経由でサーバに登録していく。指導者は，サーバの記録を参照しながら，参加者に電子メールを用いて個別に支援する。

5.3 参加者向けインタフェースの充実

ユーザーインタフェースは，簡便に体重・行動記録が登録できること，生活習慣改善につながるように行動の記録がわかりやすく把握できる機能を充実させることに配慮した。このため，1日のカロリーを自動計算させ，画面で対話的に100 kcalカードを選択できるようにした(図5参照)。

また，参加者の減量記録画面では，100 kcalカードの実施状況に応じて，「ニコニコ」または「ガッカリ」の表情をアイコンにした。体重の増減に影響のある飲み会・間食・出張・改善項目以外のスポーツ・深夜の食事・体調不良などをイベントとして登録し，体重の変動として連携して行動記録が目で見てわかりやすいようにしている。どうすれば減量し，どのようなイベントでリバウンドするかが手に取るようにわかるようになっている(図6参照)。

5.4 業務支援機能

大人数に対して「はらすまダイエット」を実施する場合，指導者のリソースの制約により，参加者全員の個別指導が難しいことと，指導者1人当たりの業務量が増大することが課題になると予測される。これらを解決すべく，指導対象者抽出技術と定型業務管理技術を新たに開発した。

指導対象者抽出技術は，体重とダイエット実施状況に基づき，減量が順調な人と，そうでない人を順位づけして，努力の割に減量がうまくいっていない支援の必要性の高い人を重点的にピックアップする。定型業務管理技術は，あらかじめ設定したタイミング，条件，処理を登録し，減量経過に応じて個別指導の要否判定や電子メール文面案の作成などを自動的に処理する。

指導者による参加者への電子メール作成・送信手順の一例を図7に示す。指導者は，まず，減量プログラム参加者一覧画面でその日にやるべき業務を確認する〔同図(1)参照〕。

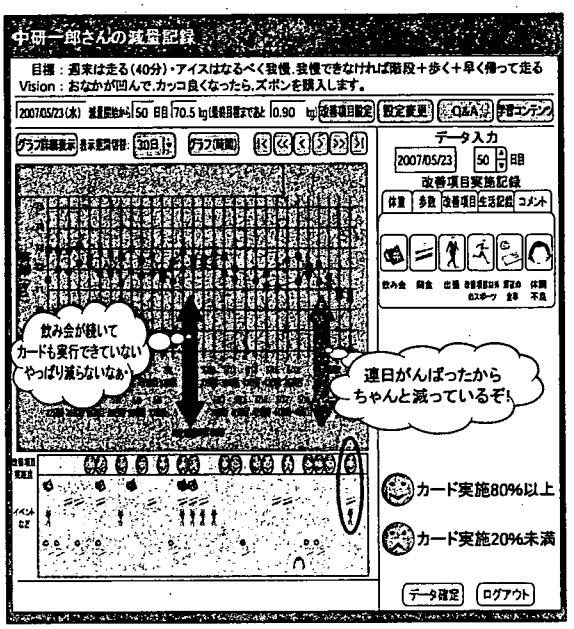

図6 参加者の減量記録画面例
減量したときやリバウンドしたときの状況が，画面を通して手に取るようにわかる。

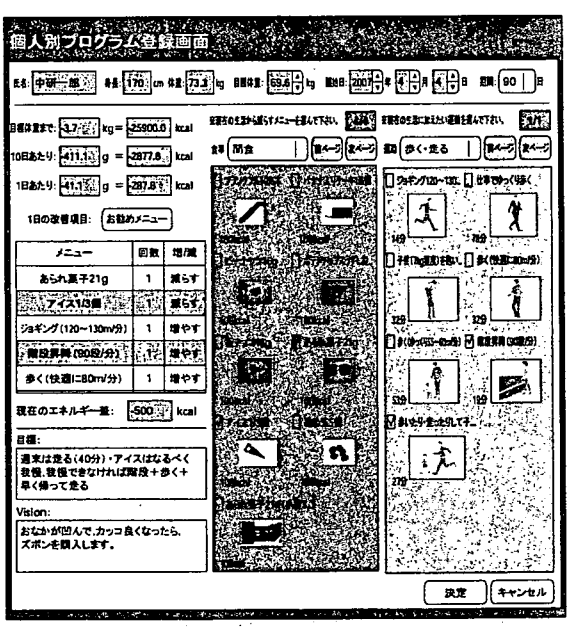

図5 「はらすまダイエット」個人別登録画面例
簡単に体重・行動記録が登録できるユーザーインタフェースに配慮している。

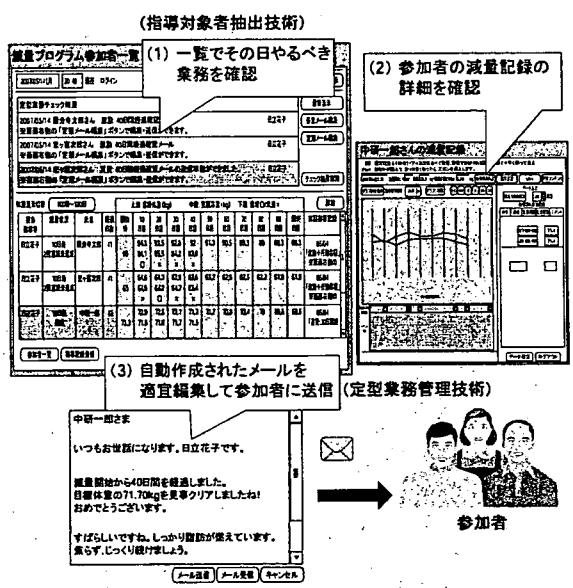

図7 指導者の指導業務の例
指導者による参加者への電子メール作成・送信手順の例を示す。

この一覧画面は，減量がうまくいっていない人が上位にリストアップされ，重点的に減量記録や行動の記録の詳細を確認することができる〔同図(2)参照〕。そして，定型業務管理技術を用いて自動作成されたメール文面を，記録の確認結果に基づき適宜編集し，参加者に電子メールを返信する〔同図(3)参照〕。このような業務の自動化を図ることにより，限られた指導者でも，より多くの参加者に「はらすまダイエット」の指導ができるようになる。

6. おわりに

ここでは，特定健診・特定保健指導の概要と，生活習慣を改善することを目的に開発された減量プログラム「はらすまダイエット」，および効率的かつ効果的に実施するための遠隔指導支援システムについて述べた。

太っていること自体が悪いのではない。実際に「ちょい太」がいちばん長生きであることはよく知られる。簡単な目安は20歳の体重の10 kg増までと言われている。血圧や血液検査で異常のない健康的でかっぷくのいい人に対して，安易にメタボリックシンドロームの疑いをかけることは厳に慎まなければならない。

ただ，残念なことに多くのメタボリックシンドロームの人たちは限度を超えて内臓脂肪が蓄積し過ぎている。そして実際に体が悲鳴を上げて警告を発している。それは，過去10年の体重，血圧，血糖，中性脂肪，尿酸，LDLコレステロールの変動を見ていけばすぐにわかる。メタボリックシンドロームと診断された人は，現在の体重の5%を減量目標にして，3か月かけてじっくりと減らすのがよい。内臓脂肪から減っていくので，必ず血圧や血糖，中性脂肪は改善する。

先進国では，肥満は疫病(しっぺい)に例えられるほど，その抑止の困難さが伝えられるが，今回のわが国での特定健診・特定保健指導がどのような展開となるか，大きな社会実験と言える。将来，日本でメタボリックシンドロームを抑止できたとしたら世界から尊敬される国になるであろう。

ヘルスケアの本質は，セルフケア，セルフコントロール，セルフコーチングであり，「心と体」をコントロールするために，客観的な目で励ますコーチを自分の中に据えることにある。客観的に見るためには，どうしても記録が必要となる。記録をすること，しかもそれを続けることがヘルスケアには欠かせない。「はらすまダイエット」で体験した90日間の記録は自身の大切な宝の情報になるであろう。

最後に，メタボリックシンドロームに向き合い，対処する「はらすまダイエット」の極意は，「無理なことはやらない。がんばらない，けれども簡単にはあきらめない。」ということである。

なお，「はらすまダイエット」は，学校法人産業医科大学公衆衛生学教室，株式会社損保ジャパン総合研究所との共同研究に基づき開発された。共同研究関係各位に，この場を借りて厚く御礼申し上げる次第である。

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ＣＴによる造船所近隣住民の胸膜プラークについての検討

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原著

CTによる造船所近隣住民の胸膜プラークについての検討

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わが国でも悪性中皮腫の増加が認められ、過去の石綿（アスベスト）の使用との関連が問題になっている。現在は、職域検診としてのアスベスト検診が中心であり、一般住民の石綿曝露への対策は不十分である。そこで、住民検診受診者に胸膜プラークがどの程度存在するのか、また、アスベスト検診におけるCTの有用性について検討した。

人口7万人の造船が基幹産業である岡山県玉野市の住民を対象とした。平成18年度に住民検診の間接X-Pを受けた8,982人のうち、アスベストによる胸膜プラークが疑われたのは172例（1.91%）であった。調査の同意が得られた144人に対して、詳しい職業歴の聴取を行い、すでに塵肺手帳をもっている29人を除いた115人にCT検診を実施した。その結果は石灰化を伴うプラーク（plc）が73例、石灰化を伴わないプラーク（pl）が21例で、間接X-Pによるプラーク正診率は81.9%であった。プラーク以外の所見として中皮腫1例、石綿肺1例、良性石綿胸水1例、びまん性胸膜肥厚2例、円形無気肺2例、器質化肺炎1例が見つかった。職歴調査からはまったくアスベスト職歴のないものは7.0%と、ほとんど何らかのアスベスト関連職業歴があった。

従来の間接X-Pによる検診でもプラークが正確に指摘できているが、早期の中皮腫やアスベスト肺などはCTによらなければ指摘できないことがわかり、アスベスト対策においても胸部ＣＴ検診が有用であることが示唆された。

キーワード：　胸部ＣＴ検診、アスベスト、胸膜プラーク、中皮腫
J Thorac CT Screen 2007;14:91-95

はじめに

欧米各国ではアスベスト被害の「指標疾患」とされている悪性胸膜中皮腫は、わが国では比較的まれな疾患といわれ、あまり注目されてこなかったが、過去のわが国のアスベストの多量の使用が原因で徐々に増加傾向を示しており、今後も増加が続き、2000から2029年までの30年間に58,000人の死亡が予想されるまでになっている[1]。中皮種は過去の石綿（アスベスト）曝露により生じると考えられており[2]、わが国で社会に大きな不安を与えている。中皮腫の発生部位は胸膜が90%近くを占めており、胸部レントゲンで発見されることが多い。胸部CT検診は肺癌の早期発見には有用であることは明らかであるが[3]、中皮腫の早期発見に有効であるかは明らかではない。また、胸膜プラークがアスベスト曝露をあらわし、しかも胸膜中皮腫の危険指標になるとも言われており[4]、胸部検診でプラークを指摘することは重要であると考え

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られている。ただし、石綿使用から中皮腫や肺癌発生までに20年以上、平均40年かかるといわれており、職域検診のみではfollow upは困難で、退職後はかなりの数の石綿曝露者が住民検診を受診することになると思われる。われわれは岡山県において、地域によっては住民検診の間接X-Pでかなり高率に石綿による変化が認められることを指摘してきたが、間接X-Pの所見が実際のプラークをどの程度正確に反映しているか、また、アスベスト検診にCTを導入することで、アスベスト関連疾患が発見できるかは不明である。そこで今回、アスベスト関連企業がある玉野市住民を対象にCT検診の石綿（アスベスト）関連疾患発見に対する有用性を検討した。

対象と方法

人口７万人の基幹産業が造船である岡山県玉野市住民のうち、2006年度に間接X-P検診を受診した8,982人を対象とした。間接X-PはHITACHI DWS-1510Jを使用して、140kV、90mAでフォトタイマーにて撮影した。間接X-Pで胸膜プラークを疑わせる所見を認めたものに対して、指定医療機関での胸部CT検診受診を勧奨した。また、同時にアスベストに関する職歴を個別に聴取した。間接X-Pは専門医２名による二重読影で胸膜プラーク所見を評価した。

一方胸部ＣＴは各医療機関での独自の条件で撮影され、低線量ではなく、通常線量による撮影となった。当施設ではSOMATOM AR. SPを使用して、感電圧110kV、感電流83mA、1.9Sで撮影した。CTでの胸膜プラークは限局性、平板状で平滑あるいは結節状の胸膜肥厚として見られ、石灰化を伴わなくてもＣＴ値はやや高く、筋層ないしそれ以上であることをめやすに判断した。

結果

間接X-P検診の結果を表１に示したが、受診者8,982人のうち1.91%にあたる172例に胸膜プラークを疑わす所見があった。性別では男性が大半を占めていた。172例のうち調査に同意が得られた144例を訪問して職歴などを聞き取り調査した。144例のうちすでに労災手帳を所得している29例を除く、115例にCT検診を施行した。

Table 1. Pleural plaque detected by miniature photofluorography

	No. of screenees	No. of cases with plaque	Positive rates (%)
Male	2,511	163	6.49
Female	6,471	9	0.14
Total	8,982	172	1.91

Table 2. Characteristics of the participants examined by chest CT

No. of participants		115
Sex	male	108
	female	7
Age	mean	76.8
	range	58-95
Asbestos plaque		94
	positive rates	81.7%
pl (pq3b and pq4)		21
plc (pq5)		73

CT検診を受けた115例の臨床的特徴を表２に示した。男性が93.9%で平均年齢が76.8歳であった。CTでもアスベストによる胸膜プラークを認めたのは94例で、間接X-Pとの一致率は81.7%であった。そのうち石灰化を認めない胸膜肥厚（pl）が21例、石灰化を認める胸膜肥厚（plc）が73例であった。

間接X-Pで誤って胸膜プラークとしたものは、CTでは前鋸筋と外斜胸壁筋が重複して生じた陰影や胸膜外脂肪組織および肋骨随伴陰影と思われた。

年齢ごとにプラーク所見をみると（図１）、

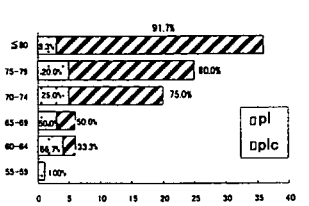

Figure 1. Asbestos plaque percent distribution by age for 94 cases detected by chest CT

若い年齢では少なく、高齢になるほど比率が高くなっていた。特に石灰化を伴う胸膜肥厚所見にその傾向が著しかった。

表３には最終的に診断されたアスベスト関連疾患を示す。肺癌は見つからなかったが、中皮腫やびまん性胸膜肥厚など一般に予後不良と言われている疾患が CT を受けることで指摘されている。肺癌と鑑別が難しい器質化肺炎が１例発見されているが、気管支鏡検査

Table 3. Asbestos-related diseases detected by chest CT screening

Disease	No. of Patients
Mesothelioma	1
Asbestosis	1
Benign asbestos pleusy	1
Diffuse pleural thickening	2
Rounded atelectasis	2
Organizing pneumonia	1

にて肺癌は否定された。このうち胸膜中皮腫例は間接 X-P ではプラーク疑い程度であったが、２ヵ月後の CT では胸水が出現しており、比較的短い間隔で進行したものと思われる。びまん性胸膜肥厚は間接X-PおよびCTともに胸膜の不規則な肥厚所見のみで、他院で過去にびまん性胸膜肥厚とすでに診断されていたことが調査で判明した。予後不良な疾患であるが、この症例の場合は数年前と比べ呼吸機能の悪化は認められなかった。

住民を対象にした検診であるが、詳細な職

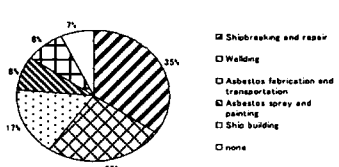

Figure 2. Distribution of Occupational exposure

業歴を聴取すると、図２に示すように異常陰影を認める大半の症例で何らかの石綿関連職歴を有していた。まったく認めないものはわずか 7%に過ぎなかった。

考察

最近、アスベスト鉱山、工場周辺、ビル破壊現場の近くでの近隣曝露や、直接アスベストをあつかう労働者ではない家族（傍職業性家庭内曝露）についての報告も増えている[5]。わが国でも熊本県での鉱山近隣居住者へのプラークの多発[6]や石綿工場近くに住んでいた主婦の中皮腫例が報告されて、今後、一般住民のアスベスト健康被害も心配されている[7]。しかし、わが国の一般住民にしめる石綿曝露者の正確な比率は明らかではなく、その発見方法についても意見が分かれている。このような状況を踏まえて、今回の研究はアスベストを多量に使う造船を基幹産業としている岡山県の玉野市で、住民検診の受診者にどの程度アスベスト曝露を示唆する所見があるかどうかを調査したものである。

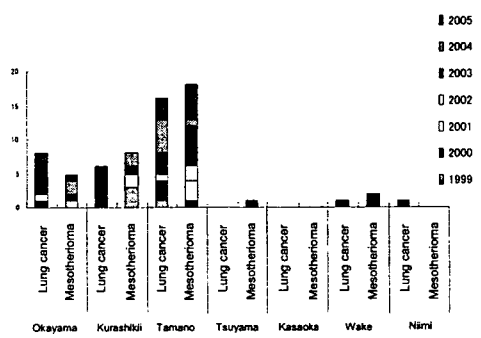

Figure 3. No. of compensation for industrial accidents by Prefectural Labor Standards Office in Okayama

図３に示すように、玉野地区は肺癌および中皮腫の労災補償件数が岡山県内の他の地区に比べて非常に多い地域である。

CT に比べ、間接 X-P での指摘率には限界があるが[8]、9000 人の受診者のうち 1.91%にアスベスト曝露歴を示すプラークを認めたことは、今後のアスベスト対策に示唆を与えるものである。

間接 X-P プラークは脂肪などを間違っているとの指摘もあるが、今回の CT による検診結果から 81.7%は正確に拾い上げていることがわかった。しかし、われわれが間接 X-P と CT を同時に行った検診の成績から、CT でのみ指摘できるプラークはこの倍近く存在することがわかっており[8]、この玉野地区全体に CT 検診を施行すれば、相当数のアスベスト曝露者が存在することを示せるであろう。もちろ

ん胸部ＣＴでもすべてのアスベスト曝露を指摘できるわけではなく、胸部CTで異常を認めない受診者の追跡も必要であろう。

いずれにしても精度の高いアスベスト検診を実施し、中皮腫や肺癌のスクリーニングに役立てるためには、従来の胸部X-P検診では不十分で、胸部ＣＴを用いた検診がぜひ必要である。

プラーク所見は年齢の高い人ほど多くなっていたが、アスベストに曝露してからプラークが出現して、それが石灰化していくのに年月を要することと、アスベスト対策が最近になるほど進んできて、若年層では曝露者が減少したことを示しているのかもしれない。

今回の115例のCT検診によって、中皮腫が1例発見できたことは衝撃的であるが、偶然の可能性も高く、アスベストCT検診を評価するためには実施症例数を増やしての検討が必要である。

今回の検診の目的のひとつに、一般住民のアスベスト曝露の比率を明らかにする目的もあったが、実際にプラーク有所見者の問診からは、アスベスト関連職歴を有している受診者が大半を占めており、環境曝露の可能性があるのは７％と極めて少なかった。

しかし、わが国では非常に多くの製品にアスベストが使用され、使用者や作業従事者がそれを認知せずにいることは問題であり、一般住民の不安を取り除くためにも、アスベスト環境曝露の実態調査は必要と思われ、現在計画されているアスベストCT検診[10]の成果に期待したい。

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Pleural plaques in residents living in a shipbuilding city in Okayama Prefecture

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The incidence of malignant mesothelioma related to asbestos exposure is increasing in Japan. It is important to know the prevalence of pleural diseases by asbestos-exposure among residents living near a manufacturer dealing with asbestos in addition to its employers. However the prevalence of pleural plaques in Japanese residents remains unclear. Therefore, this study was undertaken to assess the prevalence of pleural plaques in such residents detected by miniature photofluorography and CT. Miniature photofluorography screening was performed for 8,982 residents in Tamano city, the western part of Okayama Prefecture on 2006, in which there is a shipbuilding company. One hundred and seventy-two of the 8,982(1.91%) had pleural plaques detected by miniature photofluorography. The CT screening was performed in 115 (66.9%) of the 172 subjects, with questionnaires which provided all the data required for assessing the possibility of asbestos exposures including occupational history. As a result, we detected 73 and 21 pleural plaques with and without calcification, respectively in this CT screening. Additionally, several other radiological findings were detected in the screening; malignant pleural mesothelioma (1), asbestosis (1), benign asbestos pleusy (1), diffuse pleural thickening (2), rounded atelectasis (2) and organizing pneumonia (1). Ninety-three percents of the 94 subjects with pleural plaques had an asbestos-related occupational history. In conclusion, CT screening in asbestos-exposed residents was beneficial to detect asbestos related disease.

Key words: Thoracic CT screening, Asbestos, Pleural plaque, Mesothelioma
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Whole Blood Interferon-Gamma Assay for Baseline Tuberculosis Screening among Japanese Healthcare Students

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Background. The whole blood interferon-gamma assay (QuantiFERON-TB-2G; QFT) has not been fully evaluated as a baseline tuberculosis screening test in Japanese healthcare students commencing clinical contact. The aim of this study was to compare the results from the QFT with those from the tuberculin skin test (TST) in a population deemed to be at a low risk for infection with *Mycobacterium tuberculosis*. *Methodology/Principal Findings.* Healthcare students recruited at Okayama University received both the TST and the QFT to assess the level of agreement between these two tests. The interleukin-10 levels before and after exposure to *M tuberculosis*-specific antigens (early-secreted antigenic target 6-kDa protein [ESAT-6] and culture filtrate protein 10 [CFP-10]) were also measured. Of the 536 healthcare students, most of whom had been vaccinated with bacillus-Calmette-Guérin (BCG), 207 (56%) were enrolled in this study. The agreement between the QFT and the TST results was poor, with positive result rates of 1.4% vs. 27.5%, respectively. A multivariate analysis also revealed that the induration diameter of the TST was not affected by the interferon-gamma concentration after exposure to either of the antigens but was influenced by the number of BCG needle scars ($p = 0.046$). The whole blood interleukin-10 assay revealed that after antigen exposure, the median increases in interleukin-10 concentration was higher in the subgroup with the small increase in interferon-gamma concentration than in the subgroup with the large increase in interferon-gamma concentration (0.3 vs. 0 pg/mL; $p = 0.004$). *Conclusions/Significance.* As a baseline screening test for low-risk Japanese healthcare students at their course entry, QFT yielded quite discordant results, compared with the TST, probably because of the low specificity of the TST results in the BCG-vaccinated population. We also found, for the first time, that the change in the interleukin-10 level after exposure to specific antigens was inversely associated with that in the interferon-gamma level in a low-risk population.

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INTRODUCTION

Tuberculosis continues to be a heavy burden on human health [1]. Especially, healthcare workers are at increased risk of infection because of occupational exposure to tuberculosis patients, although in Japan this risk is lower than in the low- and middle-income countries [2–4]. Indeed, female nurses have a risk for developing active tuberculosis that is two-fold higher than that of the general population in Japan (odds ratio of 2.3) [5]. One of the important component of tuberculosis infection control is the routine screening of healthcare workers for latent tuberculosis infection and the administration of chemoprophylaxis to those who test positive. The tuberculin skin test (TST) has been the only practical means for this purpose in the past century.

At Okayama University Hospital, tuberculosis patients are incidentally referred to the hospital before a definite diagnosis has been confirmed, even though the hospital does not have a tuberculosis-specific ward. Thus, not only healthcare workers, but also healthcare students studying at our hospital are at risk of tuberculosis exposure. As an infection control policy, a TST must be taken by all healthcare students and is optional for first-year postgraduate students prior to the start of clinical training, similar to the policies of many other medical universities [6]. The main purpose for performing a TST in this situation is to identify the baseline immune status of the healthcare students in case of future contact with tuberculosis patients, rather than screening for latent tuberculosis infection, because of the very low estimated prevalence of tuberculosis infection among young people in Japan [7].

Unfortunately, the TST has several major limitations; one of them is the possible occurrence of false-positive test results in individuals vaccinated with bacillus-Calmette-Guérin (BCG) [8]. Especially, after the initial vaccination at birth, the majority of the Japanese population has been repeatedly vaccinated with BCG until recently, during childhood if a negative TST result was obtained when attending primary and junior high school. Such repeated vaccinations can lead to a significant and persistent influence of BCG on TST results [9]. Therefore, this limitation is quite problematic in Japan, especially for the precise detection of subjects without tuberculosis infections. Recently, a whole blood interferon-gamma assay has been introduced for the diagnosis of latent tuberculosis infection and active tuberculosis; this assay is based on a specific elevation in the interferon-gamma concentration that occurs as T cells respond to early-secreted antigenic target 6-kDa protein (ESAT-6) and culture

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filtrate protein 10 (CFP-10), both of which are specifically expressed by *Mycobacterium tuberculosis* but not by BCG strains. Recent studies have shown that this blood assay has a moderate sensitivity for the detection of latent tuberculosis infection in the range of 70–80%, but is highly specific (over 95%) as compared with the TST even in BCG-vaccinated patients with varying risks of infection with *M tuberculosis* or active tuberculosis [8,10]. Thus, for our healthcare students–most of who had been vaccinated with BCG, had not yet started bed-side training, and were at low risk for tuberculosis infection–a more accurate identification of their baseline immune status might be obtained using this blood test, rather than the TST. However, the usefulness of this assay as a baseline screening test for healthcare students at the time of their entry into clinical studies has not been fully evaluated in Japan. Here, we prospectively compared the accuracy of the whole blood interferon-gamma assay with that of the traditional TST in healthcare students thought to be at a low risk of infection with *M tuberculosis*.

Unlike Th1-mediated cytokines such as interferon-gamma that promote the killing of infected cells, regulatory cytokines including interleukin-10 have been implicated in the suppression of in vitro antigen-specific cellular immune responses in tuberculosis. Their down-regulatory mediators may be important players in controlling the excessive synthesis of pro-inflammatory cytokines and subsequent tissue damage [11]. However, few studies have reported how such Th2 mediators respond after stimulation with ESAT-6 and CFP-10 antigens, and all of these studies were conducted in mice and calves [12,13], not in human subjects. Therefore, we also investigated the potential influence of these specific antigens on the interleukin-10 concentration as well as the interferon-gamma concentration, and assessed the association between changes in the concentrations of these two cytokines when exposed to the above two antigens.

METHODS

Participants

All participants in this trial were prospectively recruited at Okayama University, Japan, between May and July, 2006. The subjects consisted of medical, nursing and dental students who were enrolled at the beginning of their clinical training, all of who were older than 18 years of age. First-year postgraduate students were also recruited. Subjects who received both whole blood assay and TST were considered to be eligible for this study. The protocol was approved by the ethics review committee of Okayama University. After providing written consent, the subjects were asked to complete a questionnaire about possible risk factors for exposure to *M tuberculosis*. The following data were collected: history of prior tuberculosis or exposure to a person with tuberculosis, and other tuberculosis risk factors like having an immunosuppressive condition (i.e., malignant disease or diabetes mellitus) or having taken immunosuppressive drugs in the 3 months prior to enrollment. Information regarding any clinical symptoms, previous TST results, BCG vaccination status (as reported by the prescribing physician and based on questions about past BCG vaccination and scar inspection), and demographic, clinical, and radiological data was also collected at the time of enrollment. Human immunodeficiency virus testing was not performed because of the low prevalence of positive human immunodeficiency virus expected in this young, educated population.

Test methods—TST and whole blood interferon-gamma assay

For the TST, 0.1 mL of tuberculin purified protein derivative (PPD) (Nippon BCG Manufacturing, Tokyo, Japan; equivalent to about 3 TU of PPD-S) was injected intradermally into the volar aspect of the forearm and the transverse induration diameter was measured 48 hours later, as described previously [14]. A one-step TST was then performed.

The whole blood interferon-gamma assay was performed using the QuantiFERON-TB-2G (Nippon BCG Supply, Japan) as described elsewhere [14], principally one month after the TST. A heparinized blood sample was collected from each subject by venipuncture. After overnight incubation with saline (nil control), phytohemagglutinin (mitogen-positive control), and the *M tuberculosis*-specific antigens ESAT-6 or CFP-10, the concentration of interferon-gamma in the four plasma samples from each subject was determined using an ELISA. We interpreted the test results, after subtracting the value of the negative control well from the IU amount of interferon-gamma measured in the wells stimulated by the *M tuberculosis*-specific antigens as follows: positive if the interferon-gamma concentration in either of the antigen wells was ≥0.35 IU/mL; negative if the concentrations in both antigen wells were <0.35 IU/mL and the concentration in the positive control well was ≥0.5 IU/mL; and indeterminate if the concentration in both antigen wells was <0.35 IU/mL and the concentration in the positive control well was <0.5 IU/mL in the positive-control well. The technicians performing the whole blood interferon-gamma assay had already been trained by the manufacturers and were unaware of the subjects' tuberculosis status in this study.

Test methods—Whole blood interleukin-10 assay

We measured the protein level of interleukin-10 in whole blood incubated overnight with antigens, using an ELISA kit (Biosource, Camarillo, California, USA). This assay also involved the same two steps; after the blood samples were separately incubated with CFP-10 and ESAT-6 and the nil and mitogen (phytohemagglutinin) controls for 18 hours at 37°C, the concentration of interleukin-10 in the four plasma samples from each subject was determined using an ELISA. The detection limit for interleukin-10 was <0.1 pg/mL. The results for ESAT-6 and CFP-10 were expressed as the detected concentration of interleukin-10 minus the concentration of interleukin-10 in the respective nil control plasma. This blood assay was also performed in a blind manner.

Statistical methods

Agreement between the tests was quantified using the κ statistic. A logistic regression analysis for positive TST results (induration≥15mm) and for positive blood assay results was performed to adjust the potential confounding factors including age, prior tuberculin skin test, number of BCG scars, student category and increase in the interleukin-10 concentrations when exposed to either of the antigens. The Mann-Whitney U test was used for comparisons among groups. Because our goal was to recruit all healthcare students commencing clinical training, we did not determine a formal sample size. All p-values were two-sided, and significance was set at a p-value of ≤0.05.

RESULTS

Demographics of the participants

Of the 536 students to whom the outline of this study was announced (Fig 1), 371 (69%) underwent the TST; of these students, 207 (56%) subjects also underwent the whole blood interferon-gamma assay, after providing their written informed consent. The demographics of the 207 participants are listed in Table 1. Only one of the 207 subjects had an obvious episode of close contact with a tuberculosis patient. This subject was a healthy 25-year-old nursing student who had been vaccinated with BCG soon after his

birth. At the age of about six months, his grandfather, who had lived with him, contracted pulmonary tuberculosis and died. None of the family members who had been living with this patient have since developed tuberculosis, although their TST results at the time of the grandfather's death were not available. None of the subjects enrolled in this study reported having an immunosuppressive condition. The majority of the subjects had last been screened with the TST when entering junior high school.

Comparison of the TST and whole blood interferon-gamma assay results

No treatments or interventions were undergone in the intervals between both tests. The two tests were safely conducted in all patients. Regarding the TST results, 171 subjects (82.6%) had an induration diameter of ≥5 mm, 124 (59.9%) had an induration diameter of ≥10 mm, and 57 (27.5%) had an induration diameter of ≥15 mm. The distribution of induration diameters is displayed in Fig 2. Three subjects (1.4%) had a strong reaction in addition to a large induration diameter (18 mm, 18 mm, and 15 mm), with obvious hemorrhaging in two subjects and vesicles in one subject. One of the three subjects had previously been in close contact with a tuberculosis patient, as mentioned above; since then, he had consistently exhibited a similar strong skin reaction whenever he received a TST, although his whole blood test was negative (ESAT-6: 0.03 IU/mL; CFP-10: 0.00 IU/mL). The other two subjects did not have relevant clinical symptoms, a history of close contact with a tuberculosis patient, or abnormal shadows on chest X-rays taken at the time of this study. These two subjects had received BCG vaccinations during childhood and had negative whole blood test results (ESAT-6: 0.00 IU/mL and 0.00 IU/mL; CFP-10: 0.03 IU/mL and 0.00 IU/mL).

Regarding the whole blood interferon-gamma assay (Fig 1), five subjects (2.4%) had indeterminate results because of insufficient interferon-gamma production in response to the mitogen. These indeterminate results were also used for the comparison of the two tests based on the entire sample data. The median baseline concentration of interferon-gamma (negative control) was 0.07 IU/mL (range, 0.01 to 0.79 IU/mL), while the median increase in the interferon-gamma concentration after exposure to either of the antigens was 0.01 IU/mL (range, 0 to 1.89 IU/mL). Three subjects (1.4%) had positive blood test results (ESAT-6: 0.40 IU/mL, 0.37 IU/mL, and 1.89 IU/mL; CFP-10: 0.47 IU/mL, 1.26 IU/mL,

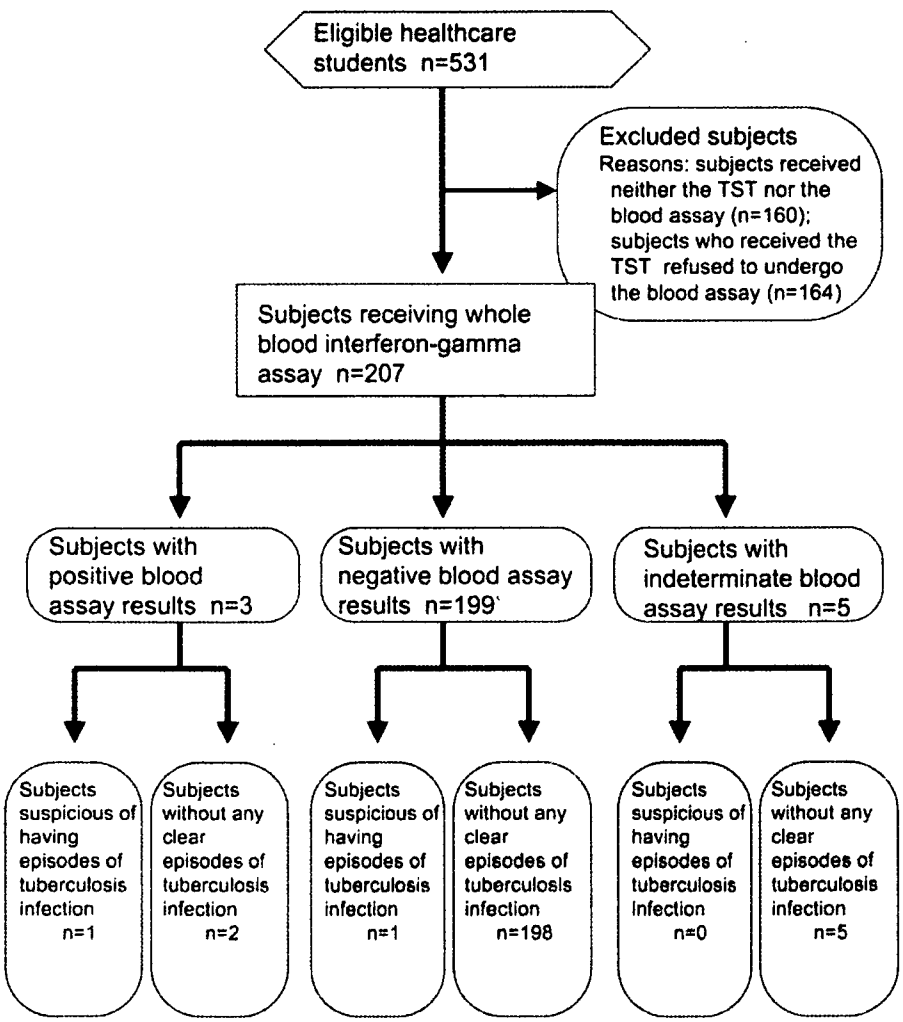

Figure 1. A flow diagram of the whole blood interferon-gamma assay in the 207 subjects.
doi:10.1371/journal.pone.0000803.g001

Table 1. Demographics of 207 participants

Gender (male/female)	75 (36%)/132 (64%)
Median age (range)	20 (18–42)
Student groups	
Medical students	42 (20%)
Nursing students	107 (52%)
Dental students	44 (21%)
Postgraduate students	14 (7%)
Prior tuberculin skin test	
Yes/No	193 (93%)/14 (7%)
Number of prior BCG vaccinations	
0/1/2/3/4	3(1%)/91(44%)/79(38%)/10(5%)/10(5%)
Unknown	14 (7%)
Median age of subjects at which BCG was finally vaccinated (range)	7 (0–16)

Abbreviation: BCG = bacillus-Calmette-Guérin.
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and 0.03 IU/mL); these three subjects all had positive skin test results (induration of 15 mm each). None of these subjects had relevant clinical symptoms, a history of recent close contact with a tuberculosis patient, or abnormal shadows on chest X-rays taken at the time of this study. Two of the three subjects had been vaccinated with BCG during childhood, while the third had a positive TST test at birth and was thus considered to have had a tuberculosis infection despite not having had an obvious episode of exposure to a person with tuberculosis or a BCG vaccination.

Considering abovementioned two subjects would have previous episodes of tuberculosis infections (the first case with the strong TST reaction and the third case with the positive blood test result), the specificity of blood assay was calculated as 96.6% (198/205) (Fig 1). Regarding TST with a cutoff of induration diameter of ≥5, ≥10 and ≥15 mm, the specificity was 17.6%, 40.5% and 73.2%, respectively. In contrast, sensitivity was not assessed because of a few subjects with obvious tuberculosis disease in our study. The association between the diameter of the TST induration and the positive whole blood interferon-gamma assay result is shown in Table 2. Using cutoff levels of an induration diameter of ≥5, ≥10 and ≥15 mm for the TST and ≥0.35 IU/mL for the whole blood assay, the overall agreement between the two tests was 18.8%, 41.1% and 72.5% ($\kappa = 0.007$, 0.020 and 0.077), respectively. A logistic regression analysis also revealed that the diameter of the TST induration (≥15 mm *vs.* <15 mm) was not affected by the interferon-gamma concentration after exposure to either of the antigens (odd ratio = 1.441, 95% confidence interval = 0.737–2.818; p = 0.285), but was influenced by the number of needle scars from the BCG vaccination (odd ratio = 3.857; 95% confidence interval = 1.022–14.555; p = 0.046). In contrast, the whole blood assay result was not affected by the number of BCG scars (odd ratio = 0.905; 95% confidence interval = 0.207–3.958; p = 0.894).

Whole blood interleukin-10 assay

This assay was assessable for all 207 subjects. The median baseline concentration of interleukin-10 (negative control) was 2.9 pg/mL (range: 0.2 to 39.9 pg/mL). This value was not associated with the

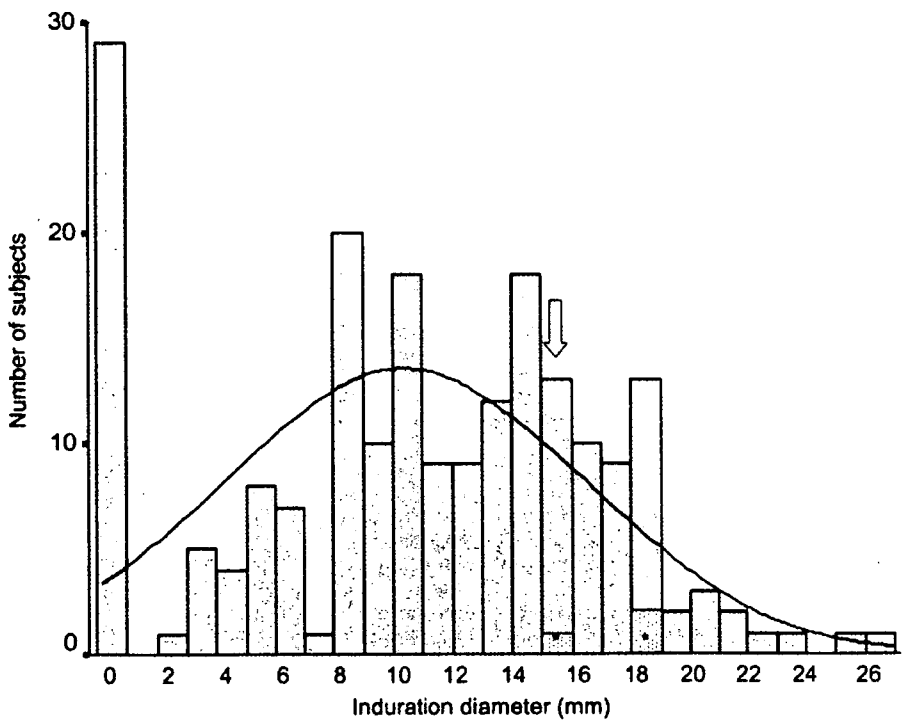

Figure 2. Distribution of tuberculin skin test and whole blood interferon-gamma assay results among 207 subjects. The bars for 15 mm and 18 mm included one and two subjects, respectively, with strong skin reactions (i.e., vesicles and hemorrhage) to the purified protein derivative (*). Three additional subjects with positive whole blood interferon-gamma assay results were included on the bar for 15 mm (arrow).
doi:10.1371/journal.pone.0000803.g002

Table 2. Agreement between whole blood interferon-gamma assay and tuberculosis skin test results

<Results*>	TST Cutpoint, mm†		
	≥5	≥10	≥5
Positive TST/positive INF-γ assay	3 (1.4%)	3 (1.4%)	3 (1.4%)
Negative TST/negative INF-γ assay	36 (17.4%)	82 (39.6%)	147 (71.0%)
Positive TST/negative INF-γ assay	163 (78.7%)	117 (56.5%)	52 (25.1%)
Negative TST/positive INF-γ assay	0	0	0
Any TST/indeterminate INF-γ assay	5 (2.4%)	5 (2.4%)	5 (2.4%)
Agreement, %	18.8	41.1	72.5
κ	0.007	0.02	0.077

†Longest transverse diameter of induration. Abbreviations: TST = tuberculin skin test, INF-γ = interferon-gamma. *IFN-γ assay cutpoint was at least 0.35 IU/mL.
doi:10.1371/journal.pone.0000803.t002

baseline concentration of interferon-gamma (negative control) (median baseline interleukin-10 levels: 2.8 vs. 3.0 pg/mL for the low (<0.07 IU/mL) and high (≥0.07 IU/mL) baseline interferon-gamma concentrations, respectively; p = 0.263; Fig 3A). In contrast, when exposed to either of the antigens, the median increase in the interleukin-10 concentration was 0.1 pg/mL (range: 0 to 11.5 pg/mL) in the 207 subjects. When the median increase in the interferon-gamma concentration after exposure to the antigens (0.01 IU/mL) was applied as a cutoff value, the median increase in the interleukin-10 level was higher in the subgroup with the smaller increase in interferon-gamma concentration (<0.01 IU/mL) than in the subgroup with the larger increase in interferon-gamma concentration (≥ 0.01 IU/mL) (0.3 vs. 0 pg/mL; p = 0.004; Fig 3B).

DISCUSSION

Historically, infection control policies in many hospitals have recommended pre- and post-employment routine screening of healthcare workers using the TST [2]; in accordance with this recommendation, the TST has generally been required for healthcare students at the start of their clinical studies [6]. In Japan, however, repeated BCG vaccination has, until recently, been performed during childhood if a negative TST result was obtained while attending primary and junior high school. Healthcare students and healthcare workers with negative TST results at the time of their entry into clinical studies or at the time of hiring might also receive BCG vaccinations in Japan, despite no clear evidence of efficacy. Also, repeated testing using tuberculin during their employment may further boost their reaction to the TST. Thus, these factors may lead to a false-positive TST result [9], producing quite discordant positive results between the two tests both in a baseline screening setting for healthcare students (1.4% vs. 27.5% for the whole blood test vs. the TST; Table 2) and in a routine screening setting for Japanese healthcare workers who have been employed for one or more years (9.9% vs. 93.1%) [2].

To overcome the high false-positive TST results, several studies have been conducted to assess the specificity of interferon-gamma assays in low-risk healthcare students without active tuberculosis [14–16]. In an Australian study, the whole blood assay was performed in 60 medical students before and five months after BCG immunization [15]. Of note, the specificity of the TST

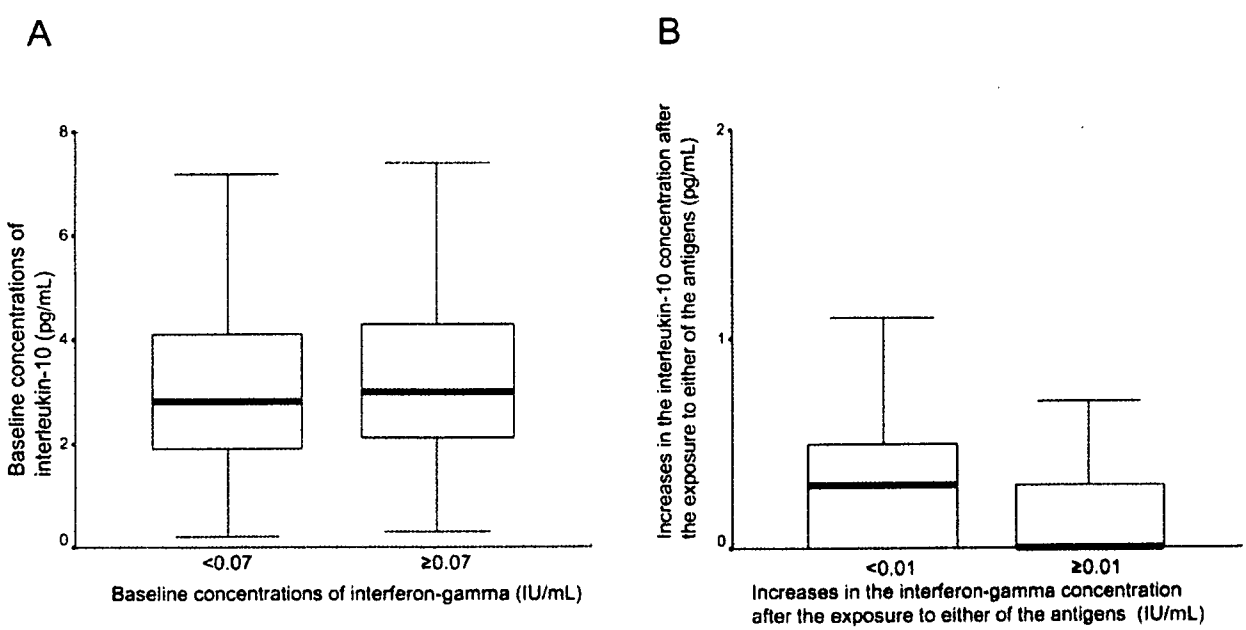

Figure 3. Interleukin-10 concentrations stratified by interferon-gamma concentrations. The median baseline concentration of interferon-gamma (negative control) was 0.07 IU/mL, while the median increase in the interferon-gamma concentration after exposure to either of the antigens was 0.01 IU/mL. A. Median baseline concentration of interleukin-10 (exposed to nil control) stratified by the baseline concentration of interferon-gamma (exposed to nil control) (2.8 vs. 3.0 pg/mL for the low [<0.07 IU/mL] and high [≥0.07 IU/mL] interferon-gamma concentrations; p = 0.263). B. Median increases in the interleukin-10 concentration stratified by increases in the interferon-gamma concentration after exposure to either of the antigens (0.3 vs. 0 pg/mL for the smaller [<0.01 IU/mL] and larger [≥0.01 IU/mL] increases in the interferon-gamma concentrations; p = 0.004).
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decreased from 100% to 87% after BCG vaccination, while a high specificity (100%) was consistently observed for the whole blood assay, irrespective of BCG immunization. A previous Japanese study screened for tuberculosis infection in 213 nursing students deemed to be truly free of infection [14], a young population that was a quite similar cohort to ours. The study reported that 98% of the students were negative for the whole blood assay, while negative TST results (induration diameter≤10mm) were observed in only 35% of the same population. In our study, 96% and 40% of the subjects had negative blood assay and TST results, respectively (Table 2), suggesting that the results of both the blood assay and the TST are almost reproducible. Both studies also indicated that the blood assay rather than the TST corresponded to the estimated cumulative prevalence of tuberculosis infection in young people in Japan [7].

In contrast, Pai et al. recently showed concordant results between the two tests in Indian medical and nursing students at the start of their clinical studies [16]. Unlike the above-mentioned studies [14,15]. and ours, this Indian study suggested that BCG vaccination had little impact on the TST results. Such minimal effect of the BCG vaccination might be more likely to occur when the vaccine is given to newborns only and in areas where the prevalence of tuberculosis is high. Considering the tuberculosis control policies of the Japanese government such as repeated BCG vaccination and the relatively low estimated prevalence of tuberculosis infection in Japan [7], the blood assay seems to be highly useful as a baseline tuberculosis screening program for our healthcare students, in whom the influence of BCG vaccination on the TST results is unavoidable. To confirm its usefulness, further comprehensive analyses are warranted, including a cost-effective study and a long-term follow-up study, especially in the three subjects who had positive blood assay results, the three subjects who had strong skin reactions to PPD but negative blood assay results, and the five subjects with indeterminate blood assay results. As a follow-up study, the Indian research evaluated serial whole blood interferon-gamma assays in medical and nursing students and found in their follow-up study that this assay showed promise for serial testing, despite the need to establish the optimal thresholds for distinguishing new infections from nonspecific variations [16]. Further research is also warranted to clarify the role of serial testing in countries with lower rates of tuberculosis incidence [17,18].

One of our limitations is that we did not fully assess the sensitivity of the blood assay because of few subjects with active tuberculosis. The recent meta-analysis revealed the sensitivity of this assay did not seem to be significantly higher than that of TST despite its high specificity [9]. Indeed, one of our cases had a negative blood assay result despite the previous close contact with a tuberculosis patient and the strong TST reaction. Overall, the discordant results between the TST and blood assay we observed in this study might partly arise from its potentially low sensitivity of the blood assay in addition to the high false positive rate of the TST. These critical points should be further assessed in carefully designed population studies.

We should note another obvious limitation in our study-the possibility of bias due to a fairly high non-response rate. Only 207 (39%) of 536 students agreed to undergo both tests, and this may have introduced a selection bias that affected the positive TST and blood assay rates. Thus, our results should be carefully interpreted.

Interleukin-10 was first detected based on its cytokine synthesis inhibitory activity, mainly on macrophages [19]. The complementary activity of interleukin-10 and Th1-mediated cytokines, including interferon-gamma, may provide a mechanism for maintaining a balance between the protective immune response and excessive cellular activation. In this study, we also found evidence of this relationship; after exposure to either ESAT-6 or CFP-10, the change in the interleukin-10 concentration was inversely correlated with that of interferon-gamma ($p = 0.004$; Fig 3B), while no association between the baseline (nil control) concentrations of the two cytokines was seen (Fig 3A). Minion et al. reported comparable data demonstrating that mice splenocytes vaccinated with *Mycoplasma hyopneumoniae* surface antigen P71 alone produced higher levels of interleukin-10, while those vaccinated with an ESAT-6:P71 fusion protein secreted higher levels of interferon-gamma but failed to produce interleukin-10 [12]. Contrary to these results, Trajkovic et al. showed that CFP-10 could stimulate the secretion of interferon-gamma in mouse J774 cells in vitro, while CFP-10 pretreatment did not affect macrophage interleukin-10 synthesis [13]. Thus, the response of interleukin-10 to these specific antigens remains controversial in preclinical studies; however, our human data might encourage further investigation of whether interleukin-10 levels after exposure to specific antigens could be a useful tool for the detection of tuberculosis infection. Our limitation is that only one regulatory factor was assessed in relation to the interferon-gamma assay; there are indeed many cytokines related to homeostatic mechanisms balancing responses to specific tuberculosis antigens [20]. Thus, a more comprehensive study of profile of many other cytokines, rather than the assessment of a solitary member like IL-10 should be done in the future.

In conclusion, we found in this prospective study that the results of an interferon-gamma assay were quite discordant with those for the TST in a medical university setting, probably because of the influence of BCG vaccination on the TST results. Given this unavoidable influence of BCG vaccination and the relatively low estimated prevalence of tuberculosis infection in Japan, the blood assay seems to be useful as a baseline tuberculosis screening test for Japanese healthcare students, despite the need for further researches. Additionally, we report, for the first time, that the interleukin-10 level after exposure to specific antigens was inversely associated with the interferon-gamma level in the reported population.

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Author Contributions

Conceived and designed the experiments: KH. Performed the experiments: KH. Analyzed the data: KH. Wrote the paper: KH TO KN TK MO YS AK KK KT MT.

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Paradoxical Discrepancy Between the Serum Level and the Placental Intensity of PP5/TFPI-2 in Preeclampsia and/or Intrauterine Growth Restriction: Possible Interaction and Correlation with Glypican-3 Hold the Key

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Abstract

There have been controversies whether maternal serum placental protein 5 (PP5)/tissue factor pathway inhibitor (TFPI)-2 is increased in the patients with preeclampsia and/or intrauterine growth restriction (IUGR). Here, we have estimated the serum PP5/TFPI-2 in these patients by a sandwich enzyme-linked immunosorbent assay with a newly developed monoclonal antibody, coupled with placental immunohistochemical studies of their placentae with semiquantitative scoring.

Serum PP5/TFPI-2 level was significantly elevated only in the patients with preeclampsia alone ($p = 0.033$), while PP5/TFPI-2 was detected significantly less intensely in the placentae of the same patients ($p = 0.035$) in immunohistochemistry, as compared to Controls. A proteoglycan present on the placental villous surface, glypican-3, showed the same pattern of staining as PP5/TFPI-2, and there was a positive correlation (C.I. = 0.506, $p = 0.004$) between the immunohistochemical scores for these. Further experiments using HepG2 cells transfected with *PP5/TFPI-2* suggested that glypican-3 could anchor PP5/TFPI-2 on the placental villi.

A possibility that a decrease in glypican-3 in the placenta increases the outflow of PP5/TFPI-2, which in turn increases its serum level, was proposed. Preeclampsia and IUGR, often regarded to have the same pathological basis in common, showed distinct distributions of PP5/TFPI-2, which could be a clue to elucidate the pathogenesis of preeclampsia and IUGR.

Keywords: Placental protein 5/tissue factor pathway inhibitor-2; Glypican-3; Preeclampsia; Intrauterine; Growth restriction; Syndecan-1

1. Introduction

Preeclampsia and intrauterine growth restriction (IUGR) are difficult to predict clinically, and are some of the severe complications of pregnancy. Although part of the mechanisms underlying these disorders has been elucidated, the ultimate causes of preeclampsia and IUGR remain unknown [1–3].

Placental protein 5 (PP5) is a soluble protein produced in the human placenta and is detected in the serum of the pregnant woman [4]. We previously have found from amino acid sequence comparisons that PP5 is identical to a 29-kDa Kunitz type proteinase inhibitor [5]. The same protein, named tissue factor pathway inhibitor (TFPI)-2, was cloned independently

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as a homologue of TFPI from a human placental cDNA library by others [6].

PP5/TFPI-2 is a potent inhibitor of trypsin, plasmin, plasma kallikrein, factor XIa and factor VIIa/TF complex, and also weakly inhibits amidolytic activity of factor Xa [7]. The expression of PP5/TFPI-2 has been demonstrated in various human tissues other than the placenta [5,8–10], and its contribution to angiogenesis [9–11] and carcinogenesis [12–15] has been the focus of several studies. Recently, the function of PP5/TFPI-2 as a mitogen for vascular smooth muscle cells [16] and retinal pigment epithelial cells [17] has been demonstrated.

Despite its abundant presence in the placenta, the function of PP5/TFPI-2 during pregnancy is not fully understood. We have demonstrated that PP5/TFPI-2 is localized on the surface of microvilli and the endoplasmic reticulum membrane of syncytiotrophoblasts by immunoelectron-microscopy, and that incubation with heparin releases PP5/TFPI-2 from the villous surface of the placenta [18,19].

TFPI is known to bind to glypican-3, a member of the transmembrane heparan sulphate proteoglycans (HSPGs), on the cell surface of hepatocellular carcinoma cell line, HepG2 cells. When HepG2 cells are incubated with heparin, TFPI is released from the cell surface into the culture medium [20]. TFPI possesses a highly positively charged region in its carboxyl terminus, for which heparin competes with glypican-3 to release TFPI [21].

As PP5/TFPI-2 has a similar structural domain to TFPI, we hypothesized that PP5/TFPI-2 might be retained on the surface of the placental villi by proteoglycans such as members of the glypican and syndecan families, and that PP5/TFPI-2 might play a role to maintain intervillous blood flow [19]. Glypican-3 is known to be expressed abundantly in the placenta [22], along with syndecan-1, a member of the HSPGs syndecan family [23].

It has been reported that the maternal serum level of PP5/TFPI-2 is elevated in the patients with severe preeclampsia [24–26]. Some investigators have reported the elevated maternal serum level of the same protein also in the patients with IUGR [25,26], while others have failed to demonstrate the elevation [27,28], using the same rabbit polyclonal antibody raised against a fraction of purified PP5/TFPI-2 as for the radioimmunoassay [29,30]. Another evaluation with new specific monoclonal antibody and with a more specific technique (sandwich ELISA) than radioimmunoassay may serve to clarify the association between the maternal serum PP5/TFPI-2 levels and preeclampsia and/or IUGR. In addition, the mechanism underlying the increase in PP5/TFPI-2 in the maternal serum remains to be elucidated. To date, there have been no reports on the in situ expression of PP5/TFPI-2 in the placentae of the patients with preeclampsia and/or IUGR as compared with their serum PP5/TFPI-2 levels.

Here we have attempted to clarify the maternal serum levels of PP5/TFPI-2, along with the in situ expression of the same protein in the placentae of the patients with preeclampsia and/or IUGR. We have also sought for the association of PP5/TFPI-2 with some proteoglycans in the placentae.

2. Materials and methods

2.1. Placental tissue and serum samples

The experimental protocol was peer-reviewed and approved by the Ethical Committee of Yokohama City University Graduate School of Medicine. Placentae, maternal and umbilical venous sera were collected from the patients who were scheduled to undergo caesarean section. After receiving a detailed explanation, each of the patients who agreed to be enrolled in this study gave written informed consent.

Preeclampsia was diagnosed according to the definition established by the National High Blood Pressure Education Program [31]. IUGR was diagnosed if the estimated weight of the fetus was less than the 10th percentile for its gestational age according to the Japanese standard fetal growth curve [32], and the presence of growth arrest and non-reassuring fetal status were inferred from the fetal monitoring. For each of the patient, the gestational age had been confirmed in the first trimester by ultrasound.

The maternal serum was sampled 10–60 min before the mothers moved to the operation room, before the administration of anesthesia. The maternal serum was also sampled 4 days after delivery. The umbilical venous serum was collected carefully from the cord to avoid contamination with maternal blood. All serum samples were stored at −80 °C until the assay.

Placental tissues were sectioned into samples of approximately 3 cm × 3 cm × whole thickness taken from five different portions, fixed in 10% neutral-buffered formalin and embedded in paraffin for histopathological studies.

2.2. Preparation of mouse monoclonal anti-PP5/TFPI-2 antibody

Monoclonal antibody was raised against a synthetic peptide antigen consisting of 14 amino acid residues, NH_2-DAAQEPTGNNAEIC-COOH, corresponding to the N-terminus of the mature PP5/TFPI-2 protein after cleavage of the putative signal peptide. Specificity of the antigenic peptide to PP5/TFPI-2 was verified by searching the peptide sequence against other proteins with the BLAST program at the National Center for Biotechnology Information, National Institute of Health, Bethesda, MD (http://www.ncbi.nlm.nih.gov/BLAST/). The cysteine residue at the carboxy terminus was conjugated to keyhole limpet hemocyanin.

To use as the standard PP5/TFPI-2 protein for screening of the hybridoma cell clones of new antibodies and for the enzyme-linked immunosorbent assay (ELISA), recombinant PP5/TFPI-2 was prepared as follows. Histidine-tagged *PP5/TFPI-2* cDNA was transfected into the yeast (*Pichia Pastoris*) by using an EasySelect *Pichia* Expression Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The expressed recombinant PP5/TFPI-2 was affinity purified against the histidine-tag by using a Ni-NTA Spin Kit (QIAGEN, Valencia, CA).

Five-week-old BALB/c mice, gained from Oriental Yeast Co., Ltd., Tokyo, Japan, were immunized with the antigenetic peptide every 2 weeks. Three days after the last injection of 250 μg of the immunogen, the mouse spleen cells were sampled and fused with a mouse myeloma cell line P3U1 using polyethyleneglycol. From the antibody produced by the hybridomas, a clone 28Aa was selected for use in the study by Western blotting against the recombinant PP5/TFPI-2 expressed in the yeast described above. The antibody of the selected clone was purified from the ascites of the BALB/c mice that had been injected intraperitoneally with the hybridoma cells by column chromatography using protein A (Amersham Biosciences Co., Piscataway, NJ).

2.3. Sandwich ELISA

Serum levels of PP5/TFPI-2 were assayed by Sandwich ELISA using the clone 28Aa mouse monoclonal antibody against human PP5/TFPI-2 as described above, and a previously described rabbit polyclonal antibody against human PP5/TFPI-2 [18].

PP5/TFPI-2 antibody clone 28Aa diluted to 10 μg/ml was applied to a 96-well plate (Greiner Bio-one, Longwood, FL). After incubation at 4 °C overnight, the plate was blocked with 1% bovine serum albumin (Sigma) in

phosphate-buffered saline (PBS) at room temperature for 1 h. Serum samples diluted five times or the recombinant PP5/TFPI-2 protein diluted to different concentrations was added to each well. The plate was then incubated at 37 °C for 1 h. After washing, the rabbit polyclonal antibody against human PP5/TFPI-2 diluted to 10 μg/ml was added to the wells, and the plate was incubated at 37 °C for an additional hour. For detection, a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig) G H+L (Molecular Probes, Invitrogen, Carlsbad, CA), diluted to 1:16 000 was added to each well. After incubation at 37 °C for 1 h, *O*-phenylenediamine (Sigma) was added for color development. Absorbance at 490 nm was read by a Benchmark Plus spectrophotometer (Bio Rad, Hercules, CA) and the results were analysed by Microplate Manager Ver. 5.2 (Bio Rad).

2.4. Immunohistochemical analyses

Paraffin sections of the placentae were routinely stained with Hematoxylin and Eosin. The samples were also subjected to immunohistochemical staining for PP5/TFPI-2 and glypican-3.

Deparaffinized and rehydrated slides were immersed in 0.01 M citrate buffer, pH 6.0 (Sigma), and heated in a microwave oven for antigen retrieval. The slides were then cooled, washed in PBS, and immersed in 3% H_2O_2 diluted in methanol at room temperature.

The clone 28Aa mouse monoclonal antibody against human PP5/TFPI-2 or a mouse monoclonal antibody against human glypican-3 (clone 1G12, Biomosaics, Burlington, VT), diluted to 5 μg/ml or 40 μg/ml, respectively, was used as the primary antibody. Histofine SAB-PO multikits (Nichirei, Co., Tokyo, Japan) were used to detect the labeled antigens. Histochemically labeled antigens were visualized by reaction with 3,3′-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Immunohistochemical staining for syndecan-1 (CD138) was also performed with a mouse monoclonal antibody against human CD138 (clone B-B4, Serotec, Oxford, UK) (diluted 1:200), as described above except for the antigen retrieval. Adjacent sections were used for immunohistochemical stainings for PP5/TFPI-2, glypican-3, and syndecan-1.

The results of the immunohistochemistry were analysed by using a modified German immunoreactive score [33–35]. The immunostaining intensity was rated as follows: 0, none; 1, weak; 2, moderate; and 3, intense. The quantity of immunohistochemically positive trophoblasts was also graded as follows: 0, none; 1, 1–10%; 2, 11–50%; 3, 51–80%; and 4, 81–100%. A score per slide was calculated as the summation of the areas of intensity multiplied by the quantity of each area. Each slide was evaluated of its score three times by two independent examiners who were blinded to its origin. The average of the scores from all of the slides of the placenta was determined as the representative data for that sample.

2.5. Transfection, immunoprecipitation and Western blotting

HepG2 cells were obtained from the Cell Bank, RIKEN BioResource Center (Tsukuba, Japan), and cultured in RPMI1640 (Kohjin Bio, Co., Itado, Japan) containing 10% fetal bovine serum (Moregate Biotech, Balimba, Australia) under an atmosphere of humidified 5% CO_2. A mammalian expression vector pcDNA3 (Invitrogen) or the vector containing the whole coding region of human *PP5/TFPI-2* cDNA was transfected to HepG2 cells with Lipofectamine 2000 transfection reagent (Invitrogen) under the manufacturer's instruction.

Forty-eight hours after transfection, the cells were lysed at room temperature for 10 min in 1 ml of a lysis buffer (25 mM Tris–Cl, pH 7.5; 100 mM NaCl; 2 mM EDTA (Sigma); 1% Triton X-100 (Sigma)) containing protease inhibitors (Complete Mini, Roch Diagnostics, Indianapolis, IN). After cell debris was removed by centrifugation, each lysate was further pre-cleared with Protein G Sepharose 4 fast flow (Amersham Biosciences).

Immunoprecipitation was carried out with 2.5 μg of the 28Aa mouse monoclonal antibody against human PP5/TFPI-2 and 50 μl of the Protein G Sepharose at 4 °C, and then the Sepharose phase was washed four times with the lysis buffer. Each immunoprecipitate was recovered by adding 50 μl of 2× SDS containing sample buffer and incubating at 70 °C for 10 min. Equal amount of immunoprecipitate (10 μl each) was subjected to SDS PAGE, followed by Western blotting for PP5/TFPI-2 (with the clone 28Aa antibody) or glypican-3 (the clone 1G12 mouse monoclonal antibody against human glypican-3, Biomosaics), respectively. An HRP conjugated sheep anti-mouse IgG (Amersham Biosciences) was used as the secondary antibody, and the signals were detected with the Supersignal West Pico chemiluminescent substrate (Pierce).

2.6. Statistics

Data are expressed as the mean ± standard error (SE). Statistical comparison was performed by either Student's *t* test, Welch's *t* test, Mann–Whitney's *U* test, or analysis of covariance (ANCOVA). The correlation index was calculated by using either Pearson's test or Spearman's test. SPSS software (Basic 11.0, SPSS Inc., Chicago, IN) was used for calculation. Significance was set at $p < 0.05$.

3. Results

3.1. Patients

Fifty-five patients who had been scheduled to undergo caesarean section at 24–39 weeks of pregnancy agreed to the collection of samples for research usage. Four patients were excluded from the study because they had previously taken medication for other pre-existing diseases. Hence, the 51 patients who had not been diagnosed of any pre-existing disease such as hypertension, renal disease, diabetes mellitus, or other chronic disease before pregnancy were enrolled in the study. There were no neonates with congenital or chromosomal abnormalities.

3.2. Maternal serum PP5/TFPI-2 levels in preeclampsia and/or IUGR

Maternal serum samples at delivery were available from the 51 patients, whose obstetrical complications are summarized in Table 1. Nineteen patients had preeclampsia, 10 of whom had preeclampsia only (Group P), and the other nine of whom had been also diagnosed as IUGR (Group P + IUGR). Seven had been diagnosed with IUGR alone (Group IUGR). The other 25 patients did not have the above-mentioned obstetric complications (the Control).

We confirmed from the clinical records that none of the patients in Group P, Group P + IUGR, and Group IUGR had been hypertensive or had proteinuria early in pregnancy, nor had they persisted hypertension or proteinuria at the time of their follow-up visits 1 month after delivery. All of the patients in Group P and Group P + IUGR had received antihypertensive medications for as long as 1–14 days.

The patients with preterm premature rupture of the membrane and premature labor in the Control had mild, if any, pathological changes in the placentae (Blanc stage [36] one, i.e., intervillositis at most), and had no clinical sign of severe chorioamnionitis or maternal systemic infection such as uterine tenderness, foul smelling amniotic fluid, maternal fever more than 38 °C, maternal tachycardia (≥120 beats/min), or maternal leukocytosis (≥20 000/μl).

The clinical features of the study groups are summarized in Table 2. The maternal mean arterial pressure (MAP) and

Table 1
Distribution of the patients

Complications/indications for C/S	Number of the patients	
Preeclampsia (Group P)		10
Non-reassuring fetal status	3	
Incontrollable maternal hypertension/renal insufficiency	6	
Both of the above	1	
Preeclampsia with IUGR (Group P + IUGR)		9
Non-reassuring fetal status	7	
Incontrollable maternal hypertension/renal insufficiency	2	
IUGR (Group IUGR)		7
Non-reassuring fetal status	7	
No above complications (the Control)		25
History of C/S	9	
Breech presentation	5	
Placenta previa	3	
Preterm PROM	4	
Preterm labor	2	
History of uterine surgery	1	
Operated atresia ani	1	
Total		51

IUGR, intrauterine growth restriction; C/S, caesarean section; PROM, premature rupture of the membrane.

urinary protein (UP) were measured at the time of blood sampling. Although none of the patients in Group IUGR had been diagnosed as hypertensive, the maternal MAP was significantly higher not only in Group P and Group P + IUGR ($p < 0.001$ for both Groups), but also in Group IUGR ($p = 0.032$), as compared with the Control. However, the maternal MAP was significantly lower in Group IUGR than in Group P ($p = 0.001$). There were no differences in the maternal MAP and UP between Group P and Group P + IUGR.

The mean gestational age at delivery was significantly younger in Group P ($p = 0.039$), in Group P + IUGR ($p = 0.005$), and in Group IUGR ($p = 0.037$) than in the Control. Even controlling for the gestational age at delivery, the neonatal birth weight was still significantly lower in Group P + IUGR ($p < 0.0001$) and in Group IUGR ($p < 0.0001$) than in the Control.

PP5/TFPI-2 has been reported to be detectable early in pregnancy, and rise to a maximum at gestational weeks 36–37 [29,30]. To adjust for the effect of gestation, we compared the serum PP5/TFPI-2 levels in the maternal samples obtained at delivery by analysis of covariance (ANCOVA) (Fig. 1), after controlling for gestational age at delivery and neonatal birth weight. The detection limit of the sandwich ELISA for PP5/TFPI-2 was 1 ng/ml, and the intra- and inter-assay coefficients of variances were 5.0% and 10.0%, respectively. The analytical recovery was 80%.

The maternal serum PP5/TFPI-2 levels were 530.8 ± 111.3 ng/ml in Group P, 362.1 ± 146.0 ng/ml in Group P + IUGR, 223.9 ± 149.8 ng/ml in Group IUGR, and 233.2 ± 83.8 ng/ml in the Control. The maternal serum PP5/TFPI-2 level was significantly higher in Group P than in the Control ($p = 0.033$), but there were no significant differences in this value between Group IUGR and the Control, and between Group P + IUGR and the Control.

The PP5/TFPI-2 levels in the umbilical serum samples and in the maternal serum samples obtained 4 days after delivery were too low to be measured (data not shown).

3.3. Immunohistochemistry for PP5/TFPI-2, glypican-3, and syndecan-1

Placental samples were available from eight patients in Group P, seven patients in Group P + IUGR, six patients in Group IUGR, and from 24 patients in the Control. We selected 12 placental samples from the patients in the Control who were matched in gestational age at delivery with the patients in the other three study groups randomly. There was no significant difference in the maternal age, body mass index, and umbilical arterial pH among the patients in the three study groups and the Control whose placental samples were subjected to immunohistochemical analysis.

Table 2
Comparison of the characteristics of the study groups

	P ($n = 10$)	P + IUGR ($n = 9$)	IUGR ($n = 7$)	Control ($n = 25$)
Maternal age (years)	31.8 ± 1.9	29.8 ± 2.1	31.0 ± 2.4	31.8 ± 1.0
Maternal BMI	24.6 ± 1.7	24.0 ± 1.7	22.7 ± 1.4	21.8 ± 0.7
Maternal MAP at delivery (mmHg)	118.2 ± 4.4*[a]	110.1 ± 6.3*[b]	88.8 ± 5.5*[c]	78.1 ± 2.0
Maternal UP (mg/dl)	296.3 ± 75.1*[d]	491.8 ± 179.5*[e]	21.4 ± 10.1	13.7 ± 7.5
% of primiparas	50.0	44.4	42.9	52.0
Gestational age at delivery (weeks)	32.5 ± 1.6*[f]	31.1 ± 1.6*[g]	31.1 ± 1.9*[h]	36.0 ± 0.8
% of male babies	30.0	44.4	57.1	48.0
UApH	7.270 ± 0.017	7.232 ± 0.019*[i]	7.295 ± 0.028	7.273 ± 0.018
Neonatal birth weight (g)	1922 ± 311	1124 ± 185*[j]	1344 ± 230*[k]	2550 ± 130

BMI, body mass index; MAP, mean arterial pressure; UP, urinary protein; and UApH, umbilical arterial pH.
Student's t test, otherwise noted.
*[a] $p < 0.001$ (compared to the Control), $p = 0.001$ (compared to Group IUGR), *[b] $p < 0.001$ (compared to the Control), *[c] $p = 0.032$ (compared to the Control), *[d] $p < 0.001$ (compared to the Control), $p = 0.009$ (compared to Group IUGR), *[e] $p < 0.001$ (compared to the Control), $p = 0.01$ (compared to Group IUGR), *[f] $p = 0.039$, *[g] $p = 0.005$, *[h] $p = 0.037$ (compared to the Control), *[i] $p = 0.037$ (compared to the Control, Mann–Whitney's U test), *[j] $p < 0.0001$ and *[k] $p < 0.0001$ (compared to the Control, ANOVA, where gestational age at delivery was set as a covariate.)

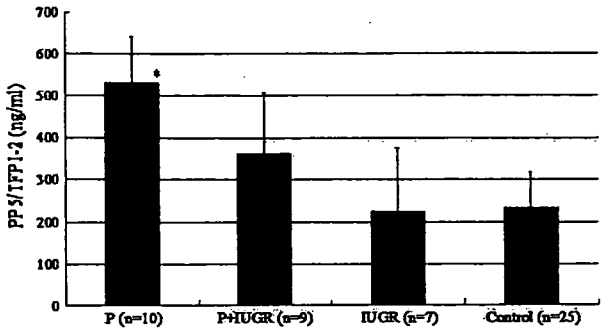

Fig. 1. Comparison of the PP5/TFPI-2 levels in maternal serum samples in the different groups obtained at delivery. Data are expressed as the mean ± SE and are adjusted for gestational age at delivery and the neonatal birth weight. *$p = 0.033$ (ANCOVA, where gestational age at delivery and the birth weight of the neonate are set as covariates).

PP5/TFPI-2 was detected in the cytoplasm of syncytiotrophoblasts, but not in any other type of cell such as cytotrophoblasts, decidual cells, stromal cells, or chorionic vascular endothelial cells (Fig. 2), as we have previously described [18,19]. Glypican-3 showed the same pattern as PP5/TFPI-2, that is, it was present only in the cytoplasm of syncytiotrophoblasts. Syndecan-1 was limited to the surface of the syncytiotrophoblasts.

3.4. Immunohistochemical evaluation

The results of the immunohistochemical analyses are summarized in Fig. 3. The coefficients of variation (CVs) of the scores from each of the two independent examiners were 19.7% and 13.8%, respectively. The CV between the scores from the two examiners was 17.9%. In contrast to the increase in maternal serum levels, PP5/TFPI-2 was detected scarcely in the placenta of Group P (Fig. 2), and so was glypican-3. The scores for PP5/TFPI-2, and also for glypican-3, in the placenta were significantly lower in Group P than in the Control ($p = 0.035$ for PP5/TFPI-2, 0.047 for glypican-3).

The scores for syndecan-1 in the placenta were significantly higher in Group P and Group IUGR ($p = 0.023$ and $p = 0.003$, respectively) than in the Control.

There was a positive correlation between the score for glypican-3 and that for PP5/TFPI-2 among the 33 placental samples examined (C.I. = 0.506, $p = 0.004$) (Fig. 4). The score for syndecan-1 correlated with neither that for PP5/TFPI-2 nor that for glypican-3 (data not shown).

3.5. Interaction of PP5/TFPI-2 and glypican-3

HepG2 cells abundantly produced both the core protein (approximately 60 kDa) and the glycated form (observed as a broad band around 97 kDa) of glypican-3, and no detectable amount of PP5/TFPI-2 was observed (Fig. 5, lanes 1 of (A) & (B)). With the antibody against PP5/TFPI-2, only the glycated form of glypican-3 and PP5/TFPI-2 were co-immunoprecipitate from the lysates of the PP5/TFPI-2 expression vector transfectants, and the core protein of glypican-3 was not detectable (Fig. 5, lanes 4 of (A) & (B)). From the lysates of the empty vector transfectants, which were prepared as a negative control, no detectable bands of PP5/TFPI-2 or glypican-3 were observed (Fig. 5, lanes 2 of (A) & (B)).

4. Discussion

First, we found that PP5/TFPI-2 interacts with glypican-3. In immunohistochemistry, glypican-3 was detected in a pattern identical to that of PP5/TFPI-2, with a positive correlation between the immunohistochemical scores for the two. The biochemical interaction of PP5/TFPI-2 with glycated glypican-3 was demonstrated in the HepG2 cells transfected with *PP5/TFPI-2*. These findings strongly support our previous proposal that glypican-3 serves as the anchor for PP5/TFPI-2 on the placental villi [19]. It is known that some proteins anchored to HSPGs can be shed together to the extracellular space [37]. Glypican-3 may not only anchor PP5/TFPI-2 but also play more roles in the secretory pathway of PP5/TFPI-2. Future studies should identify the precise localization of glypican-3 in the syncytiotrophoblasts and whether PP5/TFPI-2 and glypican-3 interact in the maternal serum.

Second, we highlighted the discrepancy that the maternal serum PP5/TFPI-2 level was increased, whereas placental PP5/TFPI-2 was detected significantly less intensely, in Group P as compared to the Control. This is the first study to investigate in parallel the maternal serum level and the placental immunohistochemistry of PP5/TFPI-2. Most glycoproteins that are produced by the placenta and detected in the maternal serum are known to be increased in the maternal serum of the patients with preeclampsia as compared to Controls [38–44]. It is thought that in preeclampsia, increased apoptosis of trophoblasts occurs in early pregnancy and that newly differentiated trophoblasts later in pregnancy overfunction as a compensation [40,42], based either on the assays of the extracts from the placenta at term [42] or on immunohistochemical studies of the placenta [41,43]. It is obvious from our data that the increase in PP5/TFPI-2 in maternal serum in preeclampsia must result from a mechanism different from that regulating other glycoproteins, which are detected strongly in the placenta in preeclampsia as compared to Controls [41,43].

Glypican-3, which was also detected significantly less intensely in the placenta of Group P as compared to the Control, may provide a clue to clarify the discrepancy in PP5/TFPI-2 levels. It is not clear whether the decreased amount of glypican-3 in the placenta in preeclampsia is caused by reduced expression of the protein through unknown mechanisms, and/or by increased cleavage of it. In either case, the amount of PP5/TFPI-2 anchored on villous surface might be decreased due to the smaller amount of glypican-3 on the villi. One could speculate that more PP5/TFPI-2 would flow out from the placenta to the maternal blood, as compared to normal pregnancy, which in turn would increase the level of PP5/TFPI-2 in maternal serum in preeclampsia. However, other mechanisms should be taken into account for the increase in PP5/TFPI-2 in