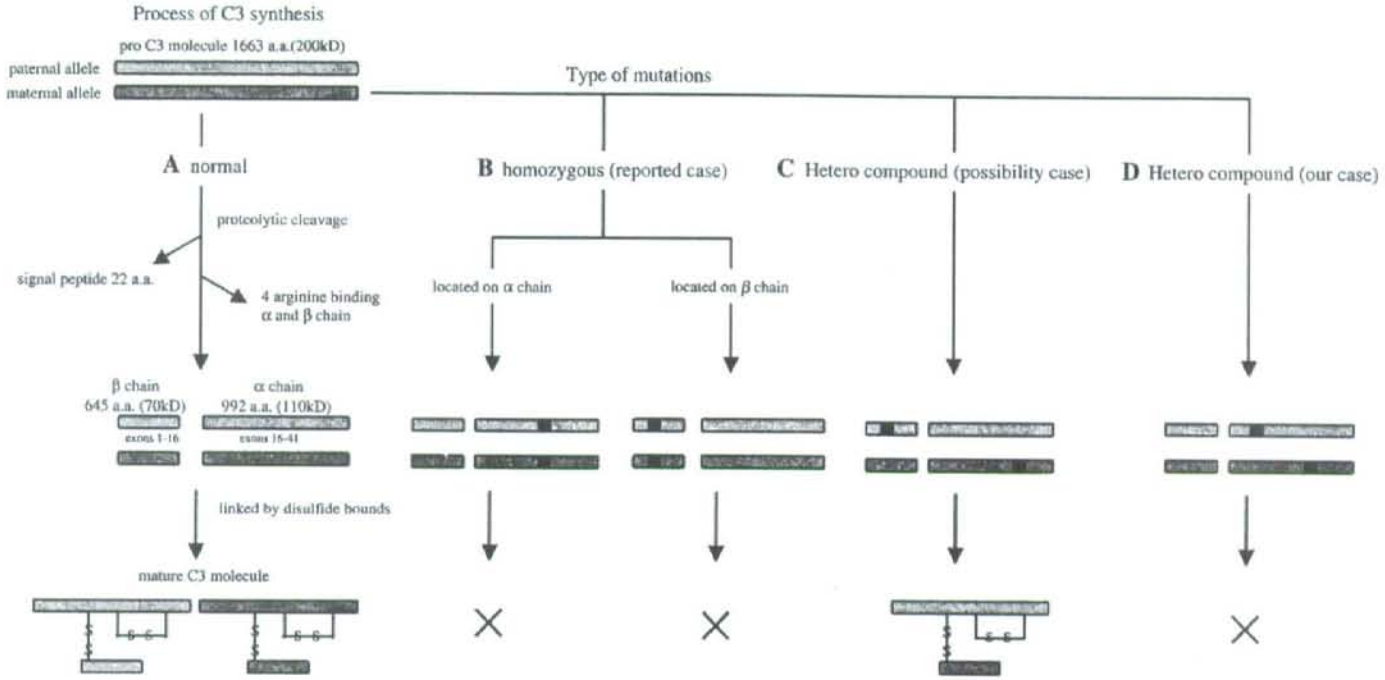

Fig. 2. The proposed hypothesis with regard to the mechanism of C3 deficiency based on the unique process of C3 synthesis. (A) Normal synthesis of the mature C3 molecule. (B–D) Various combinations of the *C3* gene mutations. (B) Homozygous mutations in the *C3* gene. No functional mature C3 molecule can be produced because both mutations ruin the same chains. (C) Hypothetical case: in some combinations of mutations in the different allele, a mature C3 molecule could be produced from functional α and β chains from the different allele. (D) Our case with compound heterozygous *C3* gene mutations. In both the mutations located in the same C3 α chain, no functional C3 molecule can be produced.

in a frameshift and a premature downstream stop codon (K1105X) in exon 26, and a nonsense mutation of C3303G (Y1081X) in exon 26, which was previously reported as homozygous mutations elsewhere [7]. These defects were not found in any unaffected family members or in 100 healthy control individuals. Both patient's PCR fragments (exon 24 and exon 26) were ligated to pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA) and transformed INVαF′ (Invitrogen, Carlsbad, CA, USA) competent cells for confirming the mutant clone sequence. It is noted that both the mutations are predicted to result in a truncation of the α chain of the C3 molecule. This finding may be linked to the unique process of C3 synthesis. The *C3* mRNA is translated into a signal peptide precursor form of pro-C3 molecule from each allele (paternal and maternal alleles), and subsequently processed by proteolytic cleavage into the β chain (13 kb from exons 1–16) and the α chain (28 kb from exons 16–41) [10], which are cross-linked by disulfide bonds to make the mature C3 molecule (Fig. 2A). Almost all cases with C3 deficiency have been reported as homozygous mutations in the *C3* gene. No functional mature C3 molecule can be produced (Fig. 2B). The hypothesis: a possible case without C3 deficiency in spite of harboring compound heterozygous *C3* gene mutations. The functional α and β chains from the different allele can be recombined to avoid severe deficiency (Fig. 2C) with the exception of nonsense mutation in β chain, in this case neither the functional α nor β chains could be synthesized from one allele with nonsense mutation. Although our case is an exceptional one with compound heterozygous *C3* gene mutations, no functional C3 can be produced (Fig. 2D). Thus, although this is the first exceptional case with heterozygous *C3* gene mutations confirmed, it is still consistent with our hypothesis. Moreover, though there might be a patient having gene mutations such as Fig. 2C, he or she avoids severe deficiency for existence of the functional α and β chains, as a result it might not be detected as C3 deficiency. The reason why the C3 deficiency is an extremely rare disease and for the high ratio of homozygous mutation occurrence might be based on such backgrounds. Further studies, for more cases with C3 deficiency that have been characterized at the molecular level, will be needed to confirm this hypothesis.

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Cell adhesion markedly increases lucigenin-enhanced chemiluminescence of the phagocyte NADPH oxidase

Futoshi Kuribayashi[1,*], Satoru Tsuruta[2], Tsuyoshi Yamazaki[3], Hiroyuki Nunoi[4], Shinobu Imajoh-Ohmi[5], Shiro Kanegasaki[6] and Michio Nakamura[1]

[1]*Department of Host-Defense Biochemistry, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*
[2]*Division of Pediatrics, Shizuoka City Hospital, 10-93 Ohtemachi, Aoi-ku, Shizuoka 420-8630, Japan*
[3]*Department of Information and Communication Technology, School of High-Technology for Human Welfare, Tokai University, 317 Nishino, Numazu, Shizuoka 410-0395, Japan*
[4]*Department of Pediatrics, Faculty of Medicine, Miyazaki University, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[5]*Department of Basic Medical Sciences, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[6]*Central Lab, Effector Cell Institute, Inc., 4-7-7 Aobadai, Meguro-ku, Tokyo 153-0042, Japan*

Lucigenin-enhanced chemiluminescence (LECL) is widely used for the detection of reactive oxygen species released from various cells and mitochondria. However, the LECL response varies depending on cell species and assay conditions at least in part by unknown factors. Here we report that cell adhesion is an important factor for increasing LECL of tetradecanoylphorbol acetate (TPA)-stimulated human neutrophils. More than 90% LECL remained even after complete removal of the cell suspension 10 min after TPA stimulation, and ~22.5% of neutrophils were adhered to the reaction tube. These results indicate that LECL by an adhering neutrophil is ~45× higher than that by a non-adhering neutrophil. LECL by leukocyte adhesion deficiency neutrophils was one-fifth of that by normal neutrophils and completely disappeared when the cell suspension was removed, confirming that LECL depends highly on cell adhesion. The oxidase activity of adhering neutrophils measured after permeabilization with Renex 30 together with NADPH addition was similar to that of non-adhering neutrophils, indicating that lucigenin and cell adhesion do not enhance the oxidase activity. Based on these findings, we propose that a mixture of adhering and non-adhering neutrophils can be used for simultaneous screenings of adhering activity and the oxidase activity of neutrophils.

Introduction

Superoxide is produced by the phagocyte NADPH oxidase of peripheral neutrophils, eosinophils, monocytes, and B lymphocytes. When neutrophils phagocytose bacteria or are activated by various artificial stimulators such as tetradecanoylphorbol acetate (TPA) or the bacteria-derived peptide, formyl–methionyl–leucyl–phenylalanine (fMLP), they produce superoxide (Kobayashi *et al.* 1990, 1995; Kuribayashi *et al.* 1995; Minakami & Sumimoto 2006; Sakamoto *et al.* 2006). The significance of the oxidase in host defense is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD), whose phagocytes are genetically defective in the oxidase (Kuribayashi *et al.* 1995; Roos *et al.* 1996).

Neutrophils of patients with leukocyte adhesion deficiency (LAD), another neutrophil disorder caused by a genetic defect in CD18 (β2-integrin), have a normal amount of NADPH oxidase to produce superoxide (Nauseef *et al.* 1986). These leukocytes do not express LFA-1, CR3, and gp150/95 on the plasma membrane as CD18 is their common subunit (Springer *et al.* 1984; Graham *et al.* 1989; Arnaout 1990), and therefore, do not adhere to ligands such as ICAM-1/2 and to artificial adhesion surfaces such as plastics (Patarroyo *et al.* 1990; Nagahata *et al.* 1995).

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**Correspondence*: kurikuri@nagasaki-u.ac.jp

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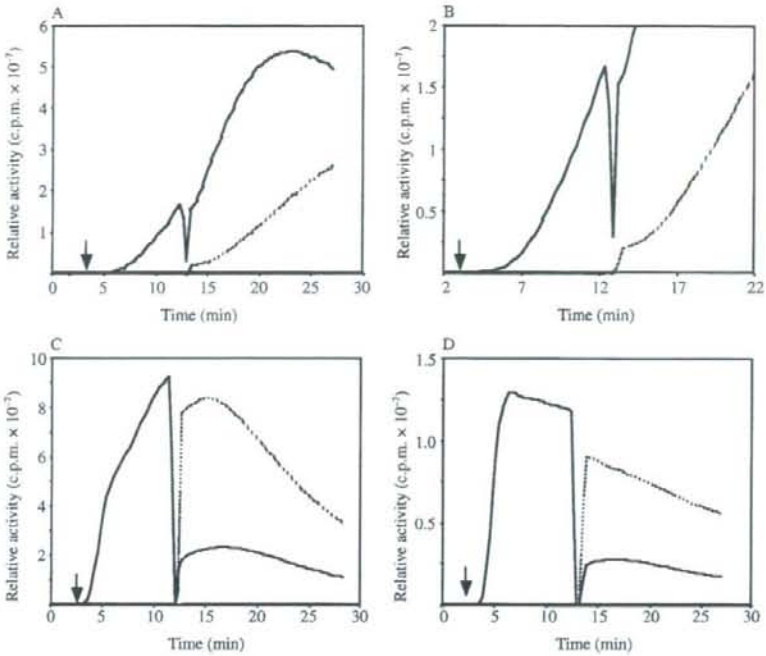

Figure 1 Effect of removal of suspension buffer from an assay tube on CL. Neutrophils (5.0×10^5) were pre-incubated in 0.5 mL reaction buffer containing lucigenin (A) and (B), luminol (C), or Diogenes (D) for 3 min before addition of TPA (200 ng/mL) (↓), and CL was monitored by LB9505C. Reaction buffer containing non-adhering neutrophils was removed from each tube at time point 13 min indicated as solid line, and transferred to a new tube indicated as dashed line. New buffer containing CL probe and TP+A was poured into former each tube, and the CL was further indicated as solid line. Note enlargement of (A) was indicated in (B) to clearly show the difference of LECL around time point 13 min.

Superoxide produced by neutrophils can be either detected by cytochrome *c* reduction, NBT reduction, or chemiluminescence (CL) responses (Baehner & Nathan 1968; Minkenberg & Ferber 1984; Kuribayashi *et al.* 2002) or calculated from cyanide-insensitive O_2 consumptions and compound III formation of peroxidases (Makino *et al.* 1986). Lucigenin-enhanced CL (LECL) is widely used because it is one of the most sensitive and convenient methods to detect reactive oxygen species (ROS) released from various cells and mitochondria (Minkenberg & Ferber 1984; Bhunia *et al.* 1997; Caldiz *et al.* 2007). It has been shown that LECL of TPA-stimulated neutrophils is abolished by superoxide dismutase (SOD) and is not seen with CGD neutrophils (Kuribayashi *et al.* 1995). However, the exact mechanism of the lucigenin response to superoxide is unknown.

In the present study, we demonstrate that LECL of neutrophils is highly increased by adhesion, and therefore, reflects both the phagocyte NADPH oxidase activity and adhesion efficiency. Thus, conventional LECL assay using a mixture of adhering and non-adhering cells can be adopted for screening disorders presenting at least one of two functions: adhesion and the oxidase activity.

Results

LECL is highly dependent on adhering cells

Neutrophils were suspended in CL probe-containing assay buffers, poured into plastic tubes, and stimulated by TPA 3 min later. The LECL of the tube was still more than 90% of the original activity after the cell suspension was removed by pipetting 10 min after TPA stimulation, and steeply increased for an additional 10 min reaching 4× high LECL in a fresh medium (Fig. 1A, solid line). Most of the disappeared LECL, ~10%, was recovered

Table 1 Cell numbers of neutrophil suspensions 10 min after TPA stimulation

CL probe	10 min
–	76.4 ± 4.3
Luminol	81.8 ± 6.1
Diogenes	76.0 ± 5.4
Lucigenin	77.5 ± 7.3

Each value is the mean ± SD obtained from five assays where the number of cells represents 100 at time point 0 min of TPA addition.

Table 2 Relative CL of an adhering neutrophil compared with a non-adhering cell defined as 1

Luminol	1.04 ± 0.42
Diogenes	0.97 ± 0.18
Lucigenin	45.1 ± 17.1

Each value is the mean ± SD of (c.p.m. for adhering cells/part of adhering cells in total cell number) divided by (c.p.m. for non-adhering cells/part of non-adhering cells in total cell number) obtained from five assays. Thus, activities of a non-adhering cell were, therefore, independently assumed to be 1 in individual conditions.

in a new tube containing transferred cell suspension (Fig. 1A,B, dashed line). Neither the remaining high CL nor its steep increase was observed in the initial tube when the CL probe was luminol (Fig. 1C), Diogenes (Fig. 1D), or Cypridina luciferin analog (CLA) (data not shown). Neutrophils remaining in the initial tube were microscopically observed to be adhering to the surface of the tube (data not shown). Accordingly, the high LECL remaining in the initial tube suggested that lucigenin in particular, increased either the number of adhering cells or the phagocyte NADPH oxidase activity of the adhering cells.

Lucigenin dose not increase adhering neutrophils

To clarify the adhesion efficiency by lucigenin, we counted the number of cells removed by pipetting (Table 1). The removed percentage of the cells in the presence of lucigenin (77.5%) was the same as that in the absence of lucigenin or in the presence of other CL probes. Therefore, the high LECL remaining in the initial tube was not due to the increased population of adhering cells but to the adhesion-specific increase of LECL. The ratio of the TPA-stimulated LECL of an adhering cell to that of a non-adhering cell was 45.1 at the time of removal of non-adhering cells, which was significantly higher when compared with ~1 for other two CL probes (Table 2). This ratio reached at least 100× at 20 min after TPA addition (Fig. 1A, solid line).

Adhesion is essential for LECL

To ascertain the importance of adhesion for high LECL, two series of experiments were designed. We artificially increased adhering cell numbers by increasing the surface area for adhesion in one experiment (Fig. 2), and decreased the numbers of adhering cells using non-adhesive

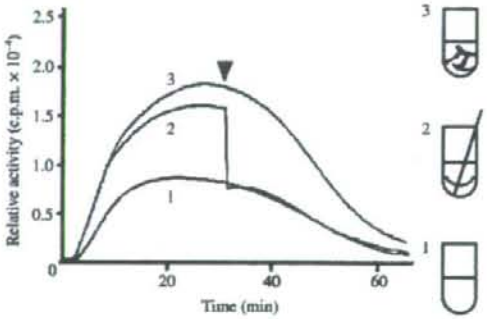

Figure 2 Effect of adherent area on CL. A suspension of neutrophils (3.6×10^5) with lucigenin stimulated by TPA was divided equally into three tubes, and CL was monitored by LB9505. Superoxide production by the original tube was detected in tube 1. To provide additional surface area for neutrophil adhesion, tube 2 contained another bottom which was removed at the indicated time (▼). Tube 3 contained broken fragments made of an identical tube.

neutrophils derived from a CD18-deficient LAD patient (Fig. 3) in the other. The surface area of a tube for adhesion was increased by providing either another piece of tube bottom (Fig. 2, tube 2) or the fragments of a cracked tube (tube 3). LECL of tube 2 was twice that of tube 1 with no plastic pieces, despite of the same numbers of cells in the two tubes. The highest LECL of neutrophils among the three tubes was observed in tube 3 (Fig. 2, curve 3).

In contrast, when adhering neutrophils were absent as shown by LAD cells (Fig. 3A (LAD) and C), the LECL of LAD neutrophils at maximal intensity was approximately one-fifth of that of normal neutrophils (Fig. 3A), and completely disappeared from the original assay tube after transferring the cell suspension to a second new one

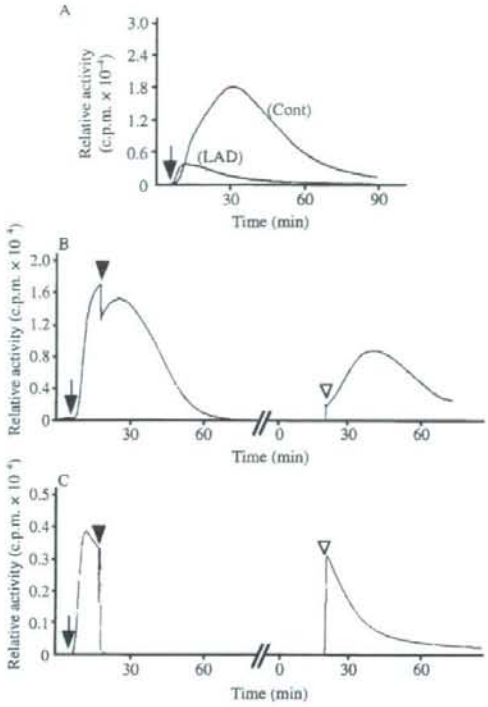

Figure 3 Comparison of LECL by control or LAD neutrophils. (A) Normal (Cont) or LAD neutrophils (2.0×10^5) were stimulated by TPA at the indicated time (▼), and CL was monitored by LB9505. Normal (B) or LAD (C) neutrophils were stimulated by TPA (▼). Neutrophils in suspension were removed from the original tube at the indicated time (▼) in the left panels and placed into another tube (▽) in the right panels. Note that the vertical scales are different in (B) and (C).

(Fig. 3C, left). Essentially the same results were observed when normal neutrophils were incubated in non-adhesive siliconized tubes (data not shown). The specific LECL activity of a normal TPA-stimulated non-adhering neutrophil of the second tube was 1.3×10^{-2} c.p.m./cell (Fig. 3B, position indicated by open arrowhead), which was comparable with the corresponding specific activity of LAD cells (1.5×10^{-2}, Fig. 3C, open arrowhead). These data imply that non-adhering normal neutrophils and non-adhesive LAD neutrophils exhibit similar LECL, which is consistent with the observations that TPA-stimulated LAD neutrophils consumed and released similar amounts of oxygen molecules and superoxide anions, respectively, as normal neutrophils (Table 3).

Table 3 O_2 consumption and cytochrome *c* reduction by TPA-stimulated normal and LAD neutrophils

	O_2 consumption	Cytochrome *c* reduction
Control	4.5 ± 0.3	4.9 ± 0.4
LAD	4.4 ± 0.1	6.0 ± 0.6

Each value (nmol/min/10^6 neutrophils) is the mean ± SD obtained from three assays.

Lucigenin does not increase the NADPH oxidase activity of adhering neutrophils

The TPA-stimulated phagocyte NADPH oxidase would not commonly be activated by adhesion as adhering cells exhibited no increased CL response when luminol, Diogenes, or CLA was used as a CL probe (Fig. 1 and data not shown). Therefore, we examined whether lucigenin in particular increased the NADPH oxidase activity of adhering neutrophils by assaying the LECL of TPA-stimulated neutrophils completely dependent on their oxidase activity.

Before the assay of the oxidase activity expressed by LECL, neutrophils were permeabilized by Renex 30 to remove NADPH, the electron-donating substrate of the oxidase, and NADPH was then supplemented exogenously (Fig. 4A, no. 1). The LECL which appeared after NADPH addition was exclusively dependent on the phagocyte NADPH oxidase as no significant CL was observed when neutrophils had not been stimulated by TPA (Fig. 4A, no. 3) nor when NADH was used as an electron-donating substrate (Fig. 4A, no 2). Furthermore, neutrophils of a CGD patient exhibited no TPA- and NADPH-dependent LECL (Fig. 4A, no. 4). This NADPH oxidase activity was proportional to the number of neutrophils applied (Fig. 4B). The high LECL of adhering cells was followed by a low phagocyte NADPH oxidase activity (Fig. 4C, upper panel). In contrast, the low LECL observed in stimulated non-adhering neutrophils was followed by a high oxidase activity. These high and low NADPH oxidase activities were 7.0×10^6 and 1.9×10^6 c.p.m., respectively, and corresponded to the high (77.5%) and low (22.5%) populations of non-adhering and adhering neutrophils (Table 1), respectively. The actual specific NADPH oxidase activities of adhering and non-adhering cells were 21.5 ± 2.0 c.p.m./cell ($n = 3$) and 24.8 ± 5.4 c.p.m./cell ($n = 3$), respectively. Furthermore, the phagocyte oxidase activities reflected by LECL were consistent regardless of whether lucigenin was added before or after the cells were permeabilized by Renex 30

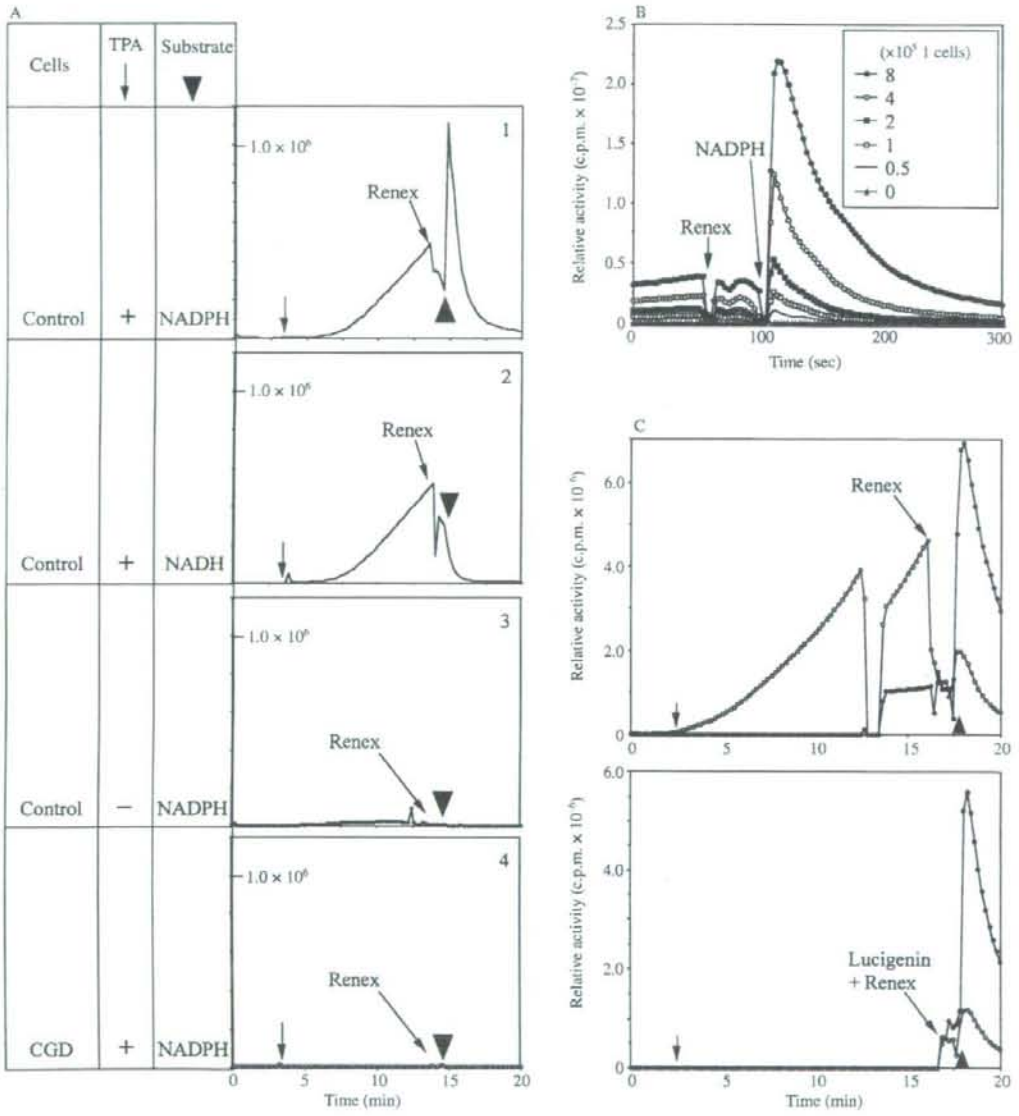

Figure 4 NADPH oxidase activity assay for adhering and non-adhering neutrophils. (A) Control neutrophils (5.0×10^4) or CGD ones (no. 4) were pre-incubated in 0.5 mL reaction buffer for 3 min before addition of TPA (↓) or buffer (no. 3), and CL was monitored by LB9505C at 26 °C. Renex 30 was added at time point 13 min followed by NADPH (150 μM) or NADH (no. 2) addition. (B) Effect of cell numbers on LECL. Renex 30 was added 10 min after TPA stimulation, and LECL after NADPH addition was monitored. Note enlargement of time scale was indicated to clearly show the oxidase activity after NADPH addition. (C) Comparison of oxidase activity between adhering and non-adhering cells. Neutrophils (5.0×10^5) were stimulated by TPA (▼) at time point 3 min, and LECL was monitored. The reaction buffer containing non-adhering neutrophils was removed from the original tube at time point 13 min indicated as open circle and transferred to a new tube indicated as closed circle. New buffer containing CL probe and TPA was poured into the original tube in upper panel. Renex 30 (▼) (together with lucigenin in the lower panel) and NADPH (▲) was added into both tubes.

(Fig. 4C, lower panel). Accordingly, lucigenin did not augment the oxidase activity of adhering cells in particular. These data clearly indicated that the oxidase activity of neutrophils was not increased by either adhesion or by lucigenin. Therefore, increased LECL of adhering neutrophils is independent of the oxidase activity *per se*, but dependent on a certain chemical process occurring under CD-18-dependent adhering situation.

Discussion

Although lucigenin is widely used to detect ROS derived from NADPH oxidase and mitochondrial membranes, the exact mechanism underlying the responses of lucigenin to ROS remained elusive. In the present study, we have shown that the LECL of the phagocyte NADPH oxidase is largely dependent on cell adhesion. When the cell suspension with luminol, Diogenes or CLA as a CL probe is removed, the CL activity is mostly disappeared as expected (Figs. 1B,C and 3C). However, LECL continues after the reaction buffer has been removed (Fig. 1A). Reaction tubes possessing larger surface areas have higher LECLs (Fig. 2), confirming that the LECL of the phagocyte NADPH oxidase is highly increased by adhesion.

The present study shows that the LECL derived from adhering neutrophils is augmented. At first, there was a possibility that LECL from cell suspension is deeply reduced through assay buffer, and with the result that LECL from adhering neutrophils becomes higher relatively. If so, artificial surroundings that LECL from adhering cells reaches photon detector through the buffer must attenuate the CL. As shown in curve 2 in Fig. 2, LECL released from cells adhering to another bottom sank in suspension buffer is as high as that to the original one, suggesting that the CL from the additional bottom reaches the photon detector through the suspension buffer without any quenching. Thus, CL generated by neutrophils in suspension has not been attenuated by an absorption because of the suspension buffer and/or cells themselves, but the LECL is augmented by the adhesion of cells. Furthermore, most of the LECL by neutrophils stimulated with fMLP remains after the cell suspension is removed (data not shown), suggesting that lucigenin together with adhesion has a specific mechanism to elevate CL regardless of the stimuli.

In this report, we have shown that lucigenin itself has a unique mechanism to enhance CL generated by adhering cells. Microenvironments enclosed by tube, cell membranes containing CD18 dependent adhesive molecules, and some secreted proteins might be important for LECL by superoxide. From our previous report (Kuribayashi *et al.* 1995) and our unpublished data, eosinophils, but not B lymphocytes, behave in the same manner as neutrophils, suggesting that some proteins derived from granules commonly shared by both neutrophils and eosinophils are necessary to enhance LECL, or the ability of B lymphocytes to adhere to the tube is weak as seen with LAD neutrophils.

The exact mechanism by which lucigenin augments CL by adhering cells has been remained unknown even after several experiments. We considered two major possibilities: One was attributed to the uneven distribution of the primary products (pP) and logarithmic interaction among them, assuming LECL to be reflecting the forming velocity of the final products (fP) giving an equation ($v = d[fP]/dt = k[pP]^m$). The other was attributed to interactions of lucigenin intermediates catalyzed by lysosomal enzymes working in strict areas observable only in adherent neutrophils. Concerning to the former possibility, xanthine–xanthine oxidase system gave at most only 2 for m, which is far less than the value observed ($> 32 = 2^5$, namely $m > 5$). Concerning to the latter possibility, we used cyanide, azide, SOD, and mannitol as inhibitors, but failed to observe any sufficiently different effects of them between adherent and non-adherent neutrophils. Even if the precise mechanism of the dramatic increase in LECL of the phagocyte NADPH oxidase is in a black box, it can be noted that our present results have demonstrated an example exhibiting the same amount of energy source, here NADPH, can emit almost two order-high light under certain conditions.

To use the LECL assay for accurate evaluation of the phagocyte NADPH oxidase activity, the assay should be performed under the conditions where either adhesion is complete or adhesion is completely blocked. However, a conventional LECL assay using a mixture of adhering and non-adhering cells can be adopted for screening disorders such as disability of adhesion known as LAD and NADPH oxidase as CGD.

Experimental procedures

Reagents

Lucigenin (bis-*N*-methylacridinium nitrate), luminol, cytochrome *c*, SOD, fMLP and TPA were purchased from Sigma. Lucigenin obtained from Boehringer Ingelheim was also examined to confirm that from Sigma. CLA was obtained from Tokyo Kasei Kogyo (Japan). Diogenes was obtained from National Diagnostics (USA).

Cell preparation

Peripheral blood was obtained from healthy volunteers, a CGD, and an LAD patient after informed consent was obtained.

Neutrophils were purified from peripheral blood as previously described (Yamauchi *et al.* 2001). Briefly, the cells were isolated by a combination of dextran sedimentation, Ficoll-Conray gradient centrifugation, and hypotonic treatment, and suspended to 10^7 cells/mL in HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 17 mM HEPES pH 7.4).

CL assay

The signal of ROS was made chemiluminescent by one of the following CL probes: lucigenin (100 μM), Diogenes (1/100× dilution from stock solution according to the manufacturer's protocol), luminol (1 mM), or CLA (1 μM). For typical measurements of LECL, 5×10^5 neutrophils were incubated in 0.5 mL of HEPES-buffered saline including BSA (0.03%) and lucigenin for 3 min before TPA (200 ng/mL) addition, and CL was monitored for 30 min by an automatic luminescence analyzer LB9505 or LB9505C (Berthold, Japan). Other LECL protocols were described in figure legends.

Cytochrome *c* reduction and O_2 consumption assays

Superoxide production by neutrophils was determined by a SOD-sensitive cytochrome *c* reduction (Kuribayashi *et al.* 2002). Typically, 1.0×10^6 cells were suspended in 1 mL of HEPES-buffered saline containing cytochrome *c* (75 μM), then pre-incubated at 37 °C for 3 min before the addition of TPA. The reduction of cytochrome *c* was measured using a UV-3000 (SHIMADZU, Japan), and superoxide production was calculated from the absorbance difference at 550–540 nm using an absorption coefficient of 21 000 M^{-1} cm^{-1}. The reduction was halted by the addition of SOD (0.1 mg/mL) to confirm the reduction to be done by superoxide. Oxygen-consuming activity was determined potentiometrically using a Clark-type electrode as described previously (Makino *et al.* 1986).

Counting neutrophils in suspension

Neutrophils (5×10^5 cells in the same tube with CL assay) were incubated in HEPES-buffered saline with or without CL probes for 3 min before TPA addition. When TPA was added to the reaction buffer, the cell number was first counted by light microscopy (PRIMO STAR, Carl Zeiss, Japan) to confirm that the numbers were not significantly different from the original ones. The suspended cells were again counted 10 min later, and the ratio of the cell numbers before and after TPA stimulation was calculated.

NADPH oxidase activity assay

This assay was performed as previously detailed with some modifications (Nakamura *et al.* 1981; Shiibashi & Iida 2001). Briefly, 10 min after TPA stimulation with lucigenin, the neutrophils were permeabilized by Renex 30 (0.015%), and the subsequent oxidase activity with NADPH (150 μM) was monitored at 26 °C.

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Case report

Segawa disease with a novel heterozygous mutation in exon 5 of the GCH-1 gene (E183K)

T. Ikeda [a,*], K. Kanmura [a], Y. Kodama [a], K. Sawada [a], H. Nunoi [a], K. Hasegawa [b]

[a] *Division of Pediatrics, Department of Reproductive and Developmental Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake-Chou, Miyazaki-Gun, Miyazaki 889-1692, Japan*

[b] *Department of Neurology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan*

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Abstract

We report a novel missense mutation in the GCH-1 gene resulting in Segawa disease. The patient, a 6-year-old girl, presented with dystonia. Her CSF biopterin and neopterin levels were reduced, suggesting Segawa disease. L-dopa administration led to clinical improvement. Genetic analysis revealed a missense mutation in exon 5 of the GCH-1 gene (E183K). Although dystonia or other movement disorders were not identified in her family, this may be explained by the low penetrance of Segawa disease.

Keywords: Segawa disease; Hereditary progressive dystonia; GTP Cyclohydrolase I; Missense mutation; Exon 5

1. Introduction

Segawa disease is known as a dopamine responsive disorder characterized by dystonia [1]. It is caused by GTP cyclohydrolase-1 (GCH-1) deficiency, a result of mutations in the GCH-1 gene [2].

We report a case of Segawa disease with a novel heterozygous missense mutation.

2. Case report

This 6-year-old girl was born of a normal pregnancy and delivery. Her subsequent psychomotor development was normal. From the age of 5, her parents noticed disturbance in her gait. Diurnal fluctuation of symptoms was observed.

On examination at age 6, her development was normal with a developmental quotient of 109 (Tumori-Inage developmental evaluation). She exhibited mild dystonic postures of the lower limbs. Dystonia emerged in the afternoon, increased with fatigue and disappeared with rest and during sleep. No other involuntary movements were found. Her deep tendon reflexes were exaggerated although without ankle clonus, and her Babinski signs were absent. Her height was within the standard range at 109.8 cm (−0.57 SD) with no stagnation of height. She was 104 cm (−0.56 SD) one year before and 98.4 cm (−0.31 SD) two years before. There was asymmetric diversity of the body.

Brain MRI, blood copper, plasma ceruloplasmin, blood amino acids, and urine organic acids were all normal. CSF pterin analysis showed low biopterin (9.7 pmol/ml; control values 25 ± 5.0 pmol/ml) and low neopterin (18.8 pmol/ml; control values 25 ± 5.0 pmol/ml).

We suspected Segawa disease. Administration of plain L-dopa (20 mg/kg/day) resulted in a prompt improvement of dystonia, and confirmed our diagnosis.

2.1. Family pedigree and genetic analysis

No dystonia or other involuntary movements were identified in her family (Fig. 1). After obtaining an

* Corresponding author. Fax: +81 985 85 2403.
E-mail address: tikeda@fc.miyazaki-u.ac.jp (T. Ikeda).

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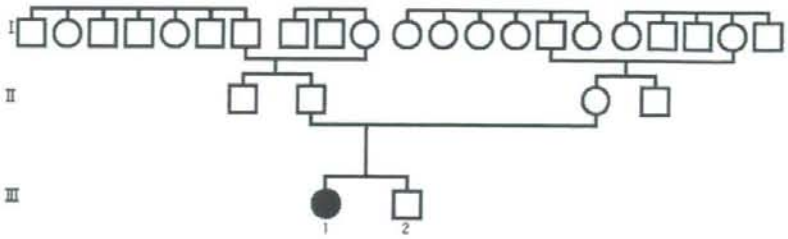

Fig. 1. Family pedigree. Black circles and squares indicate affected members.

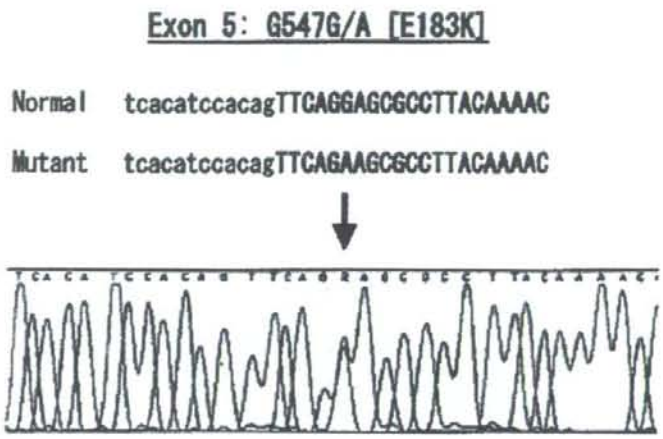

Fig. 2. Sequence analysis of GCH-1 exon 5 showing the heterozygote G547G/A missense mutation.

informed consent, we performed genetic analysis of her genomic DNA extracted from leukocytes. We analyzed exon 1–6 of the GCH-1 gene by PCR-direct sequencing according to methods previously described [2].

A heterozygote missense mutation in exon 5 (G547G/A) was discovered, resulting in E183K substitution (Fig. 2).

3. Discussion

Dopamine is synthesized by tyrosine, and tyrosine hydrolase acts as the rate limiting step. Tetrahydrobiopterin is required as a cofactor in the synthesis [1,3]. Heterozygous abnormalities in the GCH-1 gene cause a partial decrease in GCH-1, which in turn results in partial decrease in BH4. This leads to the appearance of dystonia due to low levels of dopamine [1].

Dissimilar GCH-1 mutations showed similar clinical features [4,5]. All patients of this disorder have a heterozygous mutation except for compound heterozygotes. The heterozygotic gene abnormality induces partial decrement of tetrahydrobiopterin (BH4) and affects synthesis of tyrosine hydroxylase (TH) rather selectively [5,6]. TH reduction occurs at the terminal of the nigrostriatal (NS) dopamine (DA) neurons, predominantly in the ventral area of the striatum, and hinders the D1 receptor-striatal direct pathway [6]. This consequently suppresses the inhibitory efferent pathways and develops postural dystonia via the particular descending pathways to the reticulospinal tract and postural tremor is caused by deficiency of the DA neuron innervating to the subthalamic nuclei and through the ascending pathways to the ventrolateral nucleus of the thalamus [1].

In Segawa disease the locus of mutation of GCH-1 gene differs among families but is identical in a single family. More than 80 mutations in the GCH-1 gene have been collected in the BIOMDB database [7].

Our missense mutation in exon 5 of the GCH-1 gene (E183K) has not been reported as of yet [5,7].

Segawa disease is characterized by age dependent appearance of particular symptoms, and in early childhood, as in our case, no other sign of movement disorder other than postural dystonia is seen [1]. Our patient shows diurnal fluctuation of dystonia which responded well to dopamine. The GCH-1 genes of her

parents have not been examined, and no signs of neurologic dysfunction were evident in her family. Although her parents may not share her mutation, there is also the possibility of low penetrance in Segawa disease [3]. Most patients with Segawa disease show stagnation of body length, caused by decrease of dopamine in the tuberoinfundibular neuron with D4 receptors. Though stagnation of the body length was absent, growth velocity was reduced in this case. Short stature would have resulted without treatment.

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INTERNAL MEDICINE

□ CASE REPORT □

Adult Onset X-Linked Chronic Granulomatous Disease in a Woman Patient Caused by a *de novo* Mutation in Paternal-Origin *CYBB* Gene and Skewed Inactivation of Normal Maternal X Chromosome

Takahisa Gono[1], Masahide Yazaki[1], Kazunaga Agematsu[2], Masayuki Matsuda[1], Kozo Yasui[2], Maki Yamaura[3], Fumio Hidaka[4], Tomoyuki Mizukami[4], Hiroyuki Nunoi[4], Takeo Kubota[5] and Shu-ichi Ikeda[1]

Abstract

We report a 28-year-old woman patient suffering from refractory subcutaneous abscess. Stimuli-induced microbicidal reactive oxygen metabolites formation test of the patient's neutrophils revealed that only 9.6% of the neutrophils produced H_2O_2. DNA analysis of the *CYBB* that encodes gp91^{phox} demonstrated that she was heterozygous for a nonsense mutation, ^{326}Trp(TGG)/stop(TGA) and therefore, a diagnosis of adult onset X-linked chronic granulomatous disease was made. Our molecular biological study revealed that her disease was caused by a *de novo* mutation in the *CYBB* gene on the paternal-origin X-chromosome and a skewed inactivation of the normal maternal X-chromosome.

Key words: chronic granulomatous disease, gp91^{phox}, *CYBB*, X-chromosome

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Introduction

Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency disorder, caused by a complete lack of or significant decrease in the production of microbicidal reactive oxygen metabolites (ROM) due to a defective phagocytic NADPH-oxidase (1). Approximately 70% of CGD patients have X-linked CGD caused by mutation of the *CYBB* gene that encodes gp91^{phox}. For many years the onset of X-linked CGD was thought to occur early in infancy in man patients, with a fatal outcome in adolescence due to recurrent severe bacterial or fungal infections. However, late onset cases of X-linked CGD have been recently reported in some adult woman (2-4). While it has been assumed that the late onset of woman X-linked CGD could be associated with age-related skewing of lyonization (2), the detailed mechanism of onset in adult woman patients remains obscure. Here, we report a woman patient with adult onset X-linked CGD caused by a *de novo* mutation in the paternal-origin *CYBB* gene and skewed inactivation of normal maternal X chromosome.

Case Report

A Japanese woman first noticed recurrent stomatitis and acne on her face and trunk at the age of 21. She visited our hospital, and mild hypergammaglobulinemia (IgG: 2,463 mg/dl, normal: 800 to 2,000 mg/dl) was found. Collagen disorders, such as Behçet disease and systemic lupus erythematosus (SLE), were thought to be the differential diagnoses at that time, but a definite diagnosis was not established. At

[1]Department of Medicine (Neurology and Rheumatology), Shinshu University School of Medicine, Matsumoto, [2]Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, [3]Department of Dermatology, Shinshu University School of Medicine, Matsumoto, [4]Department of Pediatrics, Miyazaki Medical College, University of Miyazaki, Miyazaki and [5]Department of Epigenetic Medicine, Faculty of Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo
Received for publication January 15, 2008; Accepted for publication March 5, 2008
Correspondence to Dr. Masahide Yazaki, mayazaki@hsp.md.shinshu-u.ac.jp

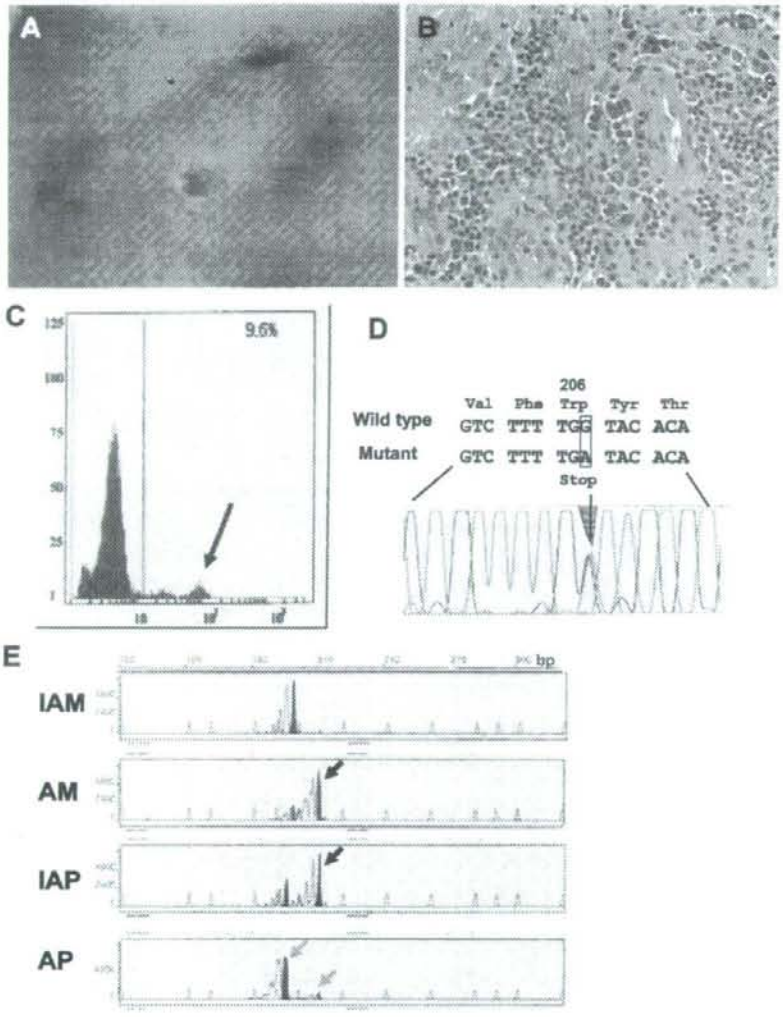

Figure 1. A: Subcutaneous ulcerative abscess in the hip (A). B: Histopathology of the subcutaneous hip abscess showing granulomatous formation and severe infiltration of plasma cells and lymphocytes. Hematoxylin and Eosin staining. C: Histogram plots of H_2O_2 production of neutrophils after stimulation with phorbol-myristate-acetate (PMA) demonstrating that only 9.6% of the neutrophils can produce H_2O_2 (arrow). D: Nucleotide sequence of exon 6 of gp91^{phox} showing that the patient is heterozygous for a nonsense mutation, 618G → A (arrow), creating a stop codon (TGA) at residue 206 instead of Trp (TGG). E. Methylation-specific PCR assay in the HUMARA locus. IAM: Inactivated X-chromosome of the patient's mother. AM: Activated X-chromosome of the patient's mother. IAP: Inactivated X-chromosome of the patient. AP: Activated X-chromosome of the patient. The maternal allele was inactivated in the patient (IAP), but activated in the patient's mother (AM) as indicated by black arrows at 210-bp. The ratio of the paternal active X to the maternal active X was skewed at 93:7 (AP, gray arrows denote the paternal active X-chromosome at 195-bp and the maternal active X-chromosome at 210-bp).

the age of 27, she suffered from refractory subcutaneous ulcerative abscess in the left inguinal region and hip (Fig. 1A). Despite therapy including oral antibiotics and draining, the abscess still persisted 10 months after onset. Skin biopsy was performed at the hip abscess and the histopathology revealed granulomatous formation and severe infiltration of plasma cells and lymphocytes, indicating the presence of chronic inflammation (Fig. 1B). A small amount

of *Pseudomonas aeruginosa* was cultured in the biopsy specimen but fungus was not detected. Acid-fast bacteria were not detected in Ziel-Neelsen staining and culture of the tissue. She was thought to have impairment of the immune system and was therefore admitted to our hospital for further examination in September 2004 at the age of 28. Physical examination showed no abnormal findings except for the subcutaneous abscess. Laboratory data demonstrated normal white blood cell count (5,850/μl) and neutrophil count (3,040/μl). Serum CRP (1.50 mg/dl, normal: <0.1 mg/dl) and CH50 (74.8 IU/ml, normal: 30 to 53 IU/ml) were elevated. Serum IgG was also high (2,850 mg/dl, normal: 680-1,620 mg/dl) but IgM, IgA, and IgD were within the normal limits. Monoclonal proteins were not detected in the serum or urine. Anti-nuclear antibody was negative and angiotensin-converting enzyme (ACE) was within the normal range. Serum anti-HIV antibody and *Treponema pallidum* hemagglutination (TPHA) were negative. Stimuli-induced ROM formation test of the patient's neutrophils with dihydrorhodamine 123 revealed that most of the patient's neutrophils (90.4%) were unable to produce ROMs after maximal stimulation with phorbol-myristate-acetate and only 9.6% of the neutrophils produced H_2O_2 (Fig. 1C). Nitroblue-tetrazolium (NBT) slide test demonstrated about 10% positive cells (data not shown). Thus, she was thought to have adult onset X-linked CGD. There were no patients with CGD in her family.

The patient had been treated with intravenous administration of sodium ampicillin/sodium sulbactam (6 g/day) for two weeks from admission and draining, and subsequently oral antibiotics were given for two weeks. The subcutaneous abscess gradually healed and she was discharged in late October 2004.

Subsequently, a prophylactic therapy with sulphamethoxazole-trimethoprim (480 mg/day) was begun and thus far, she has not suffered from severe infectious disease.

Materials and Methods

DNA sequencing of CYBB

After informed consent was obtained from the patient, DNA was purified from peripheral leukocytes. Intronic primer pairs flanking each of the 13 exons of *CYBB* gene were used to amplify each exon (5) and all exons of *CYBB* gene were analyzed by direct DNA sequencing. In addition, genomic DNA was also extracted from a buccal swab and direct DNA sequencing of exon 6 of *CYBB* gene was carried out.

Detection of X-chromosome inactivation at the HUMARA locus

After informed consent was obtained from the patient and her mother, DNA was purified from neutrophils. Using the assay of human androgen receptor (HUMARA) locus involving a methylation-specific polymerase chain reaction (M-PCR) technique (6), an X-inactivation pattern based on the ratio of the maternal inactive X to the paternal inactive X was evaluated in the neutrophils of the patient and her mother. Another X-inactivation pattern based on the ratio of the maternal active X to the paternal active X using specific primers for the unmethylated allele was also evaluated.

Results

The direct DNA sequencing of *CYBB* gene in both the leukocytes and the buccal epithelial cells revealed that our patient was heterozygous for a nonsense mutation, 618 G→A in exon 6, ^{206}Trp(TGG)→stop(TGA) (Fig. 1D), showing that she was a carrier of *CYBB* gene mutation.

The assay in the HUMARA locus revealed that the maternal allele was inactivated in the patient, but activated in the patient's mother (Fig. 1E). The paternal allele was activated in the patient (Fig. 1E). The ratio of the paternal active X to the maternal active X was skewed at 93:7 (Fig. 1E). This lyonization ratio corresponded well to the observed phenotype of only 7-9% of normal neutrophils in the patient.

Discussion

Based on the results of the DNA sequence analysis, a diagnosis of adult onset X-linked chronic granulomatous disease was made. The assay of HUMARA locus (6) strongly suggested that the paternal allele harbored the mutant *CYBB* gene. Unfortunately DNA analysis could not be performed on her father since he had already died in an accident at the age of 60. However, because he had been healthy until he died, he would not have been a CGD patient. Hence, a *de novo* mutation must have arisen on the paternal allele of the *CYBB* gene in the paternal germ line. As the maternal normal allele was gradually skewed, the neutrophils with normal function decreased and the patient developed a subcutaneous abscess as a symptom of CGD.

There have been many woman carriers of X-linked CGD to date and most of them have a balanced X inactivation ratio and are commonly healthy. However, some woman carriers with an X inactivation ratio of normal cells between 3 and 30% occasionally suffer from symptoms such as discoid lupus (DLE) photosensitivity of the skin, or aphthous stomatitis (7-9). Only massive exposure to bacteria or fungi can cause severe and life-threatening infection if the percentage of oxidase-positive phagocytes is approximately 5-10% (2). Carriers with less than approximately 3-5% are more at risk for severe infection (3). Skewing of X-inactivation as a cause of the respective diseases in women has been reported in other X-linked recessive disorders (10). To date, the presence of adult onset X-linked CGD has been reported in only 3 woman patients (2-4). The precise mechanism by which the disease occurs in adults remains unclear but a positive correlation was observed between age and degree of skewing in X-inactivation (11). Indeed, age-related skewing was revealed in one adult woman CGD patient (2) and therefore,

the late onset in woman CGD carriers including the present patient could be explained by this age-related skewing of lyonization.

Although a *de novo* mutation of the *CYBB* gene was revealed in two patients (4, 12), in the patient described by Anderson-Cohen et al (12) the patient's parents had no mutation in the *CYBB* gene. In that patient, the maternal allele of the *CYBB* gene had a nonsense mutation, leading to a premature stop codon (12). The normal paternal allele was inactivated by skewed lyonization not only in the patient's leukocytes, but also in the buccal epithelial cells. These results indicated that a *de novo* mutation had occurred on the maternal allele and skewed lyonization caused the X-linked CGD (12). In the other patient, a mutation of the *CYBB* gene, leading to a premature stop codon, was detected in the patient's leukocytes, but not in the buccal epithelial cells, indicating that somatic mutation arose in the *CYBB* gene of the hematopoietic stem cell (4).

The present patient initially presented with recurrent stomatitis before the onset of the subcutaneous abscess. Although the clinical pictures of adult onset X-linked CGD in woman patients are not fully understood, skin lesions including subcutaneous abscess, photosensitive dermatitis, and aphthous stomatitis were often seen especially before the onset of life-threatening bacterial and/or fungal infections (2, 3). The combination of DLE-like skin lesions and aphthous stomatitis, which are commonly observed in collagen diseases such as SLE and Behçet disease, were also cardinal clinical symptoms in woman carriers of X-linked CGD (7-9). Because appropriate prophylactic therapy is quite effective for prevention of severe infectious disease (4, 12), we emphasize that a careful differential diagnosis between X-linked CGD and collagen diseases is needed especially in adult woman patients with skin lesions and/or stomatitis. The stimuli-induced ROM formation test of neutrophils is necessary for the differential diagnosis.

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CASE REPORT

Successful treatment of chronic granulomatous disease with fludarabine-based reduced-intensity conditioning and unrelated bone marrow transplantation

Daiichiro Hasegawa · Masako Fukushima · Yuki Hosokawa · Hiroki Takeda · Keiichiro Kawasaki · Tomoyuki Mizukami · Hiroyuki Nunoi · Hiroshi Ochiai · Tomohiro Morio · Yoshiyuki Kosaka

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Abstract Allogeneic hematopoietic stem-cell transplantation (HSCT) for chronic granulomatous disease (CGD) with a reduced-intensity conditioning regimen can be expected to lead to less therapy-related mortality and late-onset impairment, whereas it has also been reported to increase the risk of unsustained mixed donor chimerism and late rejection after transplantation. Herein, we report a 4-year-old boy with CGD who was successfully treated with unrelated bone marrow transplantation with a reduced-intensity conditioning regimen (RIC). Fludarabine-based RIC, 4 Gy of total body irradiation, 120 mg/kg of cyclophosphamide, and 125 mg/m^2 of fludarabine, was adopted for transplantation, followed with 8.9×10^8/kg mononucleated donor cells infused without T-cell depletion. Although hematopoietic engraftment was rapidly obtained by day +17, he developed unstable donor chimerism. After tacrolimus withdrawal, the patient showed grade III acute graft-versus-host disease (GVHD), and subsequently reached full donor chimerism by day +61. Twelve months post-transplant, the patient has remained well with stable and durable engraftment, 100% donor chimerism, and normal superoxide production, without the requirement of donor lymphocyte infusions (DLI).

Keywords Chronic granulomatous disease · Unrelated bone marrow transplantation · Reduced intensity conditioning

1 Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by impaired phagocyte killing of intracellular pathogens, characterized by recurrent, often life-threatening bacterial and fungal infections and by granuloma formation in vital organs. It results from mutation in any one of four subunits of a nicotinamide adenine dinucleotide phosphate oxidase of phagocytic cells (gp91^{phox}, p47^{phox}, p67^{phox}, and p22^{phox}) [1]. Although the prognosis of CGD has markedly improved due to prophylactic treatment for infections, including the induction of interferon-gamma therapy, annual mortality is still between 2 and 5% [2]. Allogeneic hematopoietic stem-cell transplantation (HSCT) is an alternative to conventional treatment for CGD, but a high transplantation-related mortality rate [3] and high risk of graft rejection have lowered its therapeutic efficacy [4]. We here in report a 4-year-old boy with CGD who was successfully treated with unrelated bone marrow transplantation with a fludarabine-based reduced-intensity conditioning regimen (RIC).

2 Case report

A 4-year-old boy with CGD was admitted to our hospital in August 2005. He had had recurrent bacterial and fungal infections from early infancy, and CGD was diagnosed by

D. Hasegawa (✉) · M. Fukushima · Y. Hosokawa · H. Takeda · K. Kawasaki · Y. Kosaka
Department of Hematology and Oncology, Kobe Children's Hospital, 1-1-1 Takakuradai, Suma, Kobe 654-0081, Japan
e-mail: hasegawa_kch@hp.pref.hyogo.jp

T. Mizukami · H. Nunoi
Department of Pediatrics, Miyazaki University School of Medicine, Miyazaki, Japan

H. Ochiai · T. Morio
Center for cell therapy, Tokyo Medical and Dental University, Tokyo, Japan

reduced NADPH oxidase (0%), confirmed by $gp91^{phox}$ expression analysis when he was 1 year old. His elder brother was also diagnosed with CGD, and died of fungal pneumonia at the age of 10 years old. There was no HLA-identical HSCT donor in his family. He received anti-infectious prophylaxis consisting of itraconazole and sulfamethoxazole/trimethoprim. Diagnostic imaging at 3 years of age showed intraperitonial granulation tissue formation and hyperplasia of the intestinal tract, resulting from having intussusceptions two times. Interferon gamma therapy had been given for 6 months before transplantation, but subsequently failed. Thus, allogeneic bone marrow transplantation from an HLA-matched volunteer donor was planned.

At 4 years of age, he received allogeneic bone marrow transplantation from an HLA-matched unrelated donor in March 2006. Donor and recipient HLA matching was confirmed by serotyping and molecular typing of the HLA class I and II loci, respectively. We used a RIC for transplantation with total body irradiation at a dose of 2 Gy (days −8 and −7) without use of the gonadal shield, cyclophosphamide at a dose of 60 mg/kg (days −3 and −2) and fludarabine at a dose of 25 mg/m^2 (days −6, −5, −4, −3 and −2), because the patient had been chronically ill, showing intermittent fever and moderate elevation of CRP values, which was thought to be due to chronic enterocolitis. Repeated stool and blood cultures were negative for bacteria and fungi. Just before transplantation, laboratory findings included increased C-reactive protein (2.39 mg/dl) and a normal beta-D-glucan level. Latex agglutination test for serum *Aspergillus* and serum *Candida* antigens were negative.

A cell dose of 8.9×10^8/kg mononucleated cells was infused to the patient without T-cell depletion. GVHD prophylaxis consisted of tacrolimus (0.03 mg/kg/day i.v. continuous infusion from day −1) and short-term methotrexate (10 mg/m^2 i.v. on day +1, 7.5 mg/m^2 i.v. on days +3 and +6). He was also nursed in a high-efficiency, particulate-air-filtered protected environment, and underwent oral gut decontamination. He received *Peumocystis carinii* prophylaxis by sulfamethoxazole/trimethoprim, which was interrupted after transplantation until neutrophil recovery confirmed. Post-transplant regimen also included acyclovir, ursodeoxycholic acid and intravenous immunoglobulin therapy. Chimerism was studied via the analysis of informative microsatellite DNA sequences. The oxidase-positive neutrophils were detected by flow cytometry with the use of a dihydrorhodamine oxidation assay.

During the conditioning therapy for transplantation, prolonged fever rapidly resolved and C-reactive protein values also decreased to within normal ranges. A total of 300 μg/m^2 of granulocyte-colony stimulating factor was commenced on day +5 post-transplant. The patient engrafted rapidly. He achieved an absolute neutrophil count of 0.5×10^9/l by day +17. Chimerism analysis revealed 62.5% donor cell engraftment by day +21, and 39.3% donor cell engraftment additively decreased by day +34, respectively. To achieve complete chimerism, we stopped all immunosuppressants by day +39, because he had no GVHD confirmed at that time. Subsequently, grade III acute GVHD of his skin and gut were clinically confirmed on day +55, followed by full converted donor chimerism and normal superoxidase production by day +61. He was treated again with tacrolimus and 2 mg/kg of prednisolone for GVHD, and all GVHD symptoms disappeared by day +80. Reactivation of his Cytomegalovirus antigenemia was detected on day +65, and treated with ganciclovir with good response. Flow cytometric analysis with the use of a dihidrorhodamine

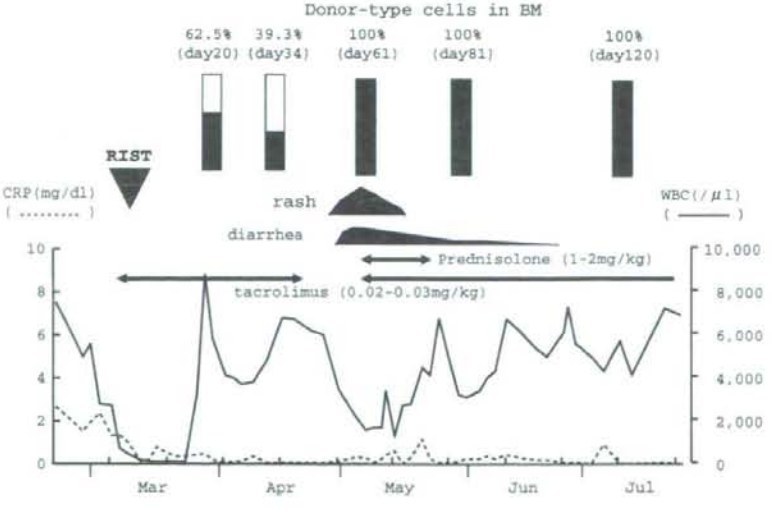

Fig. 1 Clinical course after unrelated bone marrow transplantation. RIST indicates reduced intensity stem cell transplantation. In the upper section of the figure, the donor-type cells in the bone marrow are represented as *black-lacquered*

oxidation assay showed that oxidase-positive neutrophils were detected as 100% of engrafted cells since then. Twelve months post-transplant, the patient has remained well, with stable and durable engraftment, 100% donor chimerism, normal superoxide production, without donor lymphocyte infusion (DLI) requirement (Fig. 1).

3 Discussion

Allogeneic HSCT is the curative therapy for CGD, especially in patients with no inflammatory or infectious lesions at transplant with an excellent disease-free survival rate (DFS). A survey of European Group for blood and marrow transplantation (EBMT) has advocated myeloablative regimens, mostly consisting of busulfan (16 mg/kg) and cyclophosphamide (200 mg/kg), and T-cell replete allografts from HLA-matched related donors, which provided excellent results in low-risk CGD patients (15 children and 1 adult) with no overt infectious complications at transplant and a DFS of 100% [3]. However, in the EBMT report, inadequately high rates of severe acute GVHD and pulmonary infectious complication with a transplant-related mortality of 36% (4 of 11 patients) were also observed in advanced CGD patients with active inflammation due to granulomatous colitis or active infectious disease. Thus, transplant-related mortality with standard myeloablative transplantation regimens, especially in advanced CGD, has been a major obstacle to the more widespread use of allogeneic HSCT.

Horwitz et al. recently reported promising results in the treatment of 10 advanced CGD patients with the combination of a nonmyeloablative regimen consisting of cyclophosphamide, fludarabine, and antithymocyte globulin and the use of a T-cell depleted HLA-identical allograft [5]. This US trial demonstrated that seven out of 10 patients were successfully cured of the disease, even though two patients rejected their graft and DLI led to GVHD in three patients, which was fatal in one case. There are also several reports of successful outcomes for CDG with fludarabine-based RIC [6–9], while most of them consisted of transplant from HLA-matched related donors. Furthermore, T-cell depletion could be a promising approach to reduce the incidence of GVHD, while it could be associated with an increased risk of infectious complications and graft rejection. Thus, RIC is associated with a lower toxity from the conditioning agents and may be an alternative option for CGD, while it still carries a significant risk of graft rejection and GVHD, particularly if DLI have to be used to ensure engraftment.

A national survey of HSCT for CGD in Japan has shown fairly high survival rate (22 of 28), in which the survival rate of HSCT from HLA-matched siblings were comparable to that of HSCT from HLA-matched unrelated donors, whereas that of cord blood transplantation were improperly poor (2 of 4) [10]. Recently, nonmyeloablative conditioning regimens, mostly consisting cyclophosphamide and fludarabine, have been preferred, while the myeloablative conditioning, consisted of busulfan and cyclophosphamide, have been initially performed. However, inadequately high rates of development of unsustained mixed chimerism with the requirement of DLIs were also demonstrated in the patients with RIC by cyclophosphamide and fludarabine. In current case, we adapted fludarabine-based RIC without T-cell deletion for transplantation, because it is not allowed to manipulate unrelated donor allografts for DLIs, and also increased the total body irradiation dose to 4 Gy to ensure engraftment. Taken together, although standard regimens for transplantation of advanced CGD have not been established, our present case encourages the consideration of unrelated HSCT with fludarabine-based RIC for patients with CGD, even if they have infectious complications and no suitable related donors.

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