また，バイオの今の繁栄を導いた研究者達でもあったというのは疑いがない．

サイトカインハンティングの様子の詳細は，JSICR Newsletterを参照されたい．Newsletterには先頭を駆け抜けた研究者達の熱い思いと，し残した仕事のヒントも埋まっているはず．研究に疲れたとき，道を見失いかけたとき読んでみると，これらの先達の経験から，何かを得ることができるのではないだろうか．

［日本インターフェロン・サイトカイン学会］
http://www.prevent.m.u-tokyo.ac.jp/JSICR/index.html

急性期冠状動脈炎とその後の遠隔期硬化性病変への移行-成人期動脈硬化症との違い-を考察する

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川崎病後遺症すなわち川崎病急性期に発生した冠状動脈炎の瘢痕治癒像はさまざまな形態を示すが，瘤を形成した場合には遠隔期においても新たな狭窄性病変への進展などのリモデリングが継続する．これらは後炎症性動脈硬化とよぶべき変化であり，日常内科領域で遭遇するアテローム性動脈硬化症とは発生機序，組織像ともに大きく異なる．しかし，中年期を迎えつつある川崎病既往者が増加している現在，後炎症性動脈硬化症とアテローム性動脈硬化症との関連は早急に明らかにされるべき重要な課題である．

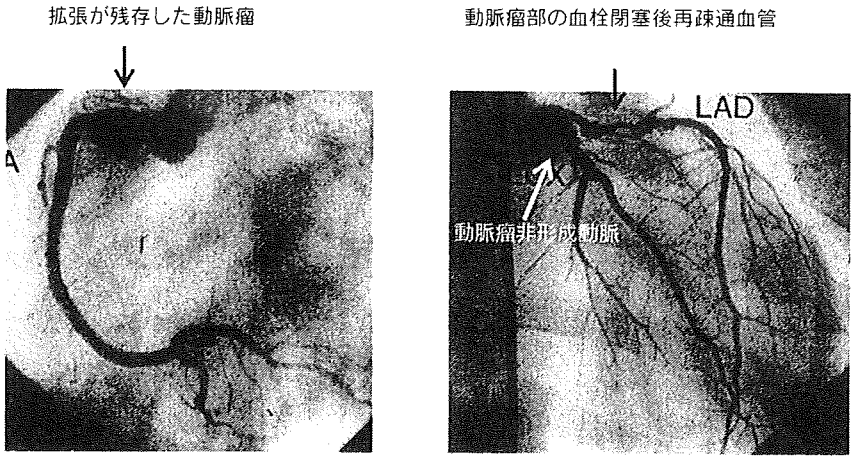
KEY WORDS
後炎症性動脈硬化症　動脈瘤　血栓閉塞後再疎通　アテローム性動脈硬化症

○ はじめに

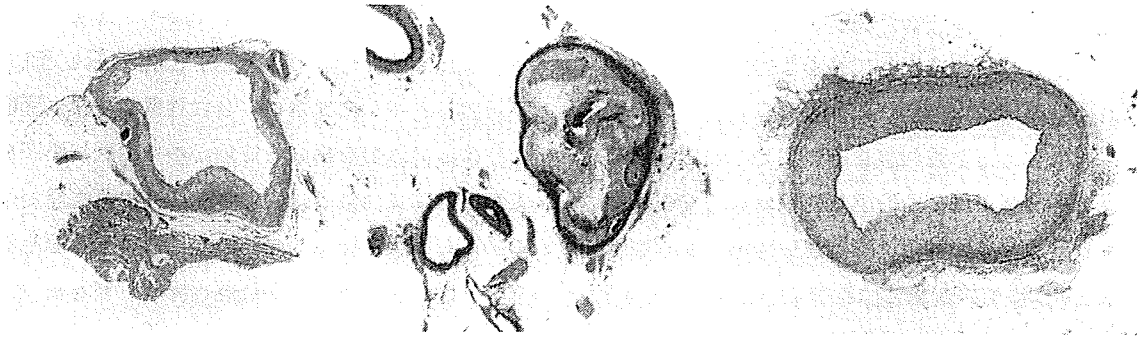
最近の川崎病全国調査成績によれば，急性期における心障害の発生率は11.0%，発症1ヵ月以降も残存する心後遺病変の発生頻度は3.2%である[1]．ともに漸減傾向にはあるものの年間1万人を超える患者が新たに発症している事実を考えると，毎年数百名の罹患児が心後遺症を残しながら治癒していることになる．さらには，川崎病の報告から40年以上が経過していることに鑑みると，後遺症を有する川崎病既往児は相当な数にのぼるといわざるを得ない．本稿では川崎病冠状動脈炎後の後遺病変の基本病理形態像について記載し，成人期に生じるアテローム性動脈硬化症との形態学的差異について言及したい．

○ 急性期川崎病冠状動脈炎は一峰性の推移を示す

病理組織学的に，川崎病冠状動脈炎は発症後6～8日ころ，炎症細胞が動脈の内腔側，外膜側から動脈壁内に侵入してはじまり，発症10日前後で血管全層に達し汎血管炎に至る．動脈壁には単球/マクロファージを主体とした炎症細胞浸潤が著明に出現しており，増殖性肉芽腫性炎症とよぶべき像を示す[2][3]．動脈の構築を保持するうえで重要な構造物である内弾性板や中膜平滑筋，そして細胞外マトリクスなどは，炎症細胞から産生，放出されるさまざまな分解酵素や活性酸素により傷害され，炎症が高度な場合には動脈壁障害の結果として動脈の拡張が



図❶　川崎病罹患後 16 年後に突然死した 20 歳男性の経過観察中に施行された冠動脈血管造影



右冠状動脈（RCA）
動脈瘤が残存

左冠状動脈前下行枝（LAD）
血栓閉塞後の再疎通血管

左冠状動脈回旋枝（LCX）
瘤非形成動脈
全周性の内膜肥厚あり

図❷　図❶と同一症例の冠状動脈ルーペ像

はじまる[4]．動脈瘤が発生するのが発症後 12 日ころである．著明な増殖性炎症は 25 病日ころまで持続したのち徐々に消退していき，40 病日ころにはほぼ消失する．そして，炎症瘢痕すなわち線維化組織は長期間残存する．とくに，動脈瘤のような動脈構築の破綻を伴った場合には後遺病変は生涯残存することになる．

この一方，急性期川崎病剖検例のなかには，冠状動脈構築の基本構築が保たれた軽度の炎症や動脈内に炎症細胞浸潤がほとんどみられないような症例も含まれており，実際にはさまざまな経過をとりうることが推測される．

○ 川崎病遠隔期にみられる冠状動脈変化について考える

遠隔期に突然死した成人男子の川崎病既往症例を提示する．症例は 20 歳男性，4 歳のときに川崎病に罹患し，心後遺症のために経過観察されていた．ある日，路上で口論をし，自転車で逃げたところ突然倒れそのまま死に至った．**図❶**に経過観察時の心血管造影写真を示す．右冠状動脈には巨大な動脈瘤が開存したまま残存している．左冠状動脈前下行枝には血栓閉塞後の再疎通像が観察される一方，左冠状動脈回旋枝は造影上明らかな変化に乏しい．剖検検索にて明らかになった本例の冠状動脈病変を示す（**図❷**）．いずれの冠状動脈の病理所見も造影所見に一致する．このように川崎病の冠状動脈後遺病変は大

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Association Between Capacity of Interferon-α Production and Metabolic Parameters

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A prompt and regulated interferon (IFN) system is critical for host defense against infectious pathogens. Although increased susceptibility to infection has been observed in subjects with diabetes or obesity, little is known about the relationship between metabolic disorders and increased susceptibility to infection. In order to evaluate the association between immune function and metabolic parameters, we examined the relationship between capacity of IFN-α production and metabolic parameters including fasting plasma glucose (FPG), lipids, uric acid, body mass index (BMI), and blood pressure in 575 healthy subjects. Linear regression analysis showed that log(IFN-α production) was positively correlated with log(triglyceride) ($r = 0.088$, $P = 0.03$) and uric acid ($r = 0.091$, $P = 0.03$), and negatively correlated with age ($r = -0.158$, $P = 0.0001$) and FPG ($r = -0.088$, $P = 0.03$). Multiple regression analysis showed that log(IFN-α production) was independently determined by age ($\beta = -0.148$, $P < 0.0001$), sex ($\beta = -0.240$, $P = 0.0003$), and FPG ($\beta = -0.096$, $P = 0.0209$), suggesting that lesser degrees of hyperglycemia also affect IFN-α production. We conclude that hyperglycemia but not BMI, hypertension, or hyperlipidemia may be associated with decreased capacity of IFN-α production and glycemic control is critical even for both subjects without any medication for diabetes and subjects under the diagnosis of diabetes on infectious diseases.

Introduction

It is widely known that individuals with diabetes mellitus are at higher risk for infections than those without diabetes mellitus. A higher prevalence of bacteriuria has been observed in subjects with diabetes compared with normal women (19% versus 8%) (Vejlsgaard 1966). In the last decade, several epidemiological studies have found an association between obesity and an increased incidence of periodontal disease (Al-Zahrani and others 2003; Genco and others 2005; Saito and others 2005; Saito and others 1998). It was reported that a higher body mass index (BMI) was significantly related to a greater prevalence of periodontal disease in Japanese healthy adults (Saito and others 2001). These observations may suggest a relationship between metabolic disorders and increased susceptibility to infection.

Interferon-α (IFN-α) is known to play an important role in the initial defense mechanism against viral and microbial diseases (Kirchner 1986). Indeed, functional immunity can be monitored using *in vitro* tests, such as natural killer cell activity and either proliferation responses or cytokine induction in lymphoid cells following mitogen stimulation. However, these measurements are found to reflect the function of natural killer cell or T cell (Biron and Brossay 2001; Stone and others 2009). As an initial response to viral and microbial infection, plasmacytoid dendritic cells (pDCs), also called type I IFN-producing cells (IPCs), is known to be central to the innate immune response of a host (Liu 2005). PDCs produce enormous amounts of IFN-α, which indirectly regulates the function of T cells and thus links innate and adaptive immune responses (Asselin-Paturel and others 2005; Fitzgerald-Bocarsly and Feng 2007). In fact, such prompt and regulated IFN system is critical for host defense against infectious pathogens. We therefore measured capacity of IFN-α production to assess the immune status of the host.

To further evaluate the association between immune function and various metabolic parameters, we examined the relationship between capacity of IFN-α production and

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fasting plasma glucose (FPG), lipids, uric acid, BMI, and blood pressure.

Materials and Methods

Subjects

Medical records of 877 individuals aged 20–79 years old, who participated in annual health examinations at 2 workplaces in Kyoto, Japan were obtained. The subject records included data on the quantification of IFN-α production in addition to routine laboratory tests. Data from the Louis Pasteur Center for Medical Research (n = 699) were reviewed from 1996 to 2004 records, and data from the Kyoto First Red Cross Hospital (n = 178) were reviewed from 2005 records.

All subjects were studied in the morning after 8 h of overnight fasting. Measurements were taken of FPG, lipid profile, uric acid, and leukocyte count, which were included in the routine laboratory blood test. BMI was calculated by the patient's weight (kilograms) divided by height (square meters). Blood pressure was measured in the patient's right arm, with the subjects in a sitting position.

After exclusion of 66 subjects with missing samples, subjects with acute infection (n = 17), chronic inflammatory disease (rheumatoid arthritis n = 15, autoimmune diseases n = 22, chronic hepatitis n = 71), and malignant tumors (n = 9) were excluded. Those who received medication for diabetes mellitus, hypertension, hyperlipidemia, or hyperuricemia (n = 102) were also excluded. Finally, a total of 575 subjects were recruited into this study. We investigated the relationships of IFN-α production to FPG, lipids, uric acid, BMI, and blood pressure. Approval for the study had been provided by the Ethics Committee of Louis Pasteur Center for Medical Research and Kyoto First Red Cross Hospital. All participants gave written informed consent.

Measurement of IFN-α production in human whole blood

Two milliliters of heparinized blood was cultured with 500 HA U/mL Sendai virus (HVJ) within 5 h after the blood was drawn. The blood–virus mixture was incubated at 37°C for 20 h. Supernatants were harvested by centrifugation at 3,000 rpm for 10 min and stored at −80°C until titration.

TABLE 1. CLINICAL CHARACTERISTICS OF THE SUBJECTS

	Mean ± SE
n	575
Age (years)	52.0 ± 0.4
IFN-α production (IU/mL)	7,237.4 ± 226.0
Systolic blood pressure (mmHg)	123.6 ± 0.7
Diastolic blood pressure (mmHg)	75.6 ± 0.4
BMI (kg/m^2)	22.6 ± 0.1
Fasting plasma glucose (mg/dL)	97.4 ± 0.9
Total cholesterol (mg/dL)	207.5 ± 1.5
Triglyceride (mg/dL)	121.0 ± 4.1
HDL-cholesterol (mg/dL)	57.9 ± 0.6
Uric acid (mg/dL)	5.2 ± 0.05

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

IFN activity in the supernatants was assayed by suppression of viral cytopathic effects in FL cells (derived from human amnion) by Sindbis virus (Kohase and others 1986). Duplicate samples of standard reference IFN were serially diluted by Eagles' MEM supplemented with 5% FCS in the wells of flat-bottomed 96-well microtiter plates (Falcon 3072) and irradiated with UV light (1,000 J/m^2) for HVJ inactivation. After UV treatment, HVJ in the blood samples could not affect the assay. FL cells (5×10^4/well) were added following incubation at 37°C for 18 h. Sindbis virus (10^5 PFU/50 μL/well) suspended in Eagles' MEM supplemented with 1% FCS was added to the FL cells after removal of the supernatant. Sindbis virus and FL cells were cultured for another 30 h. Neutral red dye (0.5%) was added 1 h before harvesting, and the amount of dye incorporated into the cells was measured in a spectrophotometer (Titertek Multiskan MCC/340) at 540 nm following extraction of dye with 0.1 M sodium phosphate and 50% ethanol solution. The results were standardized to reference IFN-α MRC 69/19B. For the characterization of IFN type, some samples were neutralized by anti-human IFN-α horse IgG (Nippon Chemical Co. Ltd., Hyougo) as described previously (Shirono and others 1990; Kuo and others 1991).

Statistical analysis

The statistical analysis was mainly performed by parametric tests. Thus, several variables with asymmetric distribution to the right were logarithmically transformed (base 10) in order to obtain substantially normal distributions before performing the tests. The log-transformed variables were IFN-α production, triglyceride. According to the analysis, male subjects were assigned 1 and female subjects were assigned 2. To describe the variables measured in this study, mean ± SE was used. Statistical analyses were conducted using the Stat View 5 system (Stat View, Berkeley, CA).

Linear regression analysis was performed to examine the relationships between IFN-α production and other variables, and also to exclude any significant collinearity among the variables before performing multivariable analysis. Multiple regression analysis was performed to assess the combined influence of variables on IFN-α production. P values <0.05 was considered as significant.

Results

Clinical characteristics of the subjects are shown in Table 1. Mean value of IFN-α production was 7,237.4 ± 226.0 IU/mL. In male subjects, the mean value of IFN-α production was 8,278.5 ± 334.0 IU/mL and female 5,884.0 ± 262.7 IU/mL, respectively. There was a significant difference between men and women (P = 0.0003). Pearson's correlation coefficients between log(IFN-α production) and other variables are shown in Table 2. There were statistically significant positive correlations between log(IFN-α production) and log(TG) (r = 0.088, P = 0.03), uric acid (r = 0.091, P = 0.03). Negative correlations were found between log(IFN-α production) and age (r = −0.158, P = 0.0001), FPG (r = −0.088, P = 0.03). No significant correlation was found between log(IFN-α production) and BMI.

Multiple linear regression analysis performed with log(IFN-α production) as the dependent variable and the candidate factors (age, sex, FPG, log(TG), uric acid) as the independent variables showed that age (β = −0.148,

TABLE 2. CORRELATION OF log(IFN-α PRODUCTION) TO MEASURES OF VARIABLES

	r	P
Age	−0.158	0.0001
Systolic blood pressure	0.04	0.34
Diastolic blood pressure	0.078	0.06
BMI	0.044	0.29
Fasting plasma glucose	−0.088	0.03
Total cholesterol	−0.028	0.5
Log(triglyceride)	0.088	0.03
HDL-cholesterol	−0.078	0.06
Uric acid	0.091	0.03

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein.

TABLE 3. MULTIPLE REGRESSION ANALYSIS ON log(IFN-α PRODUCTION)

	β	P
Age	−0.148	<0.0001
Sex	−0.240	0.0003
Fasting plasma glucose	−0.096	0.0209
Log(triglyceride)	0.057	0.2065
Uric acid	−0.054	0.2935

β, Standardized coefficients.

$P < 0.0001$), sex ($\beta = 0.240$, $P = 0.0003$), and FPG ($\beta = -0.096$, $P = 0.0209$) were independent determinants of log(IFN-α production) (Table 3).

Furthermore, we determined IFN-α production trend in subjects who had a history of health examination for a long period at the Louis Pasteur Center for Medical Research to examine a correlation among each individual. Among 64 subjects with abnormal FPG levels (≥110 mg/dL), 10 had their IFN-α production monitored once or twice per year for >5 years. During follow-up, 3 subjects developed diabetes and 7 showed increase in FPG levels, though below the diagnosis of diabetes. Although IFN-α production showed fluctuation, a simple regression line with the IFN-α production and age yielded a negative slope, while FPG levels showed increase, in all 10 subjects. The representative profile of one subject is shown (Fig. 1).

Discussion

In this study, we demonstrated that capacity of IFN-α production correlated significantly with age, sex, and FPG, but not with BMI, blood pressure, uric acid, or lipids.

Age is known to play a role in the regulation of IFN-α production, which is followed by a gradual decline with increased age (Abb and others 1984; Kita and others 1991; Katschinski and others 1994). This is consistent with the finding of Shodell et al. who reported significant decreases of the circulating pDCs in healthy aged humans, as was defined both by flow cytometry and IFN-α production (Shodell and Siegal 2002). There was also a small decline in the amount of IFN produced per pDC over the entire age range. On the other hand, no change in the total lymphocyte or monocyte counts was observed. Thus the age-related losses of IFN-α production may be due to both declining pDC numbers and a small reduction in IFN produced per pDC with aging.

Although previous studies showed no sex difference (Abb and others 1984; Kita and others 1991; Katschinski and others 1994), we observed decreased production of IFN-α in females in this study. It has been reported that the pDC numbers were not different between males and females (Shodell and Siegal 2002; Berghöfer and others 2006). Other factors like differences in innate immunity between males and females or systemic imposition of sex hormones may play a role; however, the mechanisms are not well understood (Kovats and Carreras 2008). To address these questions, further studies are required. FPG revealed to be another important determinant of IFN-α production, suggesting that lesser degrees of hyperglycemia also affect IFN-α production. And this was also observed in the history of long-term follow-up of IFN-α production among the 10 individuals with abnormal FPG levels.

Our data may coincide with a recent report by Summers et al. who described a reduced secretion of IFN-α by dentritic

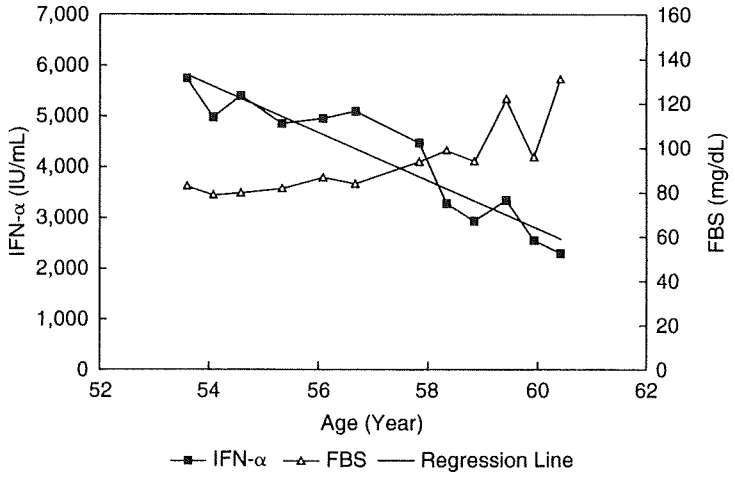

FIG. 1. IFN-α production trend in subjects with elevated fasting plasma glucose (FPG) levels, though under diagnosis of diabetes. The IFN-α values and FPG levels are plotted versus time.

cells (DC) in both type 1 and type 2 diabetes (Summers and others 2006). In their study, DC subsets in each diabetic group exhibit normal properties concerning frequency and activation state. Only low secretion of IFN-α was observed. However, the number of type 2 diabetes patients examined were quite low ($n = 7$), and the degree of glycemic control was not evaluated in their studies. Seifarth et al. reported significantly lower absolute numbers of pDC in patients with type 2 diabetes under poor glycemic control ($HbA_{1c} > 9.5\%$) compared to healthy controls (Seifarth and others 2008). Reduced absolute numbers of pDC in patients with type 2 diabetes under good glycemic control ($HbA_{1c} < 7.0\%$) was also observed compared with healthy controls, but this difference did not reach statistical significance (Seifarth and others 2008). The reason for this reduction in peripheral immune cells remains unclear; however, the decrease in the main IFN-producing cell counts may be a possible explanation of the decline in IFN-α production.

Although leukocyte count also showed correlation with IFN-α production, we did not adjust IFN-α production with leukocyte count. In this study, we aimed to investigate the immune status of each individual, but not the function of leukocyte. Moreover, the main IFN-α producer is pDC and we considered it inappropriate to overestimate the influence of leukocyte count.

There are some limitations to this study. In a bioassay of IFN, we cannot distinguish the subtypes of IFN induced by Sendai virus yet known as a potent IFN-α inducer (Klein and others 1984). However, the main subtype of IFN induced in whole blood cultures is probably IFN-α, as suggested by the results of neutralization experiments with anti-natural IFN-α antibodies, which are similar to the results of our previous study (Kuo and others 1991). As we considered it more important to determine the active forms of IFN than the subtype of IFN to prevent virus infection, titer of IFN using a bioassay of whole blood cultures was measured.

In conclusion, hyperglycemia but not BMI, hypertension, or hyperlipidemia may be associated with decrease in capacity of IFN-α production. Lesser degrees of hyperglycemia also affect IFN-α production, suggesting that glycemic control is critical even for both subjects without any medication for diabetes and subjects under the diagnosis of diabetes on infectious diseases.

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AQ1: Please check whether the citation for Table 3 is appropriate
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GAK, a regulator of clathrin-mediated membrane trafficking, localizes not only in the cytoplasm but also in the nucleus

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The ubiquitously expressed Cyclin G-associated kinase (GAK) regulates clathrin-mediated membrane trafficking in the cytoplasm. However, the association of GAK with a nuclear protein Cyclin G1 that is unrelated to membrane trafficking suggests an unidentified role of GAK in the nucleus. Indeed, we report here that GAK localizes in both cytoplasm and nucleus by immunostaining, ectopic expression of GFP-GAK and pull-down assays using dissected GAK fragments. GAK forms complexes not only with cyclin G1 but also with other nuclear proteins such as p53, clathrin heavy chain (CHC) and protein phosphatase 2A (PP2A) B′α1. Moreover, CHC associates with GAK via a different domain depending on whether it is in the cytoplasm or nucleus. Immunostaining revealed that about 20~30% of B′α1, cyclin G1 and p53 complex with nuclear GAK. CHC also displayed dots in the nucleus and almost all nuclear CHC signals colocalized with GAK. These observations together suggest an important function of GAK in the nucleus.

Introduction

GAK (cyclin G-associated kinase) is a serine/threonine kinase that was initially identified as an association partner of cyclin G (Kanaoka *et al.* 1997). As suggested by its strong homology (outside of its Ser/Thr kinase domain) to the neuronal-specific protein auxilin, the ubiquitously expressed GAK regulates clathrin-mediated membrane trafficking as it is an essential cofactor for the Hsc70-dependent uncoating of clathrin-coated vesicles (Greener *et al.* 2000). Furthermore, the knockdown of GAK by vector-based small hairpin RNA revealed that GAK not only induces clathrin exchange on clathrin-coated pits, but also mediates the binding of clathrin and adaptors to the plasma membrane and the *trans*-Golgi network (Lee *et al.* 2005; Zhang *et al.* 2005). Total internal reflectance microscopy revealed the dynamic behavior of GAK that, following transient dynamin recruitment, is transiently recruited to the clathrin puncta; this recruitment is dependent on the PTEN-like domain of GAK that binds to the phospholipids (Lee *et al.* 2006). GAK also phosphorylates the mu2-subunit of the AP2 adaptor complex, which suggests GAK plays a pivotal role in the assembly of clathrin-coated vesicles (Korolchuk & Banting 2002). Furthermore, GAK knockout mice showed that GAK deletion blocks development and causes lethality in adult animals by disrupting clathrin-mediated endocytosis (Lee *et al.* 2008). Thus, it is now well established that GAK is an essential player in membrane trafficking (Eisenberg & Greene 2007).

However, the fact that GAK associates with a nuclear protein Cyclin G1 that plays no role in membrane trafficking suggests that GAK may play other, as yet unknown, roles in cellular events other than membrane trafficking. Supporting this is the fact that the knockdown of GAK by small hairpin RNA dramatically enhanced EGFR expression and its tyrosine kinase activity, which significantly altered the spectrum of downstream EGFR signaling; it was suggested that these effects may be due to altered receptor trafficking (Zhang *et al.* 2004). Moreover, mass spectrometric analysis (MS/MS) identified GAK as a protein that interacts with nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), whose constitutive overexpression is a key oncogenic event in anaplastic large-cell lymphomas (Crockett *et al.* 2004). In addition, GAK was found to interact with androgen receptor (AR) and to enhance AR activity in a ligand-dependent manner; GAK expression was also found to be significantly increased in prostate cancers that had progressed to androgen independence (Ray *et al.* 2006). The latter report suggests

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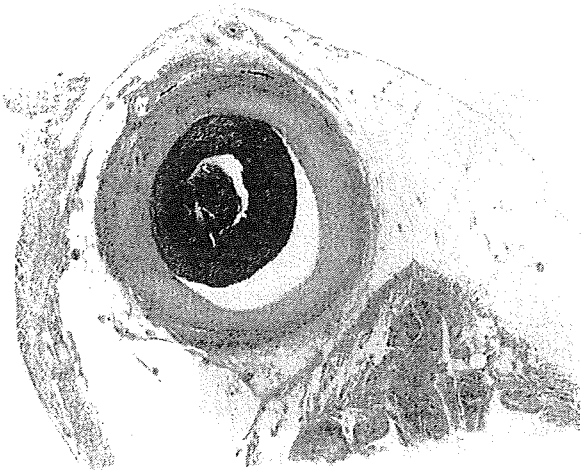
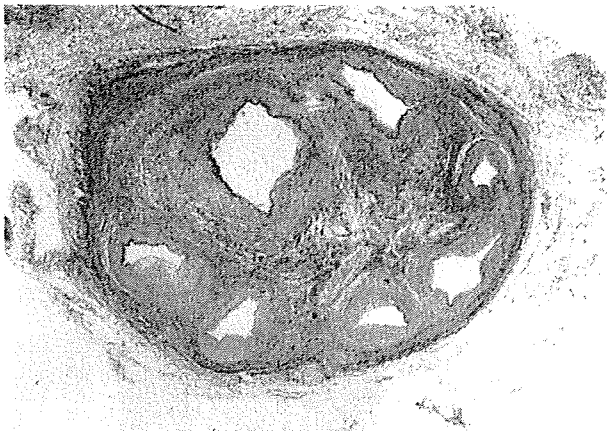


図❸　血栓が充満する動脈瘤，15 歳（AM 染色）



図❹　血栓閉塞後再疎通血管
動脈瘤内には明瞭な動脈構築を示す再疎通血管が複数認められる．12 歳女性，川崎病罹患後 11 年
（高橋啓ら，1991[5]より引用）

きく 3 つの形態に分けることができる[4]．

1．動脈瘤の拡張が継続した病変

炎症後，動脈瘤形状を残したまま器質化した病変である．瘤壁は細胞密度が著しく低下した緻密な線維組織からなる．さらに動脈瘤壁部では瘤壁を取り囲むような石灰化を伴うことが多く，剖検では石のような硬さを示す．緻密な線維組織からなる瘤の内側には平滑筋細胞が疎に分布する新生内膜肥厚が生じ，瘤内には器質化傾向を伴った新旧混在する血栓が充満することがある（**図❸**）．また，動脈瘤の前後では細胞線維性新生内膜肥厚による内腔狭窄を見，これが死因と推定された症例も存在する．

2．瘤の血栓性内腔閉塞後に再疎通が生じた病変

割面にて蓮根の切り口のような形状を示すのが特徴的である．病変の最外側には外弾性板に相当する弾性線維の取り囲みがあり，本病変は 1 本の動脈の中で生じた病変であることがわかる．さらに，罹患後，年余を経て死亡した症例の再疎通血管ではおのおのの再疎通血管を平滑筋細胞が取り囲み，あたかも正常動脈のような像を示すに至る（**図❹**）[5]．ステップ標本による観察の結果，中枢側の 1 本の冠状動脈が複数の再疎通血管に分岐し，再び合流して末梢動脈に至ること，再疎通血管は相互に連絡すること，再疎通血管から冠状動脈枝を出すことなどが確認された[5]．さらに，瘤前後では内膜肥厚による内腔狭窄が生じ，再疎通血管自体も細胞線維性組織により狭窄，閉塞することがある．

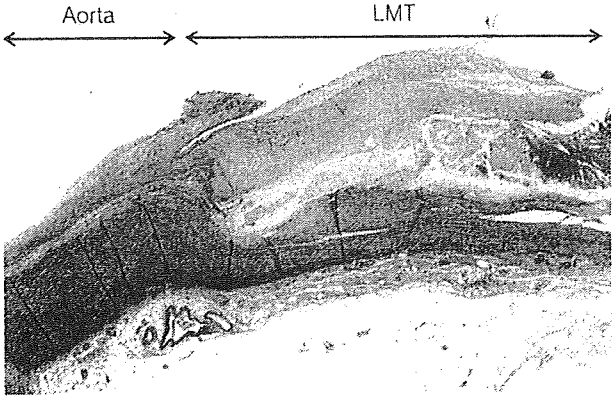
3．拡張性変化，瘤の残存を認めない動脈

冒頭に提示した症例の左冠状動脈回旋枝（**図❷右**）のように，動脈瘤を形成していなかった冠状動脈枝においても正常動脈構築とは異なる求心性内膜肥厚が観察される．この内膜肥厚は全周性に均等に発生し細胞線維成分により構成される．しかし，石灰化や壁着血栓は認められなかった．中膜は非薄化傾向にあり，外膜には線維組織の増生を伴っていた．これらの所見は瘤形成には至らなかったが本冠状動脈にも急性期に炎症が発生していたことを示すものである．

同様の変化は，川崎病に罹患した後に悪性腫瘍などの川崎病心後遺症とは直接関連しない原因で死亡した症例の冠状動脈においても高頻度に観察される[6]．一過性拡張や瘤退縮病変に対応しうる病変であると推測される．瘤が存在しない冠状動脈においても血管炎の瘢痕として矛盾しない所見が観察されたことから，多くの川崎病罹患児には急性期にさまざまな程度の冠状動脈炎が発生し，遠隔期にその瘢痕を残すと推測される．

○　川崎病後遺病変は後炎症性動脈硬化症である

上記の変化をはじめ，これまで文献に記載されている遠隔期川崎病死亡の冠状動脈変化は川崎病急性期に発生した動脈炎の瘢痕治癒像であり後炎症性動脈硬化症と総括しうる変化である．この後炎症性動脈病変には血行力



図❻ 冠状動脈起始部に生じた動脈瘤にみられたアテローム性動脈硬化症，複合病変，39歳男性
（Takahashi K *et al*, 2001[8]より引用）

今後，内科領域で後炎症性動脈病変を疑う症例に遭遇する機会が増すことが予想される．今回提示したような動脈病変をみた場合には，川崎病の後遺病変を疑い詳細な病歴の聴取をおこなう必要がある．さらに，アテローム性動脈硬化症による修飾についても注意深く観察する必要がある．

最後に，貴重な川崎病剖検例の病理組織像の検索をお許しくださいました諸先生方に深謝いたします．

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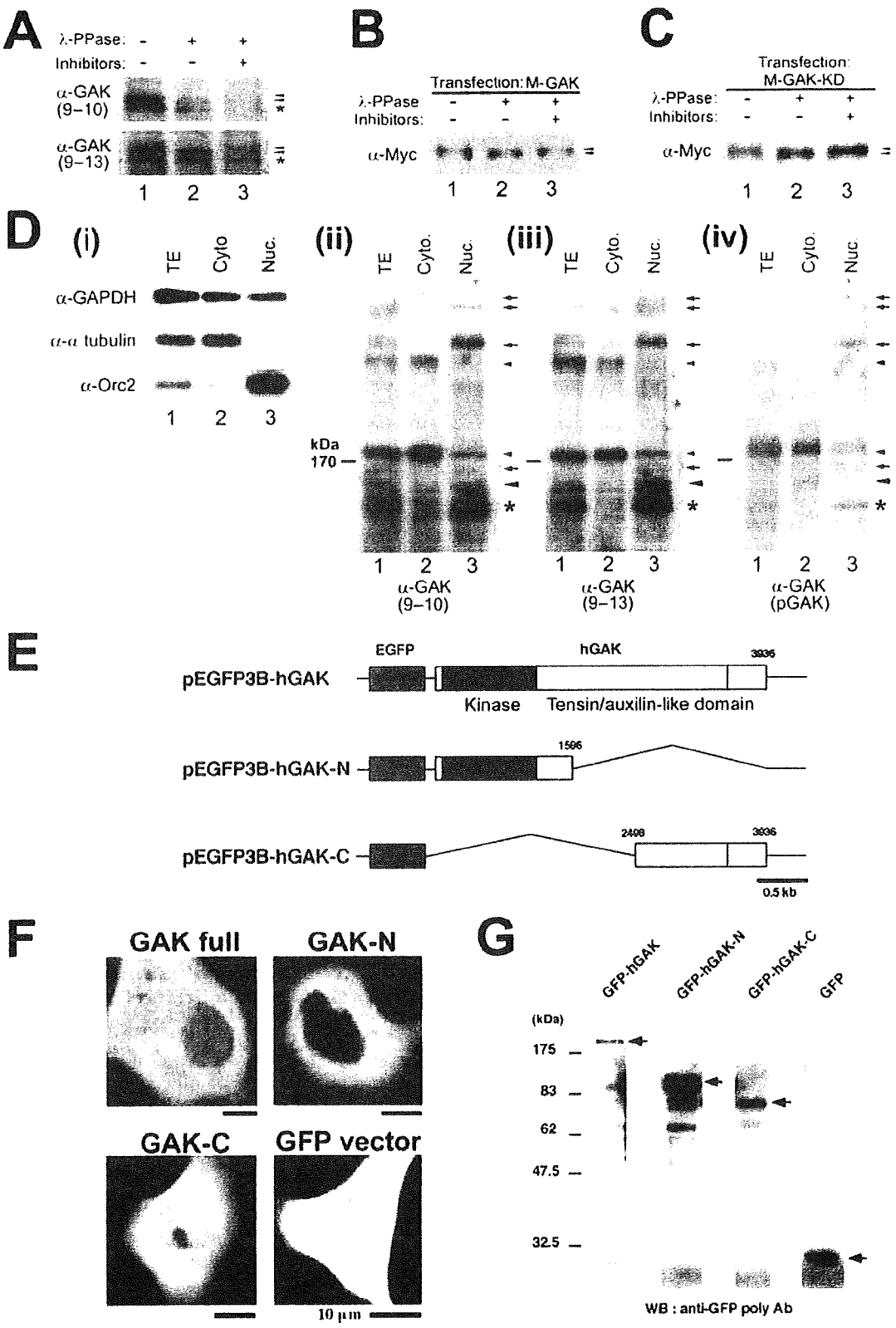

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