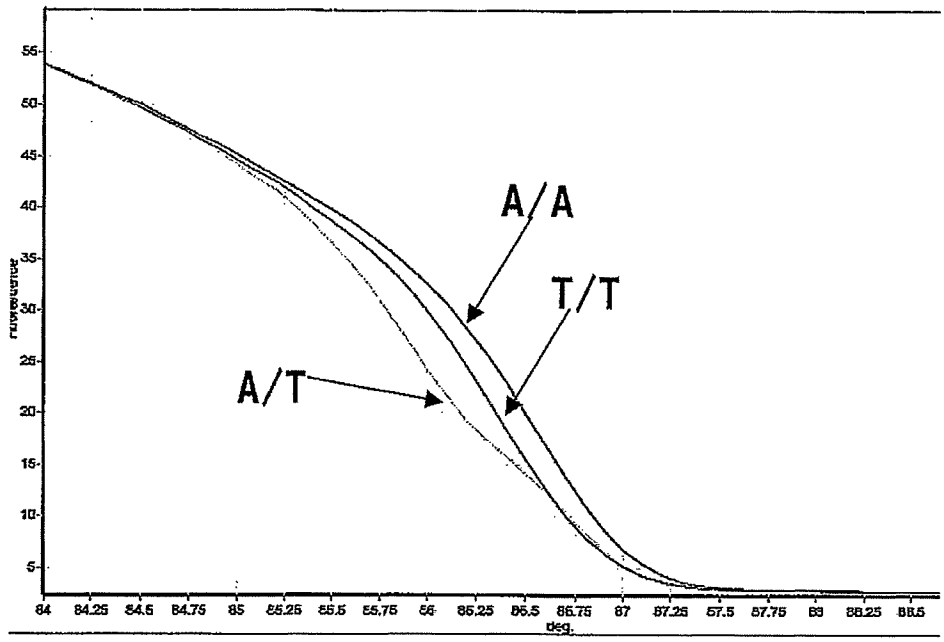

変異を熱変性の過程の変化として視覚的にとらえることができる

図2 HRMによるSNPの検出

キットと比較して少なくとも2桁以上上昇した。このツールを用いることでCAリピートキットでは広いマーカー間の隙間に埋もれていた遺伝子座が描出できるようになってきた。また，常染色体劣性遺伝性疾患を対象にすると，Lod値3.0以上とならなくても，ホモザイゴシティーマッピング法により，遺伝子局在部位が観察できるようになってきた。例えば，同祖性（Identical By Descent）を手がかりに小家系を解析すれば全部で3家系4症例程度であっても遺伝子座の特定と責任遺伝子単離が可能となり始めている。われわれもWaardenburg無眼球症候群等で本アプローチを採用し，その効果を実感している。

ハイレゾリューションメルト（HRM）法

責任遺伝子や候補遺伝子の安価な迅速スクリーニング法として採用し，その有用性は明らかである。本法は，基本的に遺伝子異常がヘテロ接合性で存在する場合に形成されるミスマッチを有するDNAヘテロ二重鎖を高感度に検出する用法である。二重鎖DNAに飽和的に結合する第二世代蛍光色素LC Green PlusやSYTO 9等の登場により可能となった検出法である。リアルタイムPCR後に熱変性を行い，ヘテロ二重鎖を有する検体はホモ二重鎖のみの検体と異なるHRMパターンを呈することで異常が検出可能である（図2）。HRM曲線のパターン変化を視覚的に捉えることで慣れると異常検出は極めて容易である。コストはダイレクトシーケンスと比較すると1/4程度であり，大量の候補遺伝子スクリーニングなどでその威力は絶大である。もちろん異常を検出した検体では，改めてシーケンスで確認する必要がある。PCR増幅からHRM解析まで約90分で終了し1回で72～384検体の処理が可能である。

リシーケンスアレーを用いた候補遺伝子解析

Affymetrix社などが提供しているハイブリダイゼーションを基盤とする塩基配列決定技術である。これは1枚のチップ上に固着させたあらゆる遺伝子点変異パターンに対応したオリゴDNAに対して症例ゲノムをハイブリさせ，大量の塩基配列情報（最大30Kb分）を短時間で解析できるプラットフォームである。このシステムを用いて，大動脈瘤・大動脈解離症例に対して，同症状を呈する関連メンデル遺伝性疾患の8責任遺伝子（*FBN1*, *TGFBR1*, *TGFBR2*, *COL3A1*, *PLOD1*, *MYH11*, *ACTA2*, *GLUT10*）の網羅的解析を行っている。現行では1症例当たり実働2～3日で全遺伝子解析が可能である。これまでに，病的な遺伝子異常を数例において同定しており，大動脈瘤・解離症例の少なくと

も数パーセントでメンデル遺伝性疾患の関与が疑われる。しかし，このシステムも完全ではなく，特定の領域や，1-数bpの塩基欠失・挿入は検出できない。コスト削減には極めて有用で，8遺伝子のダイレクトシーケンスでは，少なく見積もっても40~50万円/1例かかるが，リシーケンスアレーならその15%程度の費用しかかからない。もちろん検出した異常は，ダイレクトシーケンスで確認する必要はある。本システムは精度の問題があり診断法には使用できないが，研究レベルでは有益な手法である。

次世代シーケンサーを用いた疾患ゲノム解析

次世代シーケンサーは従来のキャピラリーシーケンサーと原理もコンセプトも全く異なるが，ヒトゲノム解析においてもこれまで不可能であった高出力の解析が期待される。筆者らは，Illumina社ゲノムアナライザーIIを導入し，疾患ゲノム解析を開始した。現時点でのシーケンス産出能はペアエンド法で20Gb，同社のアナウンスでは2009年度中に100Gbまでシーケンス出力が向上する予定である。100Gbの出力なら，ヒトゲノムを1回解析することで全ゲノム33カバレッジ分の解読が可能で理論的には変異解析にも耐えうるデータであることが期待される。現時点では，全ゲノムを解析することはコスト的にも膨大なため，解析したい領域や遺伝子群を抽出する技術を介在させるというアイデアは妥当である。そのためにマイクロアレー上や液体中でゲノム領域を選択する技術が開発され，われわれもトライアルを開始している。本稿で紹介できる十分なデータは持ち合わせていないため，いずれかの機会に改めて紹介できればと考えている。また，前述したリシーケンスアレーと次世代シーケンサーでの産出データとを比較検討することで，両プラットフォームの特徴を明らかに出来ればと考えている。

終わりに

本稿は，平成21年6月27日に第44回日本小児腎臓病学会学術集会で特別講演として発表した内容を基にまとめたものである。発表の機会を与えていただいた五十嵐隆大会長に深甚の謝意を表する。また，未発表データを割愛したため，講演内容と若干異っている。本稿が今後の読者の研究展開に少しでもお役に立てれば幸いである。

「日本小児腎臓病学会の定める基準に基づく利益に関する開示事項はありません。」

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Advance in genome analysis for human diseases

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Recently new technologies to analyze human genome in diseases emerged, which enabled us to isolate genes responsible for diseases efficiently and to screen rapidly a large number of candidate genes. As for mapping disease loci, genome microarray and high density SNP array are quite useful (even for small-sized families). As for rapid screening of many candidate genes, high resolution melting curve method, resequencing array, and next generation sequencing are expected to yield high throughputs. All the technologies presented here possess more advantage than any other conventional technologies, but each has some drawbacks. We should utilize these new technologies to attain reasonable goals, but proper understanding of their weak points is essential.

Key words: genome microarray, SNP array, high resolution melting curve method, resequncing array, next generation sequencer

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Interleukin-6 inhibits early differentiation of ATDC5 chondrogenic progenitor cells

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ABSTRACT

Interleukin (IL)-6 is a causative agent of systemic juvenile idiopathic arthritis (sJIA), a chronic inflammatory disease complicated with severe growth impairment. Recent trials of anti-IL-6 receptor monoclonal antibody, tocilizumab, indicated that tocilizumab blocks IL-6/IL-6 receptor-mediated inflammation, and induces catch-up growth in children with sJIA. This study evaluates the effects of IL-6 on chondrogenesis by ATDC5 cells, a clonal murine chondrogenic cell line that provides an excellent model for studying endochondral ossification at growth plate. ATDC5 cells were examined for the expression of IL-6 receptor and gp130 by fluorescence-activated cell sorting analysis. Recombinant murine IL-6 was added to ATDC5 cultures to observe cell differentiation, using a quantitative RT-PCR for the chondrogenic differentiation markers type II collagen, aggrecan, and type X collagen. To block IL-6, the anti-mouse IL-6 receptor monoclonal antibody MR16-1 was added. As a result, the cells expressed IL-6 receptor and gp130. The expression of chondrogenic differentiation marker gene was reduced by IL-6, but this was abrogated by MR16-1. We conclude that IL-6 inhibits early chondrogenesis of ATDC5 cells suggesting that IL-6 may affect committed stem cells at a cellular level during chondrogenic differentiation of growth plate chondrocytes, and that IL-6 may be a cellular-level factor in growth impairment in sJIA.

1. Introduction

Longitudinal bone growth occurs at the growth plates, located at the ends of the long bones, by endochondral ossification, a two-step process in which cartilage is first formed and then remodeled into bone. Firstly, mesenchymal cells differentiate into chondrocytes, which form cartilage anlagen of the future bone. The chondrocytes proliferate, mature, and become hypertrophic, and eventually calcify. This calcified cartilage is then invaded by osteoclasts, osteoblasts, and blood vessels that resorb the cartilage and replace it with bone matrix. These two processes, chondrogenesis and ossification, are regulated by the multitude of genetic and hormonal factors, growth factors, environment, and nutrition. The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis particularly is considered to have an important regulator effect on the growth plate chondrogenesis.

Growth impairment is a major complication of patients with systemic juvenile idiopathic arthritis (sJIA). In sJIA, markedly elevated interleukin (IL)-6, which binds to gp130 and to IL-6 receptor to elicit the inflammatory response, appears to be the main causative agent of the inflammation, and we have already reported that the anti-IL-6 receptor monoclonal antibody, tocilizumab, effectively blocks the inflammatory manifestations and the surrogate markers, CRP and serum amyloid A, and that it also induces catch-up growth in sJIA patients [1–3].

Recently the effects of inflammatory cytokines on growth plate chondrocytes have been studied [4–6]. Tumor necrosis factor-α (TNF-α), and IL-1 are considered to directly affect growth plate chondrocytes, but IL-6 is reported to have no direct effect on growth plate chondrocyte dynamics. IL-6 has been considered to affect growth through systemic mechanisms that alter the growth hormone/insulin-like growth factor-1 (GH/IGF-I) axis [7,8].

The purpose of the present study was to examine whether IL-6 in fact has direct effects on growth plate chondrogenesis or, as currently considered, dose not. We investigated the effect of IL-6 on the chondrogenic differentiation of the murine chondrocyte embryonal carcinoma cell line, ATDC5. This cell line has been shown to undergo a sequence of events—namely, cell proliferation, synthesis of the extracellular matrix, cellular hypertrophy, mineralization of matrix, localized vascular invasion and apoptosis—that occur during longitudinal bone growth in vivo and, thereby, provides an excellent model for studying the molecular mechanisms underlying the regulation of growth plate maturation and endochondral bone formation [9]. Our investigation of the effect of IL-6 on the ATDC5 cell line has made possible a considerable extension of our understanding of the effect of IL-6 alone at a cellular level on growth plate chondrocytes. In the presence of insulin, ATDC5 cells differentiate into chondrocytes to form cartilage nodules (chondrogenesis), accompanied with the progressive expression of type II collagen, which is the predominant

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extracellular matrix in the proliferating stage, and then they differentiate into the cells that express type X collagen in the hypertrophic stage [10].

In this study, we examined the effects of IL-6 on cell proliferation by MTT assay, and on differentiation by examination of the expression levels of chondrogenic marker genes including type II collagen, aggrecan, and type X collagen, using quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR).

2. Methods

2.1. Reagents

A murine chondrogenic cell line, ATDC5, was obtained from Riken cell bank (Tsukuba, Japan). Mouse recombinant IL-6 was purchased from R&D systems (Minneapolis, MN, USA). Anti-mouse IL6 receptor antibody (MR16-1) [11] was a generous gift from Dr. Osugi Chugai Pharmaceutical Co., Ltd. (Shizuoka, Japan). PE-conjugated rat anti-mouse IL-6 receptor monoclonal antibody was from BD Biosciences (San Diego, USA) and biotinylated rat anti-mouse gp130 monoclonal antibody and streptavidin PE were from R&D systems. 1:1 mixture of DMEM and Ham's F-12 medium and 5% fetal bovine serum were from Invitrogen (Tokyo, Japan), human transferring, sodium selenite, bovine insulin, ascorbic acid and tripsin/EDTA were from Sigma–Aldrich (Tokyo, Japan). MTT working solution was from Cosmo Bio Co., Ltd. (Tokyo, Japan) and DSMO was from Sankyo Chemical Co., Ltd. (Nagoya, Japan).

2.2. Analysis of IL-6 receptor and gp130 expressions on ATDC5 cells

FACS analysis was performed to detect IL-6 receptor and gp130 on ATDC5 cells. Following detachment of ATDC5 cells with trypsin/EDTA at Day 0 and Day 2, the cells (1×10^6 cells/ml) were incubated with PE-conjugated rat anti-mouse IL-6 receptor monoclonal antibody and biotinylated rat anti-mouse gp130 monoclonal antibody, which was revealed using streptavidin PE. Mouse spleen cells were assessed as controls. The flow cytometer used in this study was a FACSCAN (BD Biosciences), and the software used for collection and analysis of the results was the program CellQuest ProTM (BD Biosciences).

2.3. Cell culture and stimulation

ATDC5 cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 5% fetal bovine serum, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite, and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) at 37 °C in a humidified atmosphere of 5% CO_2 in air. In the present study, ATDC5 cells were plated at an initial cell density of 1.0×10^4 cells/cm^2 in 12-multiwell plastic plates (Corning, New York, USA), grown to 70–80% confluency in the culture medium, and then differentiation was induced by the addition of bovine insulin (10 μg/ml) and ascorbic acid (20 μg/ml) to the culture medium (culture Day 0). (To examine the effect of IL-6 on proliferation and differentiation of ATDC5 cells,) the culture medium was replaced every other day from Day 2 to Day 14 with or without the medium containing mouse recombinant IL-6 at various concentrations (1–100 ng/ml). Cultures at least in triplicate were used for each test.

For the experiment of interrupting the complex formation between IL-6 and IL-6 R, ATDC5 cells were preincubated (from Day 2 to Day 14) with anti-mouse IL-6 receptor monoclonal antibody, MR16-1, for 30 min added at concentrations from 0.1 to 10 μg/ml before each time that the culture medium was replaced. Every time, after this was done, fresh medium containing 100 ng/ml mouse recombinant IL-6 was added.

Table 1
Primer sequences for mouse type II collagen, aggrecan, type X collagen and GAPDH. Sequence of the primers used for RT-PCR experiments.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)
Type II collagen	AAGACCGTCATCGAGTACCGA	ACTGCGGTTGGAAAGTGTTTG
Aggrecan	AACTTCTTTGCCACCGGAGA	GGTGCCCTTTTTACACGTGAA
Type X collagen	GCAGCATTACGACCCAAGAT	TCTGTGAGCTCCATGATTGC
GAPDH	CAAAATGGTGAAGGTCGGTGTG	ATTTGATGTTAGTGGGGTCTCG

2.4. Cell proliferation assays

Cell proliferation was assessed by MTT assay, conducted at Days 0, 2, 6, 10, and 14. ATDC5 cells were plated in 12-well plates and cultured as previously described in the presence of mouse recombinant IL-6 (100 ng/ml). Culture wells were incubated with 1 ml of MTT working solution for 2 h at 37 °C. After removal of MTT working solution, culture wells were incubated with 1 ml of DMSO for 30 min at 37 °C, then 200 μl of supernatants were transferred into each well of a new 96-well microplate and the absorbance at 570 nm was measured with a spectrophotometer (Bio-Rad Model 550). The experiment was performed at least six times, and the results are expressed as means ± standard deviations.

2.5. Cartilaginous nodule formation under a phase-contrast microscope (×100)

Cartilaginous nodule formation was assessed under a phase-contrast microscope (Olympus CKX41) at Day 10.

2.6. Quantitative real time reverse-transcriptase polymerase chain reaction (qRT-PCR) of chondrogenic marker gene expression

The expression levels of chondrogenic marker genes; type II collagen, aggrecan and type X collagen mRNAs were measured at Days 6, 10, and 14 by means of qRT-PCR in ATDC5 cells cultured with 100 ng/ml mouse recombinant IL-6. Total RNA was prepared from the cultures using an RNeasy Mini Kit (Qiagen), and then qRT-PCR was carried out in an ABI Prism 7500 (Applied Biosystems, Foster, CA, USA) with a SuperScript III platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). Primer sequences are listed in Table 1. The cycling profile was 95 °C for 15 s, 55 °C for 30 s, 72 °C for 34 s for 40 cycles. The number of template copies present at the start of the reaction was determined by comparison to a standard scale prepared from mouse genomic DNA. For normalization of the RNA loading, an RT-PCR of GAPDH was also performed in each RT-PCR reaction as an internal control. The abundance of each gene was determined relative to GAPDH. The expression level of each target gene was calculated by standardizing the target gene copy number with the GAPDH copy number in a sample. The analysis of the results is based on triplicate (or more) samples.

2.7. Statistical analysis

Statistical significance was assessed by one-way analysis of variance and Mann–Whitney's *U*-test. Data are reported as the mean ± SD, and are considered significantly different at $P < 0.05$.

3. Results

3.1. Expression of IL-6 receptor and gp130 on the surface of ATDC5 cells

We assessed whether ATDC5 cells express IL-6 receptor and gp130 on their surface by flow cytometry. Although the levels of membrane-anchored IL-6 receptor on ATDC5 cells were much

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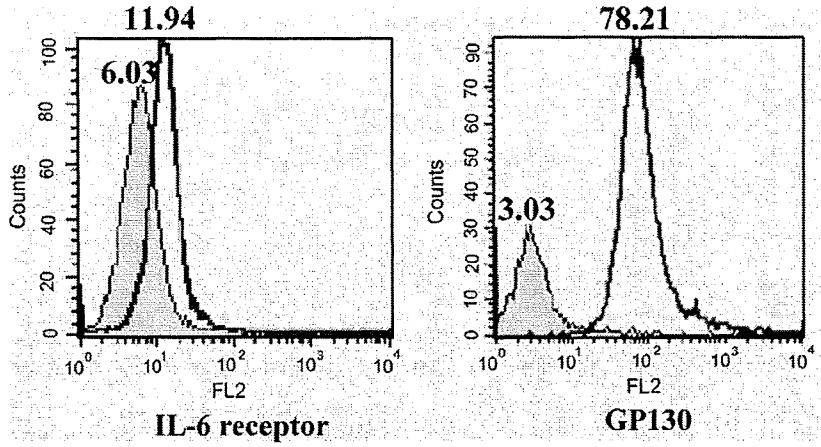

Fig. 1. FACS analysis for expression of IL-6 receptor and gp130 on the surface of ATDC5 cells.

lower than those on mouse spleen cells, we confirmed that ATDC5 cells express both IL-6 receptor and gp130 on their surface. (Fig. 1).

3.2. Molecular changes during the process of differentiation in ATDC5 cells

Chondrogenic differentiation of ATDC5 cells was further characterized by expression of cartilage-characteristic extracellular matrix genes such as aggrecan, type II collagen and type X collagen. In Fig. 2, we outlined the molecular changes of ATDC5 cells during the process of differentiation in the presence of insulin. On Day 2, ATDC5 cells express neither type II collagen nor aggrecan mRNA, but on Day 6, they express both type II collagen and aggrecan mRNA. On Day10, the expression levels of type II collagen and aggrecan gradually decrease while the expression level of type X collagen increases. On Day14, ATDC5 cells express type X collagen substitute for type II collagen.

3.3. Effect of IL-6 on chondrogenic marker gene expression in ATDC5 cells at various concentrations (1–100 ng/ml)

The results of qRT-PCR of the chondrogenic marker gene showed that IL-6 reduced type II collagen and type X collagen gene expression in a dose-dependent manner on Days 6 and 14, respectively (Fig. 3).

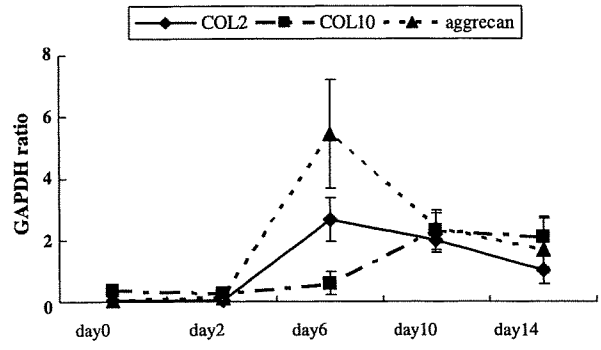

Fig. 2. Molecular changes during the process of differentiation in ATDC5 cells. The molecular changes of ATDC5 cells during the process of differentiation in the presence of insulin were outlined. On Day 2, ATDC5 cells express neither type II collagen nor aggrecan mRNA, but on Day 6, they express both type II collagen and aggrecan mRNA. On Day 10, the expression levels of type II collagen and aggrecan gradually decrease while the expression level of type X collagen increases. On Day 14, ATDC5 cells express type X collagen substitute for type II collagen.

3.4. Effect of IL-6 on cell proliferation in ATDC5 cells

The results of the MTT assay revealed that IL-6 has no inhibitory effect on cell proliferation in ATDC5 cells. The absorbance each day did not differ between the cells cultured with and without IL-6 ($P > 0.05$) (Fig. 4).

3.5. Effect of IL-6 on cartilaginous nodule formation in ATDC5 cells

In control cultures, cartilage nodules were formed at Day 10, but continual exposure of 100 ng/ml of mouse recombinant IL-6 to the culture caused cell flattening and completely inhibited cellular condensation and subsequent formation of cartilage nodules. Pretreatment of undifferentiated ATDC5 cells with 10 μg/ml of MR16-1 neutralized the effect of IL-6, resulting in the formation of cartilage nodules, but pretreatment with 0.001 μg/ml of MR16-1 did not block the effect of IL-6 (Fig. 5).

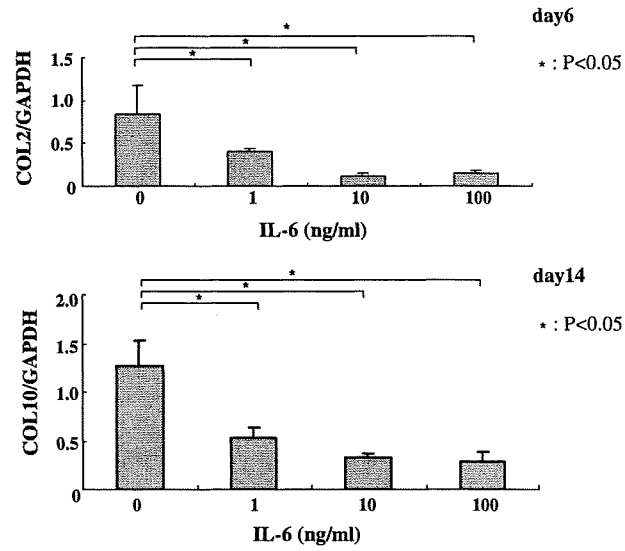

Fig. 3. Effect of IL-6 on the expression of type II collagen and type X collagen at various concentrations (1–100 ng/ml). IL-6 reduced type II collagen and type X collagen gene expression in a dose-dependent manner on Days 6 and 14, respectively. The data represents the mean ± SD from tests of samples in triplicate. $P < 0.05$ vs. control.

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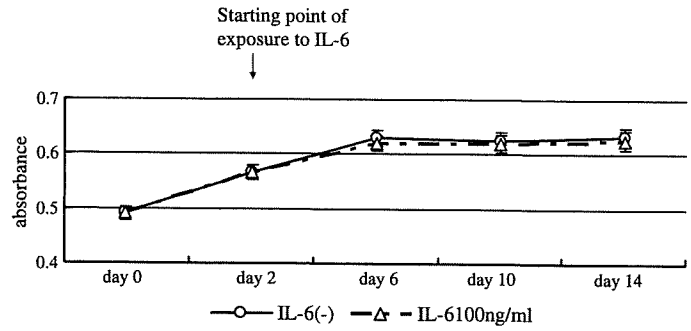

Fig. 4. MTT assay of ATDC5 cells cultured with or without IL-6. The absorbance each day did not differ between the cells cultured with and without IL-6.

3.6. Effect of IL-6 on chondrogenic marker gene expression in ATDC5 cells

The results of qRT-PCR of the chondrogenic marker gene showed that IL-6 (100 ng/ml) markedly reduced type II collagen gene expression on Days 6, 10 and 14, and reduced aggrecan gene expression on Day 6. The results also indicated that MR16-1 blocked the effect of IL-6 to increase both type II collagen and aggrecan gene expression in a dose-dependent manner (Fig. 6A and B). IL-6 (100 ng/ml) also markedly reduced type X collagen gene expression on Days 10 and 14, but MR16-1 inhibited the reduction of type X collagen gene expression dose-dependently (Fig. 6C).

4. Discussion

The present study found that IL-6 inhibited the differentiation of ATDC5 cells. This is the first report describing the inhibitory effect of IL-6 on differentiation of the chondroprogenitor cell line, ATDC5.

Generally, the ATDC5 cell line is an excellent model for studying the molecular mechanisms underlying the regulation of growth plate maturation and endochondral bone formation [9]. At the epiphyseal end of the growth plate, the reserve zone, also called the germinal or stem cell zone, contains the resting chondrocytes [10]. These cells have recently been shown to be crucial for orientation of the underlying columns of chondrocytes and therefore for unidirectional bone growth, probably through the secretion of a growth plate-orienting factor [12]. Upon some unknown trigger, the stem cells enter the proliferating zone, and local and systemic factors regulate longitudinal bone growth, which involves the differentiation of committed stem cells into proliferating chondrocytes (early chondrogenesis); after a finite number of cell divisions, these cells finally differentiate into the hypertrophic phenotype that deposits a matrix that is mineralized and eventually replaced by bone [13,14]. The ATDC5 cell line allows the study of two critical events during cartilage formation: the early differentiation of committed stem cells into chondrocytes and the terminal differentiation of proliferating to hypertrophic chondrocytes [15]. We showed the molecular changes during the process of differentiation of ATDC5 cells in the presence of insulin in Fig. 2. In this study, we investigate the effect of IL-6 especially on early chondrogenesis; the differentiation of commit-

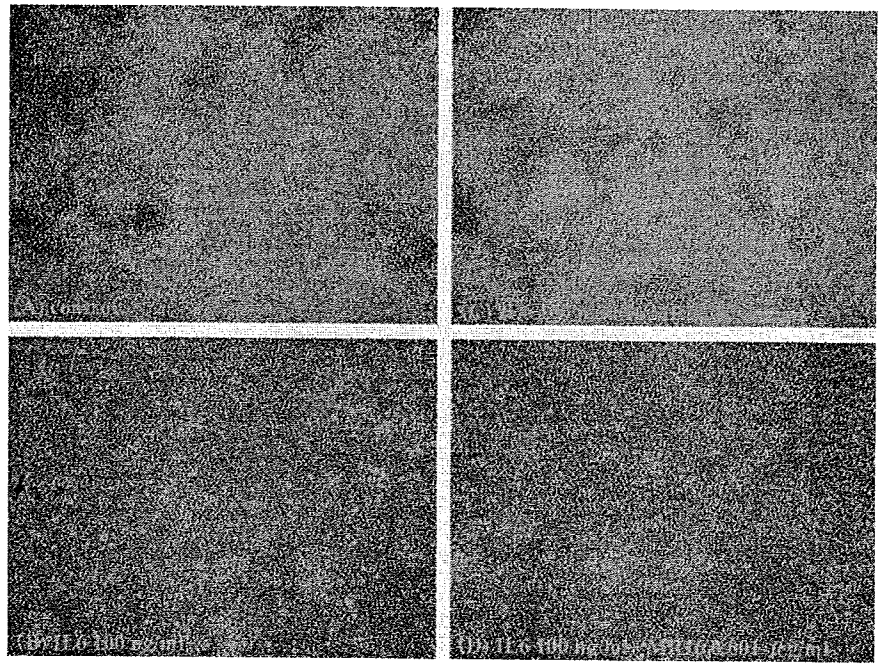

Fig. 5. Phase-contrast micrographs of ATDC5 cells with and without IL-6 at Day 10. In the absence of IL-6 (control), typical cartilage nodules are formed (A). The cells having continual exposure to 100 ng/ml of mouse recombinant IL-6 are flattened, and their cellular condensation and subsequent formation of cartilaginous nodules are completely inhibited (B). After pretreatment with 10 μg/ml of MR16-1, cartilage nodules are formed (C), but those cells pretreated with 0.001 μg/ml of MR16-1 are flattened (D).

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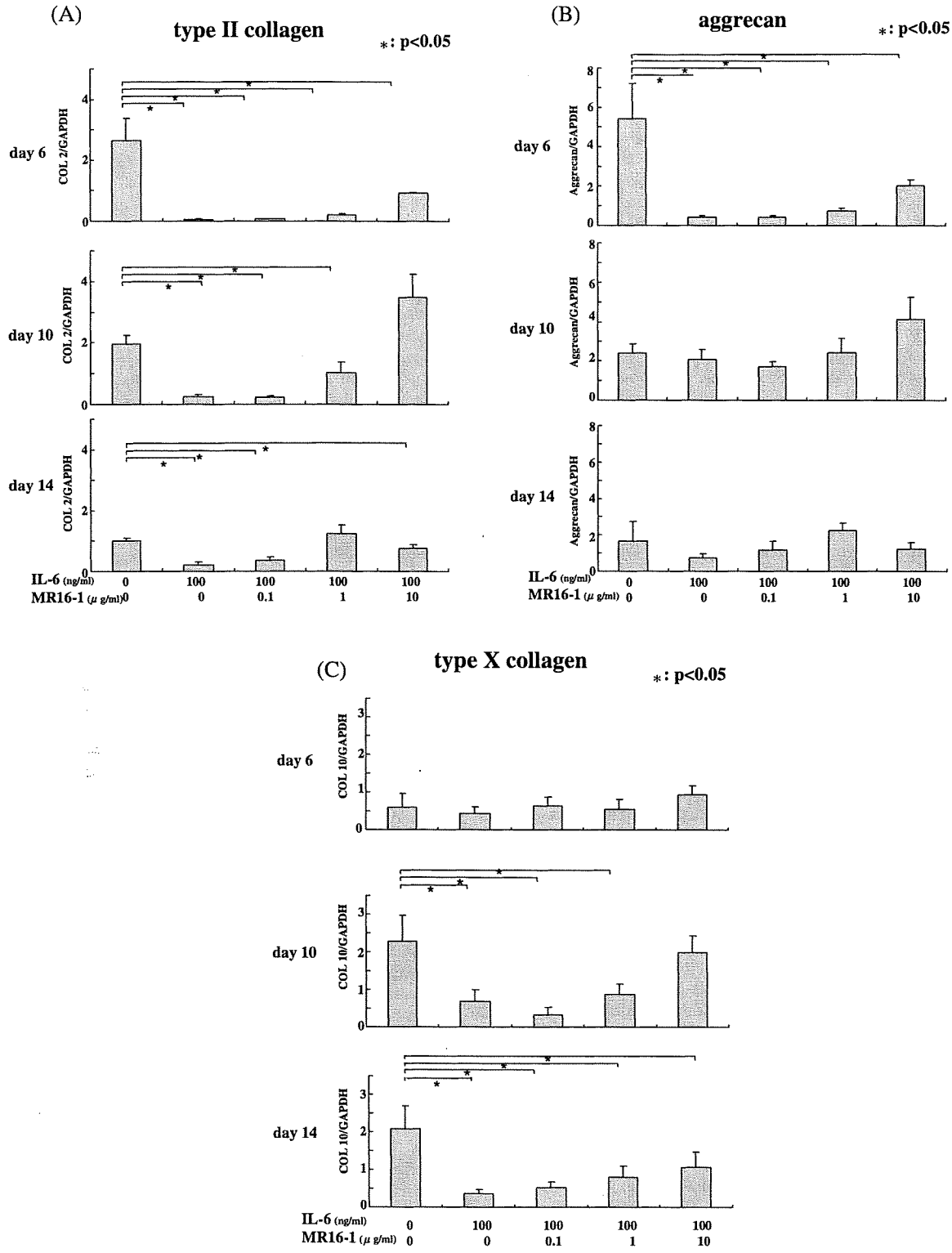

Fig. 6. Effect of IL-6 on chondrogenic marker gene expression in ATDC5 cells. IL-6 (100 ng/ml) markedly reduced type II collagen gene expression on Days 6, 10 and 14, and MR16-1 blocked the effect of IL-6 to increase type II collagen gene expression in a dose-dependent manner (A). IL-6 markedly reduced aggrecan gene expression on Day 6 and MR16-1 blocked the effect of IL-6 to increase aggrecan gene expression in a dose-dependent manner (B). IL-6 also markedly reduced type X collagen gene expression on Days 10 and 14 but MR16-1 inhibited the reduction of type X collagen gene expression dose-dependently (C). The data represents the mean ± SD from tests of samples in triplicate or more. COL 2: type II collagen, COL 10: type X collagen.

ted stem cells into proliferating chondrocytes in the ATDC5 cell line.

The first evidence that IL-6 inhibits the differentiation of ATDC5 cells is that IL-6 was found to inhibit cartilaginous nodule forma-

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tion. The processes of cellular condensation and subsequent cartilaginous nodule formation are important prerequisites for initiation of chondrogenesis in mesenchymal cell cultures. In the presence of insulin, ATDC5 cells lose contact inhibition of movement and grow beyond confluence to produce two or three layers of cells in a process known as cartilaginous nodule formation. The results shown in Fig. 5 indicate that IL-6 inhibits cartilaginous nodule formation. The second piece of evidence for the inhibitory effect of IL-6 on the differentiation of ATDC5 cells is that IL-6 markedly reduced the expression of type II collagen, aggrecan and type X collagen, when IL-6 was continuously added to the culture system of ATDC5 from Day 2.

Some previous studies have assessed whether pro-inflammatory cytokines including IL-1, TNF-α, and IL-6 could act at a cellular level on the growth plate chondrocytes [4–6]. IL-6 has been considered not to have any direct effect on growth plate chondrocyte dynamics, but to inhibit growth plate chondrogenesis and longitudinal growth by reducing the systemic effects of IGF-1 [7,8]. But we have shown that IL-6 has directly inhibited the differentiation of the chondroprogenitor cell line, ATDC5 in its early stage. Our observations in the present study raise the possibility that IL-6 may affect committed stem cells at a cellular level during chondrogenic differentiation of growth plate chondrocytes.

There is a possible reason that the results of the present experiment differed from those of earlier experiments, namely, that the timing of the addition of IL-6 to the culture system was different. In the present study, to assess the effect of IL-6 on committed stem cells, IL-6 was added to the culture medium from Day 2, a time when ATDC5 cells have not yet expressed much type II collagen mRNA—in short, they have not differentiated into proliferative chondrocytes. In the previous study, IL-6 was added to the culture medium, in which ATDC5 cells have already differentiated and have entered the proliferative stage, expressing a large amount of type II collagen mRNA [6]. We also recognized that when we added IL-6 to the culture medium from Day 6, ATDC5 cells differentiated into hypertrophic cells and expressed type X collagen mRNA on Day 14 in the same way as the control. On Day 6, ATDC5 cells had already differentiated into proliferative chondrocytes, expressing type II collagen mRNA. After all, IL-6 has no effect on ATDC5 cells, when they have already differentiated and reached the proliferative stage. In this respect, we recognized the same process as in the previous study. The main finding of our study is that only undifferentiated cells, that is, committed stem cells, are inhibited from differentiation into proliferative and subsequent hypertrophic chondrocytes by IL-6.

Horan et al. investigated the effects of pro-inflammatory cytokines on both rat costochondral resting zone chondrocytes and growth zone chondrocytes [4]. They evaluated the effect of IL-6 on (H3)-thymidine incorporation and alkaline phosphatase-specific activity for proliferation and differentiation, respectively, and they reported that IL-6 had no effect on either (H3)-thymidine incorporation or alkaline phosphatase-specific activity in either type of chondrocytes. First, in our study, the results of the MTT assay revealed that IL-6 has no inhibitory effect on cell proliferation in ATDC5 cells. Our result was the same as their observation in this respect. In addition, they reported that IL-6 had no effect on alkaline phosphatase-specific activity, in contrast to the effects observed with IL-1, which promoted the differentiation of resting zone cells. These results will be supported by our observation that IL-6 inhibits the differentiation of committed stem cells into the proliferative stage in ATDC5 cell line.

It was reported that a reduction in metatarsal growth was not noticed after 8 days' exposure to IL-6, although a reduction in metatarsal growth was seen after exposure to TNF-α and IL-1β [6]. IL-6 did not affect longitudinal growth in an organ culture system of neonatal mouse metatarsal bones in that study. However, we suspect that a reduction in metatarsal growth with exposure to IL-6 could have occurred given a longer time period of observation. IL-6 affects only committed stem cells, which do not contribute directly to longitudinal growth because the chondrocytes do not proliferate [16,17]. Moreover, the cells already differentiated into proliferating and hypertrophic zones can continue their differentiation. For the reasons stated above, a reduction in metatarsal growth might not be noted immediately after 8 days exposure to IL-6. On the other hand, TNF-α and IL-1β are already known to affect proliferative zone cells, which contribute directly to longitudinal growth.

Growth impairment is a major complication for chronic inflammatory diseases, and many reasons for growth impairment in chronic illness have been considered, such as malnutrition, hormone deficiency, glucocorticoids and inflammatory cytokines [18,19]. Systemic JIA is one of the chronic inflammatory diseases characterized by severe multi-organ diseases and growth impairment. In sJIA, IL-6 is one of the major causative agents for systemic inflammation and growth impairment. Recent trials of the anti-IL-6 receptor monoclonal antibody, tocilizumab, for patients with sJIA indicated that tocilizumab blocks IL-6/IL-6 receptor-mediated inflammation, and that the growth impairment is overcome, resulting in catch-up growth [1,2,20]. Moreover, in a recent study, the transgenic mice over-expressing IL-6 experienced a stunted growth rate, and neutralization of IL-6 activity by a MAb produced a partial improvement of the animals' growth rate [7]. De Benedetti et al. reported that IL-6 inhibits growth plate chondrogenesis and longitudinal growth by reducing the systemic effects of IGF-1 [21]. But the ATDC5 cell line that we used in the present study, is a simple culture model for investigating the cellular-level effect of IL-6 on the differentiation of growth plate chondrocytes without considering any other factors including glucocorticoids or systemic IGF-1. Also, the results of our study using this simple culture model indicated that IL-6 directly inhibited early differentiation of ATDC5 cells at a cellular level.

5. Conclusion

This study demonstrates that IL-6 inhibits the early differentiation of ATDC5 cells, suggesting, in clinical terms, that IL-6 directly inhibits early differentiation of growth plate chondrocytes, and that growth impairment in sJIA may be brought about in partially through the direct inhibitory effect of IL-6 on committed stem cells in the growth plate. In sJIA patients, committed stem cells in the growth plate may be inhibited by IL-6 from differentiation into proliferative chondrocytes, and consequently, the normal processes of bone development followed by differentiation into hypertrophic chondrocytes and the mineralization of matrix may also be inhibited. We believe that the therapeutic strategies using tocilizumab to target the IL-6/IL-6 receptor process, which effectively block the inflammatory manifestations in sJIA, are likely to achieve return to normal growth.

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Comparison of markers for fetal inflammatory response syndrome: Fetal blood interleukin-6 and neonatal urinary β_2-microglobulin

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Abstract

Aim: Chronic lung disease (CLD) is a major component in the morbidity of premature infants suffering from fetal inflammatory response (FIRS). The aim of the present study was to compare the value of measuring neonatal urinary β_2-microglobulin (β_2-MG) levels with fetal blood interleukin (IL)-6 levels in premature infants at risk of developing CLD.

Methods: Premature infants (gestational age <30 weeks) without CLD ($n = 19$) and with CLD ($n = 10$) were enrolled. We measured IL-6 levels in umbilical cord blood and β_2-MG levels in urine obtained within 48 h after birth.

Results: IL-6 and β_2-MG levels were significantly higher in infants who developed CLD than in those who did not (median IL-6, 54.7 *vs* 7.6 pg/mL; $P < 0.005$; β_2-MG 17.7 *vs* 9.3×10^4 μg/gCr; $P < 0.05$). The sensitivity and negative predictive value of β_2-MG at the cut-off value at 10.0×10^4 μg/gCr (0.90 and 0.92) were comparable to IL-6 at 16 pg/mL (0.90 and 0.94).

Conclusion: We suggest that measuring urinary β_2-MG in premature infants soon after birth can monitor FIRS and may provide information on the risk of subsequent CLD development that is as clinically important as information derived from umbilical cord blood IL-6.

Key words: β_2-microglobulin, bronchopulmonary dysplasia, chronic lung disease, fetal inflammatory response syndrome, interleukin-6.

Introduction

Various inflammatory cytokines in umbilical cord blood have been reported in infants born to mothers with histological or clinical chorioamnionitis (CAM).[1–5] Gomez *et al.* have proposed that a fetal systemic inflammatory response syndrome (FIRS) determined by inflammatory cytokine elevation (interleukin (IL)-6 >11 pg/mL) in fetal blood is a risk factor for severe neonatal morbidity.[6] Chronic lung disease (CLD) is a major component in the morbidity of premature infants suffering from FIRS. Recent investigations have demonstrated that IL-1β, IL-6, IL-8, tumor necrosis factor-α, and soluble tumor necrosis factor receptor-I levels in amniotic fluid or umbilical cord blood are high in premature infants who subsequently develop CLD.[6–15] Jobe reported that the effect of pro-inflammatory cytokines arising in the uterus could predispose premature infants to new CLD characterized by an arrest of lung development.[10] It is important to

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identify premature infants at risk of CLD; however, measuring inflammatory mediators is currently a difficult and costly process in Japan.

We have previously reported that urinary β_2-microglobulin (β_2-MG) levels are significantly higher in premature infants within 48 h after birth with maternal CAM and CLD.[16] It is suggested that the inflammatory response leads to interferon (IFN)-γ secretion, stimulating β_2-MG synthesis in blood and increasing β_2-MG excretion in urine. Therefore, if umbilical cord blood analyses of cytokines are not available, we hypothesize that neonatal urinary β_2-MG after birth can be used to retrospectively monitor FIRS and risk of an infant developing CLD. The aim of the present study was to compare the value of urinary β_2-MG with umbilical cord IL-6 in the prediction of premature infants at risk of subsequent CLD development.

Methods

Patients

The present study was conducted in premature infants admitted to our neonatal intensive care units between October 2004 and September 2005. Infants were eligible for study entry if they met the following criteria: (i) gestational age at birth <30 weeks; (ii) umbilical cord blood samples obtained at birth and urine samples before 48 h of age; (iii) absence of major intraventricular hemorrhage (grades III and IV), congenital anomalies, early-onset neonatal infection and renal abnormalities and diseases; and (iv) permission to participate in the investigation given by the infant's parents. Postnatal steroids were not administered to the infants during the study period. Approval for the study was given by the Committee for Ethics of Yokohama City University and informed consent was obtained from each infant's family.

We defined CLD as a requirement for oxygen for 28 days after birth or longer to maintain a PaO_2 >50 mmHg or an arterial oxygen saturation >90%, with symptoms of persistent respiratory distress and an abnormal chest radiograph (a hazy or emphysematous and fibrous appearance). CAM was diagnosed by pathological testing of the polymorphonuclear leukocyte infiltration of the placenta and membranes.

IL-6

Blood samples were obtained from the umbilical cord of each infant immediately after delivery. After centrifugation at 3000 *g* for 10 min, plasma was collected and stored at –70°C until it was ready for use. The concentration of IL-6 in cord blood was determined by the use of a cytometric bead array (CBA) kit (Human Th1/Th2 Cytokine CBA Kit II, BD PharMingen, CA, USA).

β_2-MG

Spontaneously voided urine samples were collected using urine bags within 48 h after birth. No pathological urine sample tests (proteinuria, hematuria, pH <6.0, infection, or abnormal sediment test) were included. Urinary β_2-MG levels were measured by latex agglutination reaction. The urinary levels of creatinine (Cr) were analyzed by the creatinine picrate reaction and used for the normalization of β_2-MG excretion.[17]

Statistics

Values are presented as median and range because they were not normally distributed. Results were analyzed by Mann–Whitney *U* test for comparisons between the two groups, and by Fisher's exact probability test for comparison of proportions. Differences were considered to be significant if the *P*-value was <0.05. To determine the cut-off value, receiver operating characteristic (ROC) curve analysis for relationship was used.

Results

Patients

We divided 29 infants into the following groups (Fig. 1): (i) infants without CLD (non-CLD group, $n = 19$); and (ii) infants with CLD (CLD group, $n = 10$).

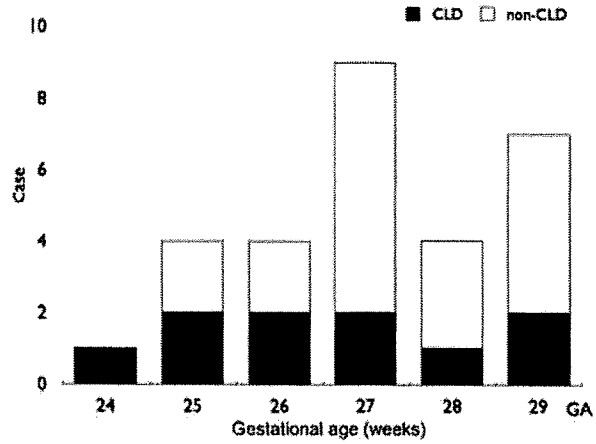

Figure 1 Number of infants with and without chronic lung disease (CLD). There was no significant difference in gestational age between the CLD and non-CLD groups (26.5 *vs* 27.0 weeks).

Table 1 Clinical characteristics of study infants with and without chronic lung disease (CLD)†

	Non-CLD group ($n = 19$)	CLD group ($n = 10$)	*P*-value
Gestational age (weeks)	27.0 (25–29)	26.5 (24–29)	NS
Birth weight (g)	1 036 (734–1 346)	817 (662–1 248)	NS
CAM	12/15 (80.0%)	6/8 (75.0%)	NS
Maternal steroid	7 (36.8%)	4 (40.0%)	NS
RDS	8 (42.1%)	9 (90.0%)	<0.05
Mechanical ventilation	13 (68.4%)	10 (100%)	NS
WBC (/μL)	8 300 (3 930–32 070)	11 100 (4 200–29 190)	NS
CRP (mg/dL)	0.00 (0.0–0.06)	0.02 (0.0–0.53)	NS
Ig M (mg/dL)	7.0 (3.0–52.8)	7.0 (3.0–48)	NS

†Data are presented as median values with ranges in parentheses and numbers with percentage in parentheses. CAM, chorioamnionitis; CRP, C-reactive protein; Ig M, immunoglobulin M; NS, not significant; RDS, respiratory distress syndrome; WBC, white blood cell.

Table 2 Umbilical cord blood interleukin-6 (IL-6) and urinary β_2-microglobulin (β_2-MG) in infants with and without chronic lung disease (CLD)†

	Non-CLD group ($n = 19$)	CLD group ($n = 10$)	*P*-value
IL-6 (pg/mL)	7.6 (0–622)	54.7 (4.0–1425)	<0.005
β_2-MG (10^4 μg/gCr)	9.3 (1.7–46.4)	17.7 (8.1–42.3)	<0.05

†Data are presented as median values with ranges in parentheses.

There were no significant differences in birth weight and gestational age between the groups. Maternal CAM and steroid therapy were not associated with the development of CLD. Respiratory distress syndrome (RDS) was far more common in the CLD group ($P < 0.05$). All patients diagnosed with RDS received surfactant. However, the number of infants mechanically ventilated were not significantly higher in the CLD group than in the non-CLD group (Table 1).

IL-6

The median value for IL-6 was significantly higher in the CLD group than in the non-CLD group (54.7 *vs* 7.6 pg/mL, $P < 0.005$, Table 2 and Fig. 2a). ROC curve analysis demonstrated a cut-off value of IL-6 for predicting CLD was 16 pg/mL (Fig. 3a). Nine of 13 infants (69.2%) with IL-6 ≥16 pg/mL developed CLD, whereas only one of 16 infants (6.3%) with IL-6 <16 pg/mL had CLD. The sensitivity, specificity and positive and negative predictive values were 0.90, 0.79, 0.69, and 0.94, respectively (Fig. 4).

β_2-MG

The median value for urinary β_2-MG in the CLD group was 17.7×10^4 μg/gCr, whereas the median value for urinary β_2-MG in the non-CLD group was 9.3×10^4 μg/gCr (Table 2). The difference was significant ($P < 0.05$, Fig. 2b). A value of 10.0×10^4 μg/gCr was chosen as a cut-off point by ROC curve analysis (Fig. 3b). CLD was diagnosed in nine of 16 infants (56.3%) with β_2-MG ≥10.0×10^4 μg/gCr, in contrast to only one of 13 infants (7.7%) with β_2-MG <10.0×10^4 μg/gCr. CLD was predicted with a sensitivity of 0.90, specificity of 0.63, positive predictive value of 0.56, and negative predictive value of 0.92 (Fig. 4).

Discussion

We report here several meaningful findings in umbilical cord IL-6 and neonatal urinary β_2-MG levels in premature infants with or without CLD. First, umbilical cord IL-6 levels were significantly higher in premature infants with CLD. Neonatal urinary β_2-MG levels were also significantly higher in premature infants who developed CLD (Fig. 2). Second, high sensitivity and negative predictive value of β_2-MG for predicting CLD were comparable to those of IL-6. The specificity and positive predictive value of β_2-MG were lower compared with those of IL-6 (Fig. 4). We suggest that urinary β_2-MG measured within 48 h after birth may be clinically useful for monitoring footprints of FIRS and predicting the risk of CLD development. Urine sample

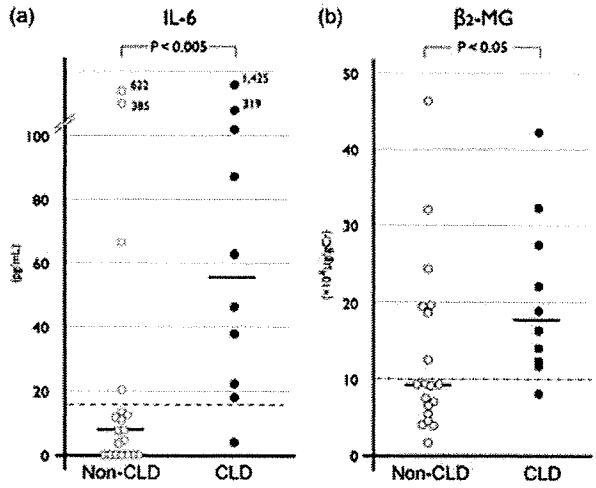

Figure 2 Umbilical cord interleukin-6 (IL-6) and neonatal urinary β_2-microglobulin (β_2-MG) levels in the non-chronic lung disease (CLD) and CLD groups. (a) The median value for umbilical cord IL-6 was significantly higher in the CLD group than in the non-CLD group (median (range), 54.7 (4.0–1425) *vs* 7.6 (0–622) pg/mL; $P < 0.005$). (b) The median value for neonatal urinary β_2-microglobulin (MG) was significantly higher in the CLD than in the non-CLD group (17.7 (8.1–42.3) *vs* 9.3 (1.7–46.4) $\times 10^4$ μg/gCr; $P < 0.05$). The horizontal solid lines indicate the median values and dotted lines indicate the cut-off values.

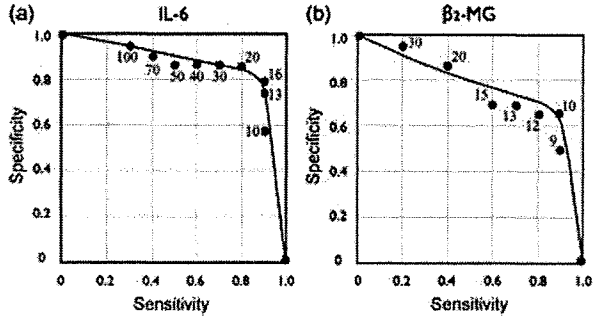

Figure 3 Receiver operating characteristic (ROC) curve analysis for the relationship between chronic lung disease (CLD) and levels of cord blood interleukin-6 (IL-6) and urinary β_2-microglobulin (β_2-MG). (a) ROC curve analysis demonstrated a cut-off value of IL-6 for predicting CLD was 16 pg/mL. (b) A value of 10.0×10^4 μg/gCr was chosen as a cut-off point by ROC curve analysis.

collection offers safe non-invasive access. In addition, we also emphasize that measurement of neonatal urinary β_2-MG is less costly than that of umbilical cord IL-6. We suggest that an elevated urinary β_2-MG level

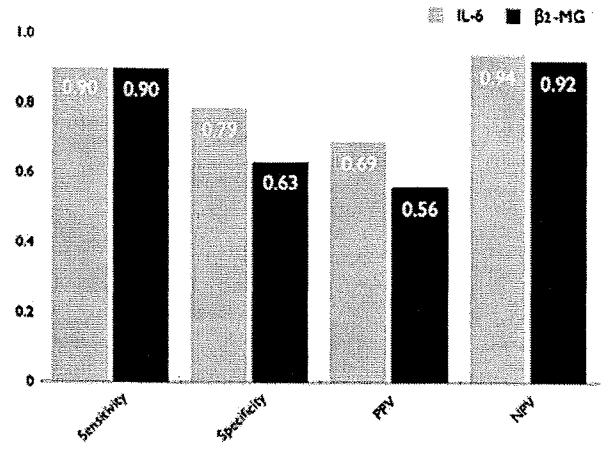

Figure 4 Diagnostic index values in the prediction of chronic lung disease (CLD). Interleukin-6 (IL-6): The sensitivity, specificity, positive predictive and negative predictive values were 0.90, 0.79, 0.69 and 0.94, respectively at the cut-off value (16 pg/mL). β_2-microglobulin (MG): The sensitivity, specificity, positive predictive and negative predictive values were 0.90, 0.63, 0.56 and 0.92, respectively at the cut-off value (10.0×10^4 μg/gCr). NPV, negative predictive value; PPV, positive predictive value.

($\geq 10 \times 10^4$ μg/gCr) may be an early predictor of subsequent CLD development in premature infants. It is clinically important to identify fetuses and/or infants at risk of FIRS and subsequently CLD development. Further immunological studies may lead to new therapeutic strategies for the patients with FIRS with the potential to protect lung tissue and prevent the development of CLD.

Increased urinary β_2-MG levels are seen in proximal tubular dysfunction because β_2-MG freely passes through glomeruli and is almost completely reabsorbed at renal proximal tubules.[18] β_2-MG is a low molecular weight protein that is produced by the expression of major histocompatibility complex (MHC) class I proteins on the surface of most nucleated cells.[19] In inflammation, IFN-γ is known to stimulate β_2-MG synthesis in blood, and then urinary β_2-MG levels rise when blood levels are above the renal tubular reabsorption threshold.[17] In addition to increased urinary β_2-MG excretion (in renal dysfunction), therefore, urinary β_2-MG elevation is also induced by increased blood β_2-MG production (in inflammation).[17] Moreover, a previous study has shown an increased IFN-γ production even in premature infants with maternal CAM.[20] Therefore, we submit that urinary β_2-MG

elevation observed in premature infants within days after birth may be associated with FIR.

A limitation of our study was that we did not collect neonatal urine samples immediately after birth. It was not clear whether the β_2-MG levels in urine obtained within 48 h after birth are associated with the IL-6 levels in umbilical cord blood. To resolve the limitation, urine samples should be collected using urine catheters just after delivery. We emphasize that spontaneously voided urine sample collection using urine bags imposes no stress on infants. A second limitation was that we did not consider the possibility that urinary β_2-MG elevation may be due the renal dysfunction associated with FIRS. More research is needed to evaluate the contribution of FIRS to the increase of urinary β_2-MG. However, any increase in urinary β_2-MG due to renal dysfunction is a consequence of FIRS, and thus the existence of FIRS can be inferred indirectly.

In conclusion, urinary β_2-MG levels measured within 48 h after birth were significantly higher in premature infants who developed CLD than in those who did not. High sensitivity and negative predictive value of urinary β_2-MG for predicting CLD were demonstrated. Although the total number of subjects studied was small, our results suggest that urinary β_2-MG levels in premature infants soon after birth can be predictive of FIRS and may give us information about the risk of subsequent CLD development and that is as clinically important as information derived from umbilical cord blood analyses of IL-6.

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Original Article

Whole blood interferon-γ assay for tuberculosis in children in Japan

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Abstract ***Background***: Whole blood interferon-γ assay QuantiFERON-TB2G (QFT-2G), which is a new specific method for diagnosing tuberculosis (TB), has been developed and used in the clinical field. The aim of the present study was to assess the usefulness of QFT-2G as an indicator, both for diagnosing childhood TB and for assessing therapeutic effectiveness.

Methods: The subjects were 61 children introduced to the TB outpatient department for the first time between June 2004 and March 2006. QFT-2G, the tuberculin test and chest computed tomography (CT) were performed for all patients.

Results: Ten patients having typical characteristics of primary tuberculosis (PTB) on chest CT, and diagnosed as having tubercle bacillus infections, all had positive reaction on QFT-2G. Of seven patients who had no abnormalities on diagnostic imaging but who reacted positively on QFT-2G, one developed TB later, and no TB was detected over the period of observation in 44 patients with negative QFT-2G at their first consultation. Moreover, four patients with non-tuberculous acid-fast bacilli in which *Mycobacterium avium* or *Mycobacterium gordonae* was detected had negative reaction on QFT-2G. In addition, all 10 patients with positive reactions on QFT-2G in whom the subsequent course of the disease was observed had decrease on QFT after treatment.

Conclusions: QFT-2G is a powerful tool with a wide application both in diagnosis and in assessment of treatment effectiveness in PTB.

Key words bacille Calmette–Guerin, child, diagnosis of tuberculosis, interferon-γ, treatment effectiveness.

The main symptoms seen at the initial stage of childhood tuberculosis (TB) are, as in adult TB, no different from those of the common cold, and if the possibility of TB is not considered in the differential diagnosis, it can be overlooked. In addition, after close or prolonged contact with a pulmonary TB patient it is essential to take early measures, including appropriate diagnosis and treatment measures, and so an understanding of the nature of pediatric TB is necessary, as is an effort to identify it in its early stages. Diagnosis of pediatric TB (especially of the infant type)[1] has hitherto been made through an overall assessment of the combined results of patient interviews (with special attention to questions about family history and history of bacille Calmette–Guerin [BCG] vaccinations), the diameter of the tuberculin reaction, blood tests, testing for acid-fast bacilli, and diagnostic imaging (in particular, computed tomography [CT] of the chest).[2,3] The tuberculin test is a powerful diagnostic technique for TB infection, but at 1–2 months after BCG vaccination even if no TB infection has occurred, and many false-positives appear, this reaction cannot offer clear and definite diagnostic evidence of an infection.[4,5] The purified protein derivative (PPD) used for the tuberculin reaction contained several hundred antigens of tubercle bacilli. Most of them bore a close resemblance to BCG antigens or non-tuberculous acid-fast bacteria antigens, so that there were positive tuberculin reactions due to BCG vaccination or to these similar non-tuberculous acid-fast bacteria. Especially in Japan, because BCG vaccination is conducted on a large scale, the diagnosis of tuberculous infections has been made more difficult.

Recently, as a result of gene cloning, two specific antigens have been identified:[6,7] early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are present in the tubercle bacillus group, namely, *Mycobacterium tuberculosis, M. bovis, M. africanum* and only a few non-tuberculous acid-fast bacteria, and which are in the *M. tuberculosis*-specific region of difference 1 (RD-1 region), a region not present in BCG. As a result of these discoveries an entirely new method that uses these antigens not seen in BCG was developed for the diagnosis of TB infections.[6,8,9] The method is as follows: using the interferon-γ (IFN-γ) measurement reagent QuantiFERON-TB2G (QFT-2G; Cellestis, Carnegie, Vic, Australia), and with ESAT-6 and CFP-10 as stimulatory antigens, the patient's whole blood was stimulated within 12 h after being drawn. Culturing was continued at 37°C for 16–24 h. The amount of IFN-γ produced by this induction was then determined using enzyme-linked immunosorbent assay. QFT-2G offers the advantages of speed and more objective results, and it is not necessary to consider the booster effect as with the tuberculin reaction, or, because a

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measured result is obtained, for the patient to have another consultation.

In the Tuberculosis Outpatient Department at Yokohama City University Hospital we carried out tuberculin and QFT-2G testing on pediatric patients, and studied the usefulness of these tests for diagnosis and their significance in the treatment of TB in children.

Methods

The subjects were 61 children (27 boys and 34 girls) visiting the Tuberculosis Outpatient Department at Yokohama City University Hospital for the first time between June 2004 and March 2006. They ranged in age from 1 month to 16 years, and the reasons for their attendance were the presence of symptoms (eight patients), family medical checks (36 patients), contact with a TB patient (five patients), abnormal chest shadows on chest X-ray (one patient), a spontaneous positive reaction in the tuberculin test (eight patients), strongly positive tuberculin tests (one patient), and a positive Koch's phenomenon (two patients). All 53 patients who did not have spontaneous positive reaction on tuberculin testing had had BCG vaccinations (Table 1).

The QFT-2G tests were performed on the whole blood of all patients at first consultation. The method for determining the levels of QFT-2G was as follows. First, using heparin as an anticoagulant, at least 1.5 mL of blood was drawn from each child, and was quickly divided into a 24-well tissue culture plate. A few drops of each of the stimulatory antigen, the negative control and the positive control were added to separate wells, and mixed for 1 min. Next, an 18 h still culture was carried out at 37°C, the resulting supernatant was drawn off and, after the temperature had returned to 17–27°C, was placed in the wells of a human IFN-γ antibody solid-phase plate that already contained horseradish peroxidase-labeled anti-human IFN-γ antibody solution. The resulting solution was then mixed and left to stand for 2 h. Finally, after the plate was washed with washing buffer, the reaction liquid was mixed and then left to react for 30 min in complete darkness. The absorbance was determined and the amount of IFN-γ produced was calculated. The cut-off point of ESAT-6 and CFP-10 was set at 0.35 IU/mL, so that any level above that point for either of them was judged to indicate a positive reaction, but judgment was reserved for results between 0.1 and 0.35 IU/mL.[10] In such cases the tuberculin test, chest X-ray, chest CT, and hematological tests (erythrocyte sedimentation rate [ESR], and leukocyte counts) were performed. A positive diagnosis of active TB was defined as the presence of tubercle bacilli demonstrated in tests of the sputum or gastric juice; or on CT, typical abnormal shadows of TB such as cavitations or nodular shadows, swelling of the hilar lymph nodes, and hypertrophy of the pleura, even when tubercle bacilli cannot be identified. On the basis of these criteria the patients were divided into three groups: group 1, those with typical findings of primary tuberculosis (PTB) on chest CT, diagnosed as having TB, and having positive reaction on QFT-2G; group 2, those without a definitive diagnosis of TB, but with positive reaction on QFT-2G; and group 3, those with negative reaction on QFT-2G. The clinical features and the course of the disease were studied in these groups. We saw the patients every 1–6 months. Also, throughout the course of treatment we followed QFT-2G and CT in 10 patients who had positive reaction on QFT-2G. Seven of the 10 belonged to group 1, and the other three to group 2. And we studied the usefulness of QFT-2G as a method of assessing treatment effectiveness.

The treatment was decided on the basis of imaging and hematology, with reference to the treatment techniques recommended by the American Thoracic Society and the Centers for Disease Control and Prevention.[11] In all cases, every 3–6 months from the start of treatment, depending on the course of the disease, image-based assessments were made using chest radiograph or CT at the same time as the changes in QFT-2G level. Moreover, in relation to the determination of the QFT-2G values, in compliance with the ethics regulations of this hospital, the consent of the child or of the parents was obtained. Each patient's data were kept under strict security.

Results

The 61 patients were divided into three groups, as shown in Table 1.

Group 1 consisted of 10 patients. They ranged in age from 5 months to 16 years (average age, 8.6 ± 5.0 years), and the reasons for their attendance were presence of symptoms in four, family medical checks in two, contact with a TB patient in two, and a spontaneous positive reaction in the tuberculin test in two. Tuberculin reactions were positive in nine patients. Average ESR was 26.3 ± 20.8 mm/h. In pediatric TB the rate of bacterial elimination is low, and tubercle bacilli were seen in only five of these 10 patients. In the other five, cavitations or nodular shadows, swollen hilar lymph nodes, thickening of the pleura, and other characteristic findings of TB were present, and an overall diagnosis of TB infection was made. These 10 patients had positive reaction on QFT-2G. Average QFT-2G (ESAT-6 and CFP-10) was 7.098 ± 7.337 IU/mL and 6.713 ± 7.421 IU/mL, respectively.

Group 2 consisted of seven patients who were QFT-2G positive, but whose other tests did not point to a diagnosis of TB. They ranged in age from 1 month to 12 years (average age, 5.4 ± 4.7 years), and the reasons for their attendance were family medical checks in six, and a spontaneous positive reaction in the tuberculin test in one. Tuberculin reactions were positive in all seven patients. Average ESR was 10.6 ± 3.6 mm/h. Average QFT-2G (ESAT-6 and CFP-10) was 14.368 ± 4.470 IU/mL and 6.074 ± 6.119 IU/mL, respectively. In none of the seven could TB be diagnosed from chest X-ray or CT, and chemoprophylaxis was started. Patient 14 was given isoniazid internally for chemoprophylaxis from the outset because the mother had contracted TB, but during the course of observation an infiltrative shadow appeared in the left lung field on chest CT and TB was diagnosed. In the other six patients, however, no sign of TB manifested in the 6 months to time of writing, and the course of the disease was followed at regular intervals.

Group 3 consisted of 44 QFT-2G-negative children. They ranged in age from 1 month to 16 years (average age, 5.7 ± 4.6 years), and the reasons for their attendance were presence of symptoms in four, family medical checks in 28, contact with a TB patient in three, abnormal chest shadows on chest X-ray in one, a spontaneous positive reaction in the tuberculin test in five, strongly

Table 1 Patient profiles

Group	Patient ID no.	Age (years)	Reason for visiting clinic	BCG	Tuberculin test (mm)	Culture for *Mycobacterium* spp.		ESR (mm/h)	Chest CT	QFT-2G (IU/mL)	
						Sputum	Gastric lavage			ESAT-6	CFP-10
1	1	14	Fever, cough	+	0 × 0/12 × 12	*M. tuberculosis* 2 +	NP	36	Cavity formation and infiltration in left upper lobe	16.148	16.148
	2	13	Cough	+	15 × 14/19 × 16	*M. tuberculosis* 2+	NP	41	Cavity formation and infiltration in right upper lobe	17.049	16.144
	3	14	Cough	+	NP	*M. tuberculosis* 2+	NP	20	Cavity formation and infiltration in right upper lobe	2.852	8.366
	4	13	Fever, cough	+	23 × 4/60 × 25	NP	–	57	Tuberculoma in left upper lobe	8.001	–0.161
	5	8	Family examination	+	27 × 30/67 × 49	*M. tuberculosis* 2+	NP	10	Tuberculoma in left S6 area	4.66	16.003
	6	6	Family examination	+	19 × 12/52 × 29	NP	*M. tuberculosis* 2+	12	Normal	0.49	0.27
	7	8	Contact with TB patient	+	9 × 7/20 × 20	NP	–	59	Right pleural effusion, thickening of pleural membrane	0.460	0.084
	8	9	Contact with TB patient	+	7 × 7/25 × 20	NP	–	3	Tuberculoma in right upper and lower lobes	0.446	0.176
	9	0.83	Positive reaction in tuberculin test	–	10 × 10/20 × 20	NP	–	4	Infiltration in left upper lobe, swelling of left hilar lymph nodes	1.744	9.933
	10	0.42	Positive reaction in tuberculin test	–	10 × 10/27 × 27	NP	–	21	Infiltration in right upper lobe, swelling of right hilar lymph nodes	2.02	0.162
2	11	7	Family examination	+	11 × 11/24 × 23	NP	–	13	Normal	0.022	2.17
	12	6	Family examination	+	17 × 14/33 × 20	NP	–	17	Normal	1.073	1.903
	13	1	Family examination	+	0 × 0/10 × 10	NP	–	8	Normal	11.207	11.117
	14	2	Family examination	+	15 × 15/30 × 30	NP	–	8	Normal	3.284	16.684
	15	7	Family examination	+	20 × 19/20 × 19	NP	–	9	Normal	17.529	0.456
	16	12	Family examination	+	23 × 19/40 × 35	–	NP	9	Normal	1.48	8.303
	17	1	Positive reaction in tuberculin test	–	0 × 0/17 × 17	NP	–	NP	Normal	7.811	1.887

CFP-10, culture filtrate protein 10; CT, computed tomography; ESAT-6, early secretory antigenic target 6; ESR, erythrocyte sedimentation rate; NP, not performed; QFT-2G, QuantiFERON-TB2G; TB, tuberculosis.

positive tuberculin tests in one, and a positive Koch's phenomenon in two. Tuberculin reactions were positive in 24 cases. Average ESR was 8.5 ± 12.6 mm/h. Average QFT-2G (ESAT-6 and CFP-10) was 0.004 ± 0.012 IU/mL and 0.004 ± 0.019 IU/mL, respectively. On chest X-ray and CT no hilar lymph node swelling or nodular shadows were seen, and TB was not diagnosed in any patient, either at the initial consultation or during the study period of 18 months. *Mycobacterium avium* was detected in two patients, and *M. gordonae* in two patients, but QFT-2G was negative in these four patients.

We then examined the usefulness of QFT-2G for judging treatment effectiveness. More than 6 months after treatment was terminated, in the 10 QFT-2G-positive patients who could be examined, the changes in QFT-2G levels were examined (Fig. 1). Seven of the 10 belonged to group 1, and the other three, to group 2. The imaging findings in the seven from group 1 improved with treatment, and also, the QFT-2G decreased. When the three from group 2 were given isoniazid internally for chemoprophylaxis, a fall in QFT-2G was seen. Five of the 10 patients became QFT-2G negative. It was observed that, although three (two in group 1 and one in group 2) of the five patients who continued to be positive had no change in imaging findings, their QFT-2G levels rose, and then fell naturally.

Discussion

This study examined whether QFT-2G is useful as an indicator in the diagnosis and treatment of childhood tuberculosis. In all patients who had cavitations, swelling of the hilar lymph nodes, nodular shadows and other characteristic findings of pulmonary TB, and who were therefore diagnosed as having TB, QFT-2G was positive, and in one asymptomatic patient who had no abnormal imaging findings, but who had a positive result on QFT-2G, TB developed later. In patients who had negative QFT-2G at the initial consultation, TB was not found to have developed during the subsequent observation, and in patients with a history of BCG vaccination and positive tuberculin reactions, QFT-2G was negative, and it was confirmed that QFT-2G was unaffected by BCG. Moreover, in cases of non-tuberculous acid-fast bacilli in which *M. avium* or *M. gordonae* was detected, just as was stated in the published reports,[5,12] QFT-2G was negative. For patients in whom it was possible to observe the changes in QFT-2G together with the course of treatment over time, the QFT-2G level fell significantly in accordance with the improvements in symptoms.

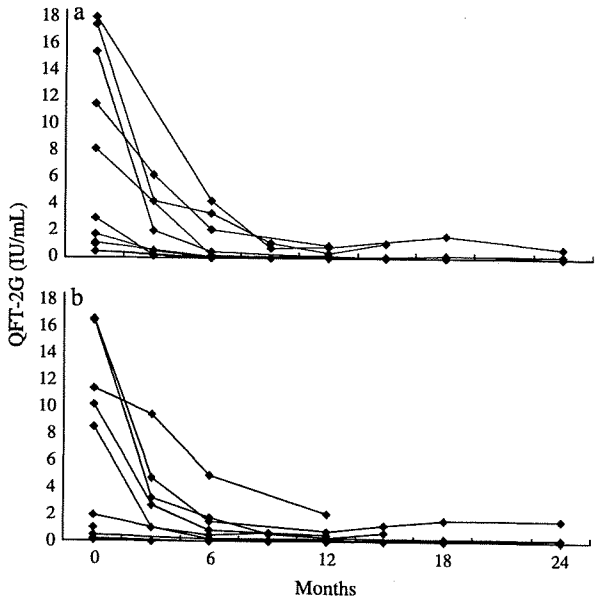

Fig. 1 Change in QFT-2G (QuantiFERON-TB2G) for (a) early secretory antigenic target 6 and (b) culture filtrate protein 10 for 10 tuberculosis patients (group 1, $n = 7$; group 2, $n = 3$).

In a clinical study conducted in the Tuberculosis Outpatient Department at Yokohama City University Hospital, 105 children with TB were examined during the 26 years between 1975 and 2001. The characteristics of childhood TB and how they differ from adult TB were clarified. The main points were as follows:[1–3] (i) childhood TB has a clinical pattern that differs from that of adult TB: in babies and infants the symptoms caused by the tubercle bacillus appear almost immediately, whereas in adults the tubercle bacillus responsible for the previous infection is reactivated and spreads through the lung by lymphogenous metastasis, forming cavities; (ii) the majority of child TB patients are aged between 0 and 6 years; (iii) most cases (>95%) of infant TB result from familial infections; (iv) out of 79 children under 7 years old, 17 had hematogenous dissemination, which causes meningitis and miliary TB, and the outcomes of these serious cases of TB were extremely poor: three children died and nine other children had severe sequelae, indeed, in infants the disease is liable to progress in this way, resulting in serious cases of TB such as meningitis and miliary TB; and (v) in infants the excretion rate of the tubercle bacilli is extremely low. In addition, some of the typical results from tests of such cases are the following: (i) there are no specific blood test abnormalities found in childhood TB, because tests such as ESR and leukocyte counts show little variation, and it is difficult to offer one of these as an index of activity; (ii) in infants, if activity is present, the serum IgM rises to more than twice the age of the child; (iii) plain chest X-ray rarely shows any detectable abnormality, and so it is safer to perform chest CT, which can give a great deal of information; (iv) attention should be given to swelling of the hilar or parabronchial lymph nodes; and (v) cavitation does not occur in pediatric pulmonary TB, and microscopy of gastric juice and sputum rarely results in the detection of tubercle bacilli. In view of the aforementioned characteristics of childhood TB, its diagnosis (especially that of infantile-type TB) has been based on an overall assessment of the medical interview (particularly replies about BCG vaccination history and family history), the diameter of the tuberculin reaction, hematological tests, tests for acid-fast bacilli, and diagnostic imaging (in particular, chest CT). A characteristic finding, however, particularly deserving of attention is that, in infantile TB, there are few cases of excretion of bacilli. The amount of excretion of bacilli is also small, and the proportion of cases in which bacteria are detected in actual microscopy is ≤approximately 8% (and even in cultures, up to approx. 35%),[1–3] and therefore, if the detection of bacteria is taken to be the criterion, it is extremely difficult to make a definite diagnosis of infantile TB.

In the past the tuberculin reaction was one of the most powerful methods of detecting whether a TB infection was present or not, but, owing to the effects of the BCG vaccination, this reaction yields false-positive results despite the absence of infection by tubercle bacilli, and therefore presents problems in a clinical setting.[4,5]

The newly developed QFT-2G is attracting much attention as an innovative tool for testing for the presence of a tubercle bacillus infection without being affected by previous BCG vaccinations.[8,9] In this method, 5 mL of blood is drawn from the subject, and the stimulatory antigens are added to the sample and left to stand overnight. Then, on quantitative analysis of IFN-γ, which is produced by the T lymphocytes (in particular, effector T-cells), it is possible to assess whether the body has produced a cellular immune reaction and, if so, the strength of that reaction.[6,8,13] The non-homologous portion of the amino acid sequence of BCG and the human-type tubercle bacillus was identified using new molecular biological techniques, and this method uses this non-homologous portion as a stimulatory antigen. In Japan, where BCG vaccinations are given during childhood, this is considered to be of great significance. In a QFT-2G trial using 118 patients with smears positive for TB and the 220 patients who had had BCG vaccinations, the sensitivity and specificity, when the cut-off for the IFN-γ produced was set at 0.35 IU/mL, were high at 89.0% and 98.1%, respectively, suggesting that this method is highly effective for diagnosing TB infection.[10] In addition, recently, many articles comparing the usefulness of QFT-2G in adult patients for treating active and latent TB with the results of the tuberculin reaction have appeared, and QFT-2G has been used widely in clinical research to indicate, for example: (i) diagnosis of active disease; (ii) differences between infection with TB or non-tuberculous acid-fast bacteria; (iii) differences between TB infections and BCG vaccine administered previously; (iv) foreknowledge of the reactivation of latent TB; and (v) assessment of treatment efficacy.[13–19] It has been proved that this method of examination has a higher specificity, and the cross-reactivity between BCG vaccine and non-tuberculous acid-fast bacteria is low.[13–15]

The number of effector T-cells is greater when antigen presentation occurs in an infectious condition than in a non-infectious condition, and the QFT-2G level, that is, the amount of production of IFN-γ, increases. When the activity of the infection is lessened by treatment, the number of effector T-cells circulating in the peripheral blood decreases, and the memory T-cells increase in number. Because the amount of IFN-γ produced by the memory T-cells is small, the measured IFN-γ falls. The culture period of QFT-2G is 18 h, and it is thought that the IFN-γ detected is provided by the effector T-cells. Theoretically speaking, if the infection is active, the QFT-2G level is high, and when the infection is inactive, the IFN-γ amount is thought to fall. In the present study also, when treatment was begun, the imaging findings improved, and at the same time, QFT-2G fell, and these changes are useful in assessing the effects of treatment. The present study also showed that, at the end of treatment, approximately half of the patients were QFT-2G negative, while the other half continued to be positive. The imaging findings are considered to be important for judging when to terminate treatment; and for diagnosing a relapse, the imaging findings are thought to be important. A long period of observation is necessary to see how the QFT-2G will change.

The question of how much blood should be drawn for QFT-2G in childhood patients has been raised, but the number of lymphocytes from whole blood required for this test is greater in infants and children than for adults, but we have found that a sample of $\geq$1.5 mL provides test results that are reliable. An examination based on a greater number of cases, however, should be done to ascertain the amount of blood suitable for a sample.

In the present paper the usefulness of QFT-2G for the purposes of diagnosis and assessment of treatment results, without distortion by BCG vaccination history, has been acknowledged. The present study is believed to have demonstrated, even in childhood TB patients in whom diagnosis is difficult because the excretion rate of bacilli is low, that QFT-2G has valuable qualities as an alternative test method for diagnosis and treatment outcome assessment. We intend to determine the significance of these results by accumulating data on more cases of childhood TB.

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