tibodies (Fig. 3B, panel b). HΔC cells showed the same pattern as the MTF cells (Fig. 3B, panels g to i). With Western blots, mouse TFAM-HA was detected by anti-HA antibodies in medium without doxycycline (double arrowheads), whereas there was no signal in medium containing doxycycline (Fig. 3C, compare lanes 2 and 3 in the lower panel). Anti-human TFAM did not react with mouse TFAM (Fig. 3C, lane 3; notice their molecular sizes). Similarly, human TFAM-ΔC-HA (triple arrowheads) was detected by both anti-human TFAM and anti-HA antibodies only in medium without doxycycline (Fig. 3C, lanes 4 and 5 in the upper and lower panels). Thus, expression of the recombinant proteins was completely regulated by doxycycline. Endogenous human TFAM (single arrowhead) was not affected by the overexpression of exogenous TFAM (Fig. 3C, upper panel). The amount of BAP37 (arrow), an inner membrane protein, was not altered (Fig. 3C, upper panel), indicating that the amount applied was essentially the same among all samples.

Overexpression of mouse TFAM-HA or human TFAM-ΔC-HA increases the amount of mtDNA. Because it takes about 10 days to stably express exogenous mouse TFAM-HA or human TFAM-ΔC-HA after removal of doxycycline (data not shown), we did experiments at least 14 days after removal of doxycycline. In medium without doxycycline, the amounts of mouse TFAM-HA and human TFAM-ΔC-HA were 0.77 ± 0.17 (mean ± standard deviation) and 1.05 ± 0.11, respectively, of that of endogenous human TFAM; i.e., the total amount of TFAM was 1.77 and 2.05, respectively, that of cells grown in medium with doxycycline (Fig. 4B). Overexpression of total TFAM by a factor of 1.77 (in MTF cells) and 2.05 (in HΔC cells) increased the amount of mtDNA by a factor of 1.89 ± 0.22 and 1.99 ± 0.18, respectively (Fig. 4B). Neither nucleus-encoded mitochondrial proteins (prohibitin and cytochrome *c*) nor an mtDNA-encoded protein, cytochrome *b*, was affected by the overexpression of TFAM (Fig. 4A). Thus, the amount of TFAM led the change in mtDNA levels in parallel.

Both mouse TFAM-HA and human TFAM-ΔC-HA can maintain the amount of mtDNA. mtDNA was increased by the overexpression of C-tail-deleted human TFAM, which is considered not to activate transcription (6), raising the possibility that the increase in mtDNA does not require the upregulation of transcription. In order to clarify this point, we performed RNAi in the TFAM-overexpressing cells. To avoid suppression of recombinant human TFAM by the RNAi treatment, silent mutations were introduced into a cDNA of human TFAM (see Methods). In medium with doxycycline, MTF cells and HΔC cells expressed little or no mouse TFAM-HA (Fig. 5A, double arrowheads; lanes 1 and 2) and human TFAM-ΔC-HA (Fig. 5A, triple arrowheads; lanes 5 and 6), respectively. However, In medium without doxycycline, the exogenous TFAM proteins were overexpressed (Fig. 5A, bottom panel, lanes 3, 4, 7, and 8). The RNAi treatment (i.e., 3 days after RNAi) suppressed the endogenous TFAM (Fig. 5A, fourth panel, lanes 2, 4, 6, and 8), but exogenous TFAMs were not affected (Fig. 5A, bottom panel, lanes 4 and 8; double and triple arrowheads), showing selective downregulation of endogenous human TFAM.

In MTF cells in which mouse TFAM is not yet expressed but endogenous TFAM is repressed by RNAi (Fig. 5B), mtDNA was reduced compared to that in the MTF cells in which endogenous TFAM is not repressed (Fig. 5B). In MTF cells in which mouse TFAM is expressed but endogenous TFAM is not repressed (Fig. 5B), mtDNA was almost doubled with the twofold increase in total TFAM. mtDNA was reduced to the control level by the RNAi treatment in the mouse TFAM-expressing cells (Fig. 5B). The reduced amount of mtDNA by RNAi in medium without doxycycline roughly equaled that in medium with doxycycline (compare the difference between RNAi with and without doxycycline) (Fig. 5B). A similar pattern was observed in HΔC cells (Fig. 5C). These results suggest that both exogenous TFAMs are as competent in the maintenance of mtDNA as endogenous human TFAM under conditions in which endogenous human TFAM remains at about 15% of the control level.

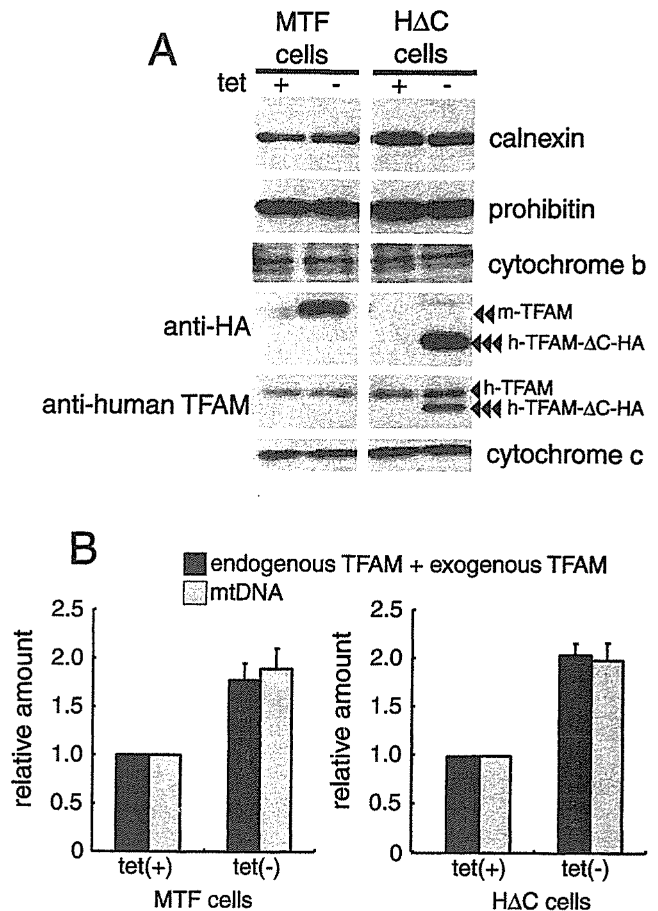

FIG. 4. Amount of mitochondrial proteins and mtDNA in MTF and HΔC cells. (A) Total cell lysates of cells cultured with doxycycline [tet (+)] or without doxycycline [tet (−)] were used for Western blotting. Prohibitin and cytochrome *c* are mitochondrial proteins encoded by the nuclear genome. Cytochrome *b* is a mitochondrial protein encoded by mtDNA. Endogenous human TFAM (arrowhead), mouse TFAM-HA (double arrowheads), and human TFAM-ΔC-HA (triple arrowheads) are indicated. Calnexin, a microsomal protein, is shown as an internal standard. (B) Quantification of relative amounts of TFAM (black bar) and mtDNA (gray bar). Signal intensities on the Western blots were measured for estimating the number of TFAM molecules, endogenous TFAM, and exogenous TFAM. The amount of mtDNA was measured by quantitative PCR. Error bars indicate ±1 standard deviation from the mean of three independent experiments.

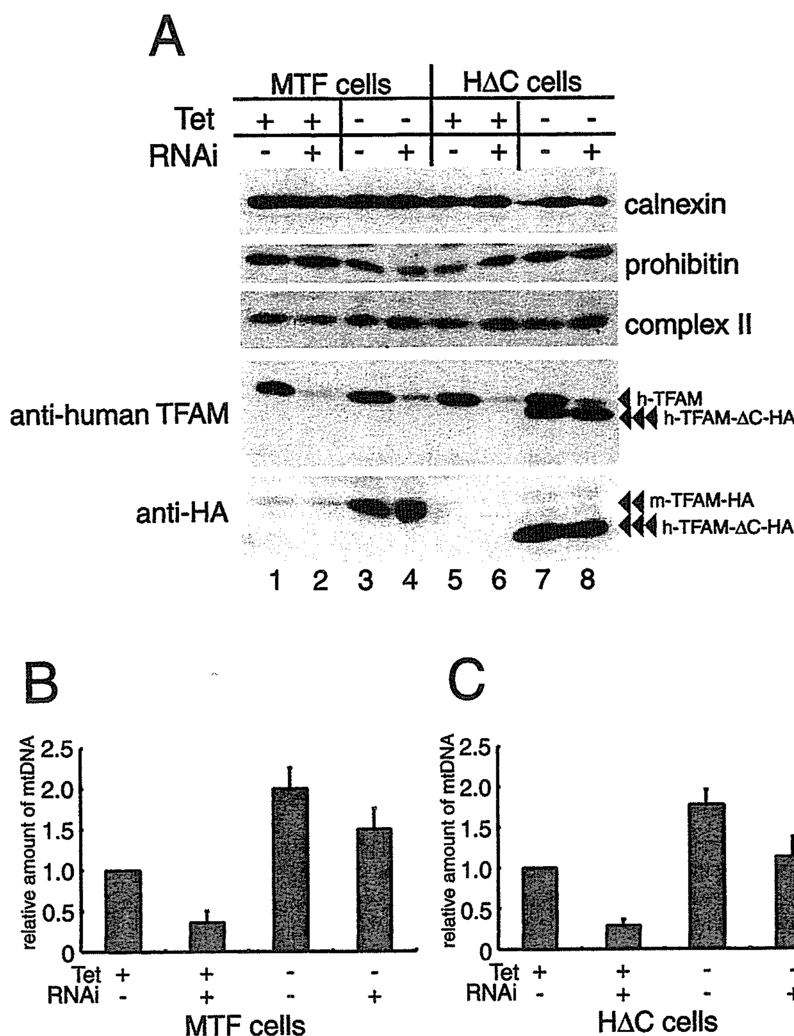

FIG. 5. (A) MTF and HΔC cells were cultured with doxycycline [tet (+)] or without doxycycline [tet (−)]. Three days after RNAi treatment (RNAi +) or without RNAi treatment (RNAi −), the cells were collected and analyzed. The expression of proteins in MTF and HΔC cells was analyzed by Western blotting. Endogenous human TFAM (arrowhead), exogenous mouse TFAM-HA (double arrowheads), and exogenous human TFAM-ΔC-HA (triple arrowheads) are indicated. (B and C) The amount of mtDNA was measured in cells with and without doxycycline and RNAi treatments, as indicated. Error bars indicate ± 1 standard deviation from the mean of three independent experiments.

In vitro and in organello transcription. The C-tail of TFAM is considered essential for initiation of transcription from LSP (6). To confirm that the HA tag does not function like the C-tail, we performed an in vitro transcription assay for human LSP. Human TFAM-ΔC without an HA tag was about 100-fold less active than full-length human TFAM (unpublished data). As expected, a transcript from LSP in the presence of His-human TFAM-ΔC-HA was about 100-fold less than that in the presence of His-human TFAM (Fig. 6A), indicating that LSP-dependent transcription is not increased by adding the HA tag to human TFAM-ΔC.

With the isolated mitochondria from HΔC cells cultured with and without doxycycline and with and without RNAi treatment, we measured transcription in organello by measuring newly synthesized transcripts. The newly synthesized transcripts were labeled with [^{32}P]UTP for 30 min in mitochondria and analyzed by urea-polyacrylamide gel electrophoresis (Fig. 6B). The transcripts were not detected in mitochondria from Rho^0 206 cells (data not shown), excluding any background contribution from genomic transcription. Between the 3,000- and 700-base size markers, the signals for transcription were seen as bands, although the bands were not identified (right panel). In addition to these bands, a smearing background was seen. When human TFAM-ΔC-HA was overexpressed, this smearing background was about 1.6-fold higher (left panel, lane 3) than in the control (lane 1). This nonspecific background was reduced by RNAi treatment (lane 2; by about 60%) and returned to the control level with expression of human TFAM-ΔC-HA (lane 4). The level of the nonspecific smear appeared to be correlated with the amount of mtDNA. The background might reflect nonspecific LSP/HSP-independent transcription or highly heterogeneous RNA processing of LSP/HSP-specific transcription. Another possibility may be RNA primers remaining in nascent mtDNA strands that initiated at diverse sites according to the strand-coupled replication model.

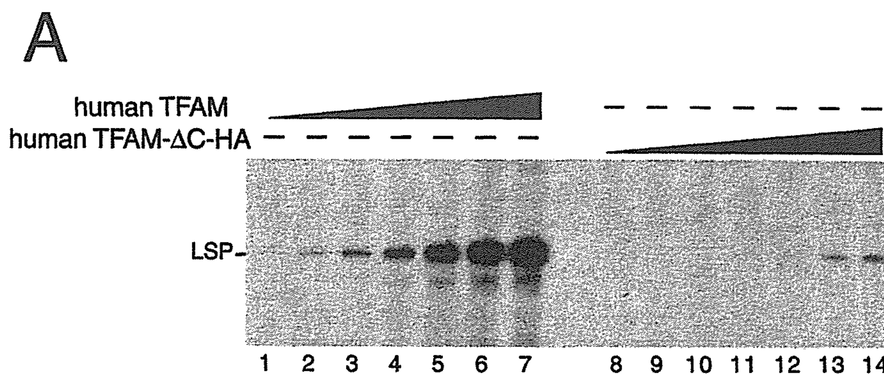

FIG. 6. In vitro and in organello transcription assays. (A) TFAM lacking its C-terminal tail only supports low levels of transcription from LSP. A template containing the light-strand promoter (LSP, 85 fmol) of human mtDNA was used for in vitro runoff transcription assays. The reactions were performed with the following pure recombinant proteins: human mitochondrial RNA polymerase (250 fmol), human TFB2 M (500 fmol), and wild-type human TFAM with an N-terminal His tag or human ΔC-tail TFAM with an N-terminal His tag and C-terminal HA tag (human TFAM-ΔC-HA) in increasing amounts (0.02, 0.05, 0.2, 0.5, 1.6, 5, and 15 pmol). (B) A representative image of five independent experiments is shown. The HΔC cells were cultured with and without doxycycline and treated or not by RNAi, and mitochondria were isolated from these cells 3 days after RNAi treatment. The newly synthesized transcripts were labeled with [α-^{32}P]UTP for 30 min in mitochondria and analyzed by urea-polyacrylamide gel electrophoresis. The approximate size of nucleotides is shown based on the [γ-^{32}P]ATP-labeled DNA size markers (left panel). The part showing bands larger than 600 bases was enlarged (right upper panel). The asterisks indicate measured bands used for estimation. The isolated mitochondria (5 μg of protein) were analyzed by Western blotting to check the protein amount (anti-BAP37 antibody, right lower panel) and the efficiency of RNAi (anti-human TFAM antibody, right middle panel).

Because processed transcripts of mtDNA are distributed mainly between bases 2000 and 700 according to their expected lengths, we hypothesized that the bands found between bases 2000 and 700 are promoter-dependent transcripts (bands marked by asterisks, right panel). The signal intensities were corrected by subtracting the background. When human TFAM-ΔC-HA was overexpressed (lane 3), the specific signals were not changed or somewhat decreased (61 to 113%; average, 77%) in spite of the twofold increase in the amount of mtDNA. This inhibition may be caused by competition between TFAM-ΔC-HA and endogenous TFAM for the promoters. When wild-type human TFAM was reduced by RNAi, the specific signals were reduced to about 46% (41 to 53%) and 49% (44 to 74%) of the control in control and human TFAM-ΔC-HA-overexpressing cells, respectively (lanes 2 and 4), while the amount of mtDNA was about 0.2 and 1 times that of the control cells, respectively (Fig. 5C). Compensatory upregulation of the transcription seems to occur in the former situation. The decrease in the latter may also be due to the competition. Thus, the amount of mtDNA was not correlated with the transcription level.

Both mouse TFAM-HA and TFAM-ΔC-HA are components of the mitochondrial nucleoid. We have previously demonstrated that mtDNA and human TFAM were mostly included in the NP-40-insoluble fraction but that other mitochondrial matrix proteins, P32 and mtSSB, were mostly recovered from the soluble fraction, while part of mtSSB was bound to mtDNA as a component of the mitochondrial nucleoid (1). To examine whether overexpressed TFAM is bound to mtDNA, we prepared mitochondria from MTF and HΔC cells cultured without doxycycline and then separated them into NP-40-insoluble (P1) and -soluble (S1) fractions (Fig. 7, lanes 2 to 3 and 11 to 12). As reported previously (1), most of the endogenous human TFAM (arrowhead) was recovered from the P1 fraction (Fig. 7, lanes 2 and 11), whereas most of the p32 and mtSSB were recovered from the S1 fraction (Fig. 7, lanes 3 and 12). Mitochondrial DNA was detected only in the P1 fraction by PCR (lanes 2 and 11). Both mouse TFAM-HA (double arrowheads) and human TFAM-ΔC-HA (triple arrowheads) were also mostly recovered from the P1 fraction (Fig. 7, compare lane 2 with 3 and lane 11 with 12).

The P1 fractions were treated with nuclease S7 or DNase-free RNase A and then separated into insoluble (P2) and soluble (S2) fractions. After treatment with nuclease S7, most of the endogenous human TFAM, exogenous mouse TFAM-HA, and human TFAM-ΔC-HA were recovered from the soluble fractions (S2) (lanes 7 and 16). However, after the RNase A treatment, the TFAMs were recovered from insoluble fractions (P2) (lanes 8 and 17). These findings suggest that both endogenous and exogenous TFAM molecules are bound to mtDNA as mitochondrial nucleoid components.

DISCUSSION

We showed that the amounts of TFAM and mtDNA were reduced in parallel (Fig. 2B). There are already two reports on suppression of TFAM in vertebrate cells: heterozygous targeted disruption of the *Tfam* gene in mice (19) and in chicken DT40 cells (22). In both cases, both TFAM and mtDNA were decreased by about 50%. These reports, however, showed only steady-state values a long time after the 50% decrease in TFAM, whereas we measured the daily change in the amount of TFAM and mtDNA after the start of RNAi treatment. In the present study, we first showed that the amount of mtDNA was strongly correlated to that of TFAM on a time scale that appears to be less than 1 day. Taking into account that the changes in TFAM and mtDNA were nearly the same at each day, mtDNA levels may reach the corresponding levels of TFAM within hours.

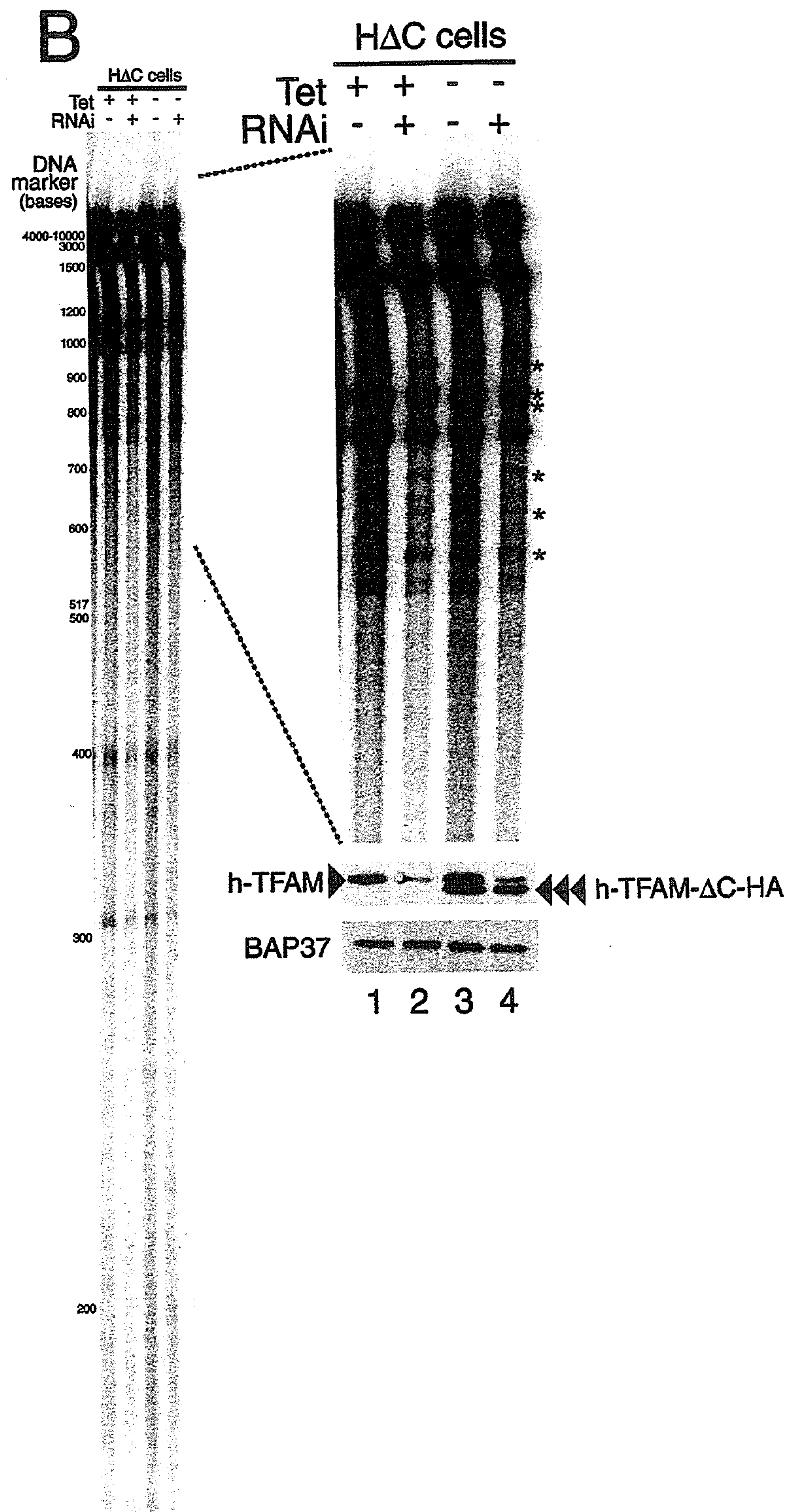

FIG. 6—*Continued*

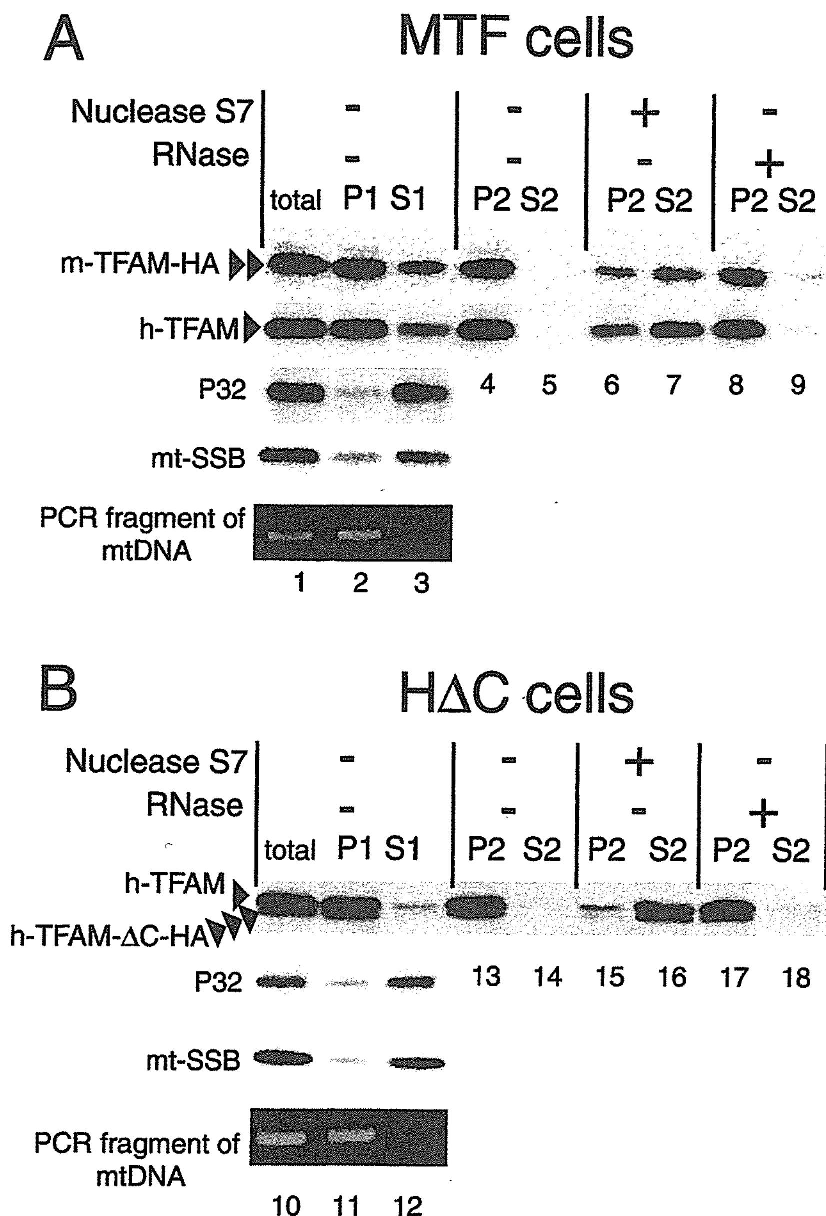

FIG. 7. Mitochondria were prepared from MTF (A) and HΔC (B) cells cultured without doxycycline. The mitochondria were solubilized with 0.5% NP-40 (total) and separated into pellet (P1) and supernatant (S1). The P1 fractions were treated with nuclease S7 or DNase-free RNase A and then separated again into pellet (P2) and supernatant (S2). Each sample was analyzed by Western blotting with anti-human TFAM, anti-P32, anti-mtSSB, and anti-HA antibodies. mtDNA in total, P1, and S1 were detected by PCR. Human TFAM (arrowhead), mouse TFAM-HA (double arrowheads), and human TFAM-ΔC-HA (triple arrowheads) are indicated.

The replication of mammalian mtDNA is coupled with transcription according to the strand displacement model (34). Thus, one possible mechanism by which mtDNA decreases or increases with the decrease or increase in TFAM is that replication of mtDNA is down- or upregulated due to the down- or upregulation of TFAM-activated transcription. However, we found that the transcription level was not correlated with the amount of mtDNA (Fig. 5C and 6B). On the other hand, we noticed a striking correlation between the levels of TFAM and mtDNA. TFAM is a transcription factor (10, 11, 23, 30), but it is also an HMG protein having DNA-binding properties regardless of DNA sequence (11, 30). TFAM molecules are abundant enough to cover mtDNA entirely, and indeed most of them bind mtDNA, suggesting that mtDNA is packaged with TFAM (1, 36) and TFAM is in functional excess (33).

Such a nucleoid structure may also be required for the stability of mtDNA. In agreement with this notion, mouse TFAM and human TFAM-ΔC were as active in the maintenance of mtDNA as wild-type TFAM, at least under conditions in which promoter-specific transcription was maintained at a certain level (Fig. 5C and 6B). About 85% of full-length TFAM could be replaced without reduction in mtDNA by TFAM-ΔC-HA, which has only 1% of the LSP-dependent transcription activity of full-length TFAM (Fig. 5A and C). If the majority of TFAM

molecules maintained mtDNA through transcription-coupled replication, the amount of mtDNA would decrease. Therefore, this result raises the possibility that the majority of TFAM molecules participate architecturally in maintenance.

The overexpressed recombinant TFAM was mostly recovered from the insoluble fraction together with mtDNA (Fig. 7, lanes 2 and 11). Nuclease S7 treatment but not RNase A treatment released recombinant TFAM as well as endogenous TFAM to the soluble fraction (Fig. 7, lanes 6 to 9 and 15 to 18), supporting the idea of TFAM-mtDNA binding. Taken together with the fact that the level of mtDNA rapidly and finely corresponds to the level of TFAM (Fig. 2), one likely explanation is that the vast majority of TFAM molecules are bound to mtDNA and that only mtDNA covered with TFAM can be maintained. According to this model, the mass but not the copy number of mtDNA would be titrated by TFAM, consistent with a finding by Tang et al. that the total mass but not the copy number of mtDNA is constant among cells harboring wild-type, deleted, and partially duplicated mtDNAs (37). Conversely, when mtDNA was depleted with ethidium bromide in HeLa cells, TFAM was reduced to the same extent as mtDNA (33). TFAM and mtDNA may thus stabilize each other when bound together.

We presume that TFAM exists abundantly and that the majority of TFAM molecules are bound to sites other than the promoter regions for packaging mtDNA. Goto et al. reported that TFAM levels can be substantially reduced by RNAi without significant inhibition of transcription per mtDNA in insect cells (15). Here we also showed that transcription per mtDNA was not decreased but rather upregulated by RNAi-induced suppression of TFAM (see Fig. 5C and Fig. 6B, lane 2). These observations suggest that the amount of TFAM can be reduced without lowering the transcription level per mtDNA if the amount of TFAM is manipulated within a cell. In contrast, Garstka et al. reported that import of TFAM into isolated mitochondria significantly enhances transcription in organello (14).

The reason for these apparently contradictory observations is currently unknown. However, the differences may reflect differences in the experimental systems. Garstka et al. manipulated the amount of TFAM with isolated mitochondria, while we in the present study and Goto et al. did so in living cells. A possible but hypothetical explanation is to assume that TFAM preferentially binds to promoters with a higher affinity but can also be preferentially displaced from the promoters by an unknown mechanism. In a cell, continuous input of TFAM would compensate for this selective displacement. However, in isolated mitochondria, there is no input of TFAM unless TFAM is imported artificially, as done by Garstka et al. When the promoter regions are selectively vacant at a certain level, a small amount of TFAM could enhance transcription because TFAM has a higher affinity for the promoters. A human Lon protease homologue is one candidate for such selective displacement, because the homologue was recently reported to bind preferentially to the GT-rich sequence overlapping the LSP of human mtDNA (21).

TFAM is essential for transcription of mtDNA, and TFAM-enabled transcription may be involved in the replication of mtDNA. However, we propose that TFAM may have a dual role in the maintenance of mtDNA, transcription and nucleoid formation. It is probable that the majority of TFAM molecules are involved in architecturally maintaining the higher structure of mtDNA, because at any time most TFAM molecules should be bound to nonpromoter regions. The higher structure is called nucleoid in general. However, it might be more appropriately described as a mitochondrial chromosome or mitochromosome when based on the whole structure of mtDNA.

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Analytical goals for coagulation tests based on biological variation

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Abstract

Allowable imprecision and bias reference limits for laboratory data can be calculated based on measurements of biological variation. Although biological variation of clinical chemical data has been reported from many laboratories, there have been few reports of biological variation in coagulation tests. In this study, we calculated the biological variation of 13 coagulation tests in the clinical laboratory of Kyushu University Hospital and determined allowable imprecision and bias limits of variation. The participating subjects were 17 healthy individuals: three males and two females in their 20s, two males and two females in their 30s, one male and four females in their 40s, and two males and one female in their 50s. Monthly measurements were performed before breakfast 12 times from June 2001 to May 2002 and allowable imprecision and bias limits were calculated. Taken together with coefficient of variation of control plasma used in daily laboratory work at the hospital, the allowable imprecision limits of intra-laboratory variation determined in this study appear to be in attainable ranges.

Keywords: allowable bias; allowable imprecision; biological variation; inter-subject variation; intra-subject variation.

Introduction

In current laboratory practice, it is not rare for common reference intervals to be used among several laboratories or within a particular area. In order to share data and reference intervals among laboratories, variations in laboratory data must be maintained within allowable imprecision and bias limits. In 1970, Harris et al. (1) categorized variations of laboratory data into biological variations and analytical errors. Gowans et al. (2) proposed analytical goals for the acceptance of common reference intervals by laboratories throughout a geographical area, and biological variations of healthy individuals have been used for evaluating variations of laboratory data. Stöckl et al. (3) and Fraser et al. (4, 5) recommended methods for calculating allowable imprecision and bias. In clinical laboratory science, data on biological variations have mainly been applied to clinical chemical test items (6, 7). To our knowledge, however, there are few publications on intra-subject and inter-subject biological variation in prothrombin time (PT) and activated partial thromboplastin time (APTT) (8, 9).

Previously, we estimated allowable imprecision and bias limits using the biological variation of clinical chemical test items (10). In the present study, we further attempted to establish allowable imprecision and bias limits using biological variations of 13 coagulation tests.

Materials and methods

Materials

The participating subjects were 17 members of our department: three males and two females in their 20s, two males and two females in their 30s, one male and four females in their 40s, and two males and one female in their 50s. Monthly blood samples were obtained before breakfast from June 2001 to May 2002. Each 4.5-ml blood sample was collected into a sampling tube containing 0.5 ml of 0.107 mol/l sodium citrate and centrifuged at 3000 rpm (1980 *g*) for 15 min and the plasma was used for the coagulation tests. "Standard plasma" for determination of the reference intervals in the 13 coagulation tests was obtained by collecting blood from healthy individuals (30 males and 30 females) before breakfast. Plasma from these 60 healthy subjects was pooled to yield "standard plasma", stored at −80°C, then thawed in a water bath at 37°C for 3 min and mixed by inversion immediately before use. Separately, we also prepared "normal pooled plasma" for daily monitoring use as "control plasma" for which values of the 13 coagulation tests were within the reference intervals.

Measurements

Items included in the coagulation tests were PT, APTT, fibrinogen (Fib), thrombotest (TB), antithrombin (AT), α_2-plasmin inhibitor (α2PI), plasminogen activity (PLG), thrombin-antithrombin complex (TAT), α_2-plasmin inhibitor-plasmin complex (PIC), thrombomodulin (TM), plasminogen activator-tissue plasminogen activator inhibitor-1 complex (tPAI-C), protein S (PS) and protein C (PC). Table 1 shows the analytical methods, representation methods, reagents, calibrators and measurement apparatuses used for these factors.

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Table 1 Laboratory assays in coagulation tests.

Factors	Methods	Units	Kits manufacturers	Standards manufacturers	Automatic analyzers manufacturers
PT	Clotting time methods	seconds, %, INR	Thromborel^R S Dade Behrig, Germany	Standard plasma	MDA180 Haemostasis Bio Merieux, USA
APTT	Clotting time methods	seconds	MDA Platelin LS Bio Merieux, USA	Standard plasma	MDA180 Haemostasis Bio Merieux, USA
Fib	Clotting time methods	g/l	MDA Fibriquik Bio Merieux, USA	MDA verify reference plasma Bio Merieux, USA	MDA180 Haemostasis Bio Merieux, USA
TB	Clotting time methods	%	Thrombotest^R owren Axis-Shield Poc AS, Norway	Standard plasma	MDA180 Haemostasis Bio Merieux, USA
AT	Chromogenic substrate	%	MDA Antithrombin III Bio Merieux, USA	Standard plasma	MDA180 Haemostasis Bio Merieux, USA
α2PI	Chromogenic substrate	%	Testzym·neo APL Chromogenix, Sweden	MDA verify reference plasma Bio Merieux, USA	MDA180 Haemostasis Bio Merieux, USA
PLG	Chromogenic substrate	%	MDA Plasminogen Bio Merieux, USA	MDA verify reference plasma Bio Merieux, USA	MDA180 Haemostasis Bio Merieux, USA
TAT	EIA	ng/ml	TAT test·F Sysmex, Japan	TAT Standard Sysmex, Japan	Elsia F750 Sysmex, Japan
PIC	EIA	μg/l	PIC test·F Sysmex, Japan	PIC Standard Sysmex, Japan	Elsia F750 Sysmex, Japan
TM	EIA	TU/ml	TM test·F Sysmex, Japan	TM Standard Sysmex, Japan	Elsia F750 Sysmex, Japan
tPAI-C	EIA	ng/ml	tPAI-C test·F Sysmex, Japan	tPAI-C Standard Sysmex, Japan	Elsia F750 Sysmex, Japan
PS	Clotting time methods	%	Staclot Protein S Diagnostica Stago, France	Standard plasma	MDA180 Haemostasis Bio Merieux, USA
PC	Clotting time methods	%	Staclot Protein C Diagnostica Stago, France	Standard plasma	MDA180 Haemostasis Bio Merieux, USA

Calculation methods

Data from laboratory assays of coagulation are affected by liver dysfunction and inflammatory reactions because coagulation factors are produced in the liver and inflammatory reactions interfere with coagulation tests. To eliminate these effects, coagulation tests were performed with simultaneous measurements of white blood cell (WBC), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and C-reactive protein (CRP). Reference intervals of WBC, AST, ALT and CRP were set as 3.50–9.00×10^3/μl, ≤33 U/l, ≤42 U/l in males and ≤27 U/l in females, and ≤2 mg/l, respectively. Intra-subject variation (CV_I) and inter-subject variation (CV_G) were calculated after exclusion of subjects whose data deviated from these reference intervals.

Through a one-way analysis of variance we calculated intra-subject variance (SI^2) and inter-subject variance (SG^2), and also determined analytical variance (SA^2) using the long-term variance of the "control plasma" (normal pooled plasma). Intra-subject variance (SI^2) and inter-subject variance (SG^2) were significantly different. Therefore, using these parameters, intra-subject variation (CV_I) and inter-subject variation (CV_G) were calculated as $CV_I=[SI^2-SA^2]^{1/2}/\text{mean}\times100$ and $CV_G=[(SG^2-SI^2)/12]^{1/2}/\text{mean}\times100$, respectively (1, 11). The allowable imprecision and bias limits for intra-laboratory variation (CV_A) and inter-laboratory variation (B_A) were calculated as desirable performance $CV_A<0.5\ CV_I$ and $B_A<0.25[CV_I^2+CV_G^2]^{1/2}$, as minimum performance $CV_A<0.75\ CV_I$ and $B_A<0.375[CV_I^2+CV_G^2]^{1/2}$, respectively (3–5).

Validation of intra-laboratory allowable imprecision limits (CV_A)

The values of CV_A were compared with the latest coefficient of variation (CV%) of the "control plasma" (normal pooled plasma) used for daily analyses.

Results

Biological variation and allowable imprecision and bias limits for laboratory coagulation tests

As described in Materials and Methods, blood specimens from 17 subjects were collected monthly from June 2001 to May 2002 (total number of measurements=204) and the coagulation test items listed in Table 1 were measured for all of these specimens. To eliminate the effects of inflammation and liver dysfunction, coagulation tests were performed with simultaneous measurements of WBC, AST, ALT and CRP. Intra-subject variation (CV_I) and inter-subject variation (CV_G) were calculated after exclusion of subjects whose data deviated from the reference intervals (after exclusion, the total number of measurements used for calculation was 170). Table 2 shows CV_I, CV_G, CV_A and B_A for the coagulation test data.

Verification of allowable imprecision limits for intra-laboratory variation (CV_A)

The CV_A calculated from the data for each test item was compared with the coefficient of variation (CV%) of the "control plasma" (normal pooled plasma) used for daily analyses. In Table 2, the CV% items marked with asterisks (*) were within the CV_A of the coagulation test items. Eleven items of the coagulation tests fell within the CV_A, but four items did not (Table 2).

Discussion

We have determined allowable imprecision and bias limits based on biological variation for 13 coagulation tests in 17 healthy individuals. Data from these coagulation tests can vary depending on the condition of subjects at the time of blood sampling; for example, the presence or absence of inflammation or liver dysfunction. To exclude values that deviated from reference intervals because of such factors, we excluded subjects with abnormalities of liver function according to measurements of AST and ALT, and those with inflammation according to measurements of WBC and CRP.

Although the number of subjects in this study was relatively low, we carefully eliminated the effects of inflammation and liver dysfunction so that our coagulation test data purely reflected the variations of coagulation function within individuals. In Table 2, we compared our CV_Is with previously published results (8, 9, 12). All CV_Is reported in 1985 by Costongs et al. (8) were larger than those in the present study. This difference may be due to improved analytical methods since 1985 and to the inclusion of smokers (n=126) and women taking oral contraceptives (n=61) among the large number of subjects (n=274) examined by Costongs et al. (8). All of the subjects in the present study were non-smokers and none of the women were taking oral contraceptives. CV_Is reported in 1992 by Dot et al. (9) were relatively consistent with our results (Table 2). Chambless et al. (12) reported short-term (1–2 week) CV_Is of APTT, Fib, and AT, all of which were about half of those reported in our present study (Table 2), suggesting that short-term CV_I is smaller than long-term (1 year) CV_I.

In the present study, we examined biological variations not only of PT, APTT, Fib, AT, α2PI and PLG, but also of TB, TAT, PIC, TM, tPAI-C, PS and PC, which have not been previously examined (Table 2). It is quite valuable to determine the biological variations and the allowable imprecision and bias of these coagulation tests, as these laboratory tests are important in screening for thrombophilia (13). PT and APTT are the most prevalent coagulation tests, which have been measured by a wide variety of combinations of reagents derived from different organs, principles of measurements used in the measuring apparatuses, and standard plasma selected. According to the PT-INR survey data of the Kyushu geographical area in 2001, the coefficient of variation in specimens from healthy individuals was 4.5% in all laboratories (n=133), 3.7% with reagent A (n=51), 3.9% with reagent B (n=27) and 4.8% with reagent C (n=38) (data not shown). These coefficients of variation were larger than the values of B_A% (1.3%, 1.9%) calculated in the present study (Table 2). However, as the intra-laboratory variation (CV_A) was small (Table 2), inter-lab-

Table 2 Coagulation tests: biological variation and the allowable imprecision and bias limits for laboratories.

Coagulation tests	Units	Mean	Biological variation		Imprecision	Bias	"Control plasma"		Other reports		
			Intra-subject CV_I, %	Inter-subject CV_G, %	Intra-laboratory CV_A, %	Inter-laboratory B_A (*[2] B_A), %	2002 (1/11-30/11, n: 20)		1985 Ref. 8*[3]	1992 Ref. 9*[4]	1992 Ref. 12*[5]
							Mean	CV, %	CV_I, %	CV_I, %	CV_I, %
PT	seconds	11.3	2.4	4.0	1.2	1.2 (1.7)	11.4*[1]	1.0			
	%	99.2	5.6	9.4	2.8	2.7 (4.1)	95*[1]	2.4			
	INR	1.01	3.0	4.0	1.4	1.3 (1.9)	1*[1]	1.2			
APTT	seconds	29	3.3	7.1	1.6	2.0 (3.0)	27.7*[1]	0.6	5.8	2.3	
Fib	g/l	2.3	8.7	14.8	4.4	4.3 (6.5)	3.4*[1]	2.1	6.8	2.1	1.7
TB	%	78.4	12.9	12.9	6.5	4.6 (6.8)	73*[1]	1.9	10		6.8
AT	%	105.5	2.1	2.6	1.1	0.8 (1.2)	98	2.3	3.1		1.1
α2PI	%	115.7	4.8	7.1	2.4	2.1 (3.2)	105	4.4	5.8		
PLG	%	111.1	4.2	10.5	2.1	2.8 (4.2)	114	3.0	7.7		
TAT	ng/ml	1.0	26.0	20.0	12.9	8.1 (12.1)	4.4*[1]	2.8			
PIC	μg/ml	0.5	18.0	26.0	8.4	7.3 (11.0)	1.3*[1]	3.0			
TM	TU/ml	8.8	11.4	16.5	5.7	5.0 (7.5)	14.5*[1]	1.7			
tPAI-C	ng/ml	8.3	15.9	31.2	7.9	8.7 (13.1)	11.5*[1]	3.6			
PS activity	%	103.5	7.6	22.3	3.8	5.9 (8.8)	91	5.9			
PC activity	%	101.5	7.9	17.5	4.0	4.8 (7.2)	91*[1]	3.1			2.1 (antigen)
Number of subjects			17						274	39	39
Intervals			Monthly						Monthly	Monthly	1-2 weeks
Terms or times			Twelve months						Six months	Nine months	Three times

*[1]:Control plasma CV% < intra-laboratory CV_A%; *[2]B_A:Minimum performance for allowable bias; *[3]:Reference 8 (Netherlands), *[4]: Reference 9 (Spain), *[5]: Reference 12 (North Carolina).

oratory variation is expected to approach the value calculated in this study as the standardization of coagulation tests progresses.

Recently, the European Concerted Action on Thrombosis (ECAT) monitored plasma AT activity using data from 82 laboratories from 1996 to 1999 (14). On the basis of the reported CV_Is (8, 12), none of the laboratories in the ECAT study fulfilled the criterion of $CV_A < 0.5\ CV_I$; if the total biological variation for AT was calculated as 10.5% on the basis of the CV_I and CV_G data reported by Chambless et al. (12), 20% of the participating laboratories fulfilled the goal for diagnostic testing. This indicates that, at the present time, only a limited number of laboratories can overcome the allowable imprecision on the basis of biological variation of long-term analytical performance of coagulation tests.

In this study, we determined the allowable imprecision (CV_A) and bias (B_A) limits of 13 coagulation tests. The determined CV_A of the 15 items in the 13 coagulation tests are considered to be sufficiently attainable when compared with the coefficient of variation of the "control plasma" (normal pooled plasma) but it would not be easy to apply the CV_A to the intra-laboratory variation used in external quality assessment.

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Letter to the Editor

Development of a new colorimetric method for protein S activity measurement

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Development of a reliable method (simple, sensitive and accurate) for protein S measurement is needed (1–4), as the protein C/protein S coagulation regulatory system has been shown to be involved in thrombosis (5–16). This letter describes a new colorimetric method we have recently developed for measuring protein S activity. This method, unlike conventional ones that measure clotting time, measures protein S activity colorimetrically using chromogenic substrates. The principle of this assay system is that protein S activity is calculated by analyzing the correlation between protein S activity and thrombin production rate in the protein C/protein S coagulation regulatory system and the blood clot reaction system reconstructed in vitro (17, 18). Using this method, protein S activity was measured in a variety of samples and their values were compared to those obtained with the conventional method measuring clotting time (19).

Human activated protein C (APC), human protein S and human prothrombin, used in the assay, were obtained from Enzyme Research Laboratories, Inc. (South Bend, SD, USA), purified bovine factor Xa (FXa) from New England Biolabs, Inc. (Beverly, MA, USA), purified human factor Va (FVa) from Hematologic Technologies, Inc. (Essex Junction, VT, USA), thrombin chromogenic substrate S-2238 (20) from Chromogenix-Instrumentation Laboratory (Milan, Italy), bovine serum albumin (BSA) from Sigma-Aldrich Co. Ltd. (St. Louis, MS, USA) and porcine liver L-α-phosphatidylethanolamine (PE), porcine liver L-a-phosphatidylcholine (PC) and bovine brain L-α-phosphatidylserine (PS) from DOOSAN Serdary Research Laboratories (Yongin, Korea). A clot-time assay kit, STA®-Staclot® Protein S (Diagnostica Stago, Asnières, France), was used for comparison with our new method. A calibrator was made with purified protein S added to protein S-deficient plasma, and the activity was expressed as μg/ml PS-equivalent. Protein S-deficient plasma was prepared from fresh plasma by immunoadsorption on an anti-protein S immnoglobulin G (IgG) monoclonal antibody (3) column. Phospholipid (PE/PC/PS = 50/20/30 moles) and phospholipid (PC/PS = 80/2 moles), at the specified compositions, were taken into a test tube and, following evaporation of CHC_3 with N_2 gas, were re-suspended in distilled water and sonicated at 60°C for 10 minutes.

Protein S activity was measured as follows. Samples (4 μl) were incubated at 37° C for 10 minutes in a total volume of 100 μl containing 344 pmol/l APC and 10 μmol/l phospholipid PC/PS in buffer A (50 mmol/l Tris-HCl, pH 7.5, 0.15 mol/l NaCl, and 0.1% BSA). After addition of 100 μl containing 331 pmol/l FVa, 10 μmol/l phospholipid PC/PS and 5 mmol/l CaC_2 in buffer A, the mixture was further incubated for 10 minutes at 37° C (to degrade FVa by APC and protein S). During this incubation, 88 pmol/l FXa, 15 μmol/l phospholipid PE/PC/PS and 10 mmol/l $CaCl_2$ in 180 μl buffer A were mixed in a cuvette with 1.4 μmol/l prothrombin and 1.5 mmol/l S-2238 in 180 μl buffer A. Into this cuvette, the reaction mixture (180 μl), after incubation, was added and incubated at 37° C for measurement of non-degraded FVa activity. The absorbance at 405 nm was recorded for 40 minutes.

The principle of this method is shown schematically in Figure 1A. First, APC, protein S and FVa are incubated to hydrolyze FVa by APC and protein S (reaction 1). Since APC activity is increased by protein S, protein S activity can be measured from unhydrolyzed FVa. The remaining FVa activity is measured from the rate of thrombin production using the FXa and prothrombin reactions. Thrombin activity is obtained by measuring p-nitroaniline released from S-2238 as the absorbance at 405 nm (reaction 2). Figure 1B shows the time course of the reaction measured by the method described above. Since remaining FVa activity and protein S activity show an inverse relationship, the absorbance decreases as protein S activity increases.

Since thrombin is produced at a constant rate in this system, p-nitroaniline production increases at a constantly increasing rate. Therefore, the absorbance increases in proportion to the square of the reaction

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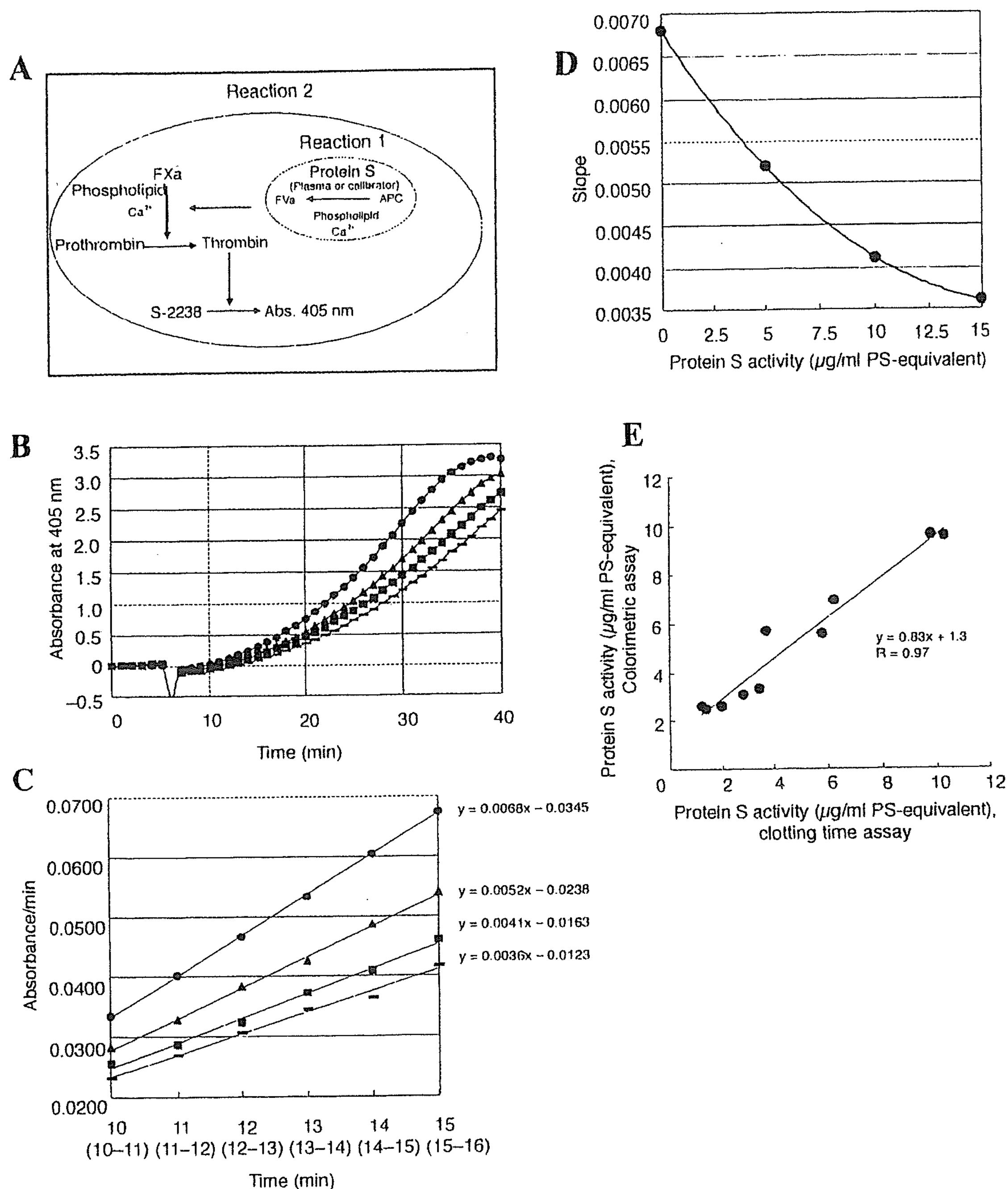

Figure 1 Method for protein S activity measurement using chromogenic substrates. A: Schematic diagram of protein S activity assay system. FVa degradation is produced by the reaction of APC, protein S and FVa (reaction 1). The reaction mixture is then added to a mixture of FXa, prothrombin and S-2238 to induce the enzymatic reaction cascade. Thrombin, thus generated, catalyzes the release from S-2238 of p-nitroaniline, which is measured as absorbance at a wavelength of 405 nm (reaction 2). The rate of p-nitroaniline generation depends on remaining FVa activity, which depends on protein S activity. B: Time course of thrombin production measured by the protein S assay system for constructing a protein S calibrator. FXa, prothrombin and S-2238 in 360 μl were pre-incubated for 5 minutes and then added to 180 μl reaction mixture containing APC, protein S and FVa. Absorbance was recorded for 40 minutes. Protein S activity was as follows: 0 (●), 5 (▲), 10 (■) and 15 μg/ml PS-equivalent (–). C: Differentials of reaction curves in (B). Absorbance changes within 1 minute are plotted from 10 to 16 minutes after the start of the reaction. Protein S activity was as follows: 0 (●), 5 (▲), 10 (■) and 15 μg/ml PS-equivalent (–). D: Calibration curve of protein S activity. The slopes of straight lines in (C) are plotted against standard protein S activity. E: Comparison of measurements of plasma protein S activity between colorimetric assay and clotting time assay.

time, as shown in Figure 1B. When the differentials of absorbance increases in a unit time are plotted against time, straight lines are obtained, as shown in Figure 1C. The calibration curve for protein S activity is obtained when the slopes of these lines are plotted against protein S concentration (Figure 1D).

Protein S activity was calculated from the calibration curve for plasma samples measured similarly. The day-to-day reproducibility was examined by five measurements of the plasma from a healthy person and a pregnant woman. In the former plasma, the mean activity was 11.0 μg/ml PS-equivalent with a

variable coefficient of 2.7%, and in the latter, 4.3 μg/ml PS-equivalent with a variable coefficient of 7.2%.

Ten plasma samples containing different amounts of free protein S antigen were prepared, and protein S activity was measured by the new method and the conventional clotting method. These measurements showed excellent correlation ($r=0.97$) (Figure 1E).

The method we have developed for measuring protein S activity will contribute to routine operations and standardization of protein S activity measurements as well as screening for protein S abnormalities, although the sensitivity, reproducibility and cost must be further improved. In addition, it could be applied to measurement of other blood coagulation factors.

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Relation of Serum Total Cholesterol and Other Risk Factors to Risk of Coronary Events in Middle-Aged and Elderly Japanese Men With Hypercholesterolemia
—— The Kyushu Lipid Intervention Study ——

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for the Kyushu Lipid Intervention Study (KLIS) Group

Background The role of serum total cholesterol (TC) in the development of coronary heart disease (CHD) may differ in different age groups.
Methods and Results The relation of serum TC and other risk factors to CHD events was examined in middle-aged (<65 years) and elderly (≥65 years) men separately in the Kyushu Lipid Intervention Study (KLIS). Subjects were 4,349 men aged 45–74 years with serum TC of 220 mg/dl or greater who had no history of myocardial infarction, coronary angioplasty, or stroke. There were 123 CHD events (ie, myocardial infarction, coronary bypass surgery, coronary angioplasty, cardiac death, and sudden death) in a 5-year follow-up period. The Cox proportional hazards model was used with baseline and follow-up serum TC, baseline high-density lipoprotein (HDL) cholesterol, hypertension, diabetes mellitus, and other factors as covariates. Serum TC concentration during the follow-up, not at baseline, was associated with an increased risk of CHD events, especially in elderly men. High concentrations of serum HDL cholesterol were associated with a modest, statistically nonsignificant decrease in the risk among middle-aged men. An increased risk of CHD events associated with diabetes mellitus was greater in middle-aged men. Hypertension and smoking were not measurably related to the risk in either middle-aged or elderly men.
Conclusions Both the serum TC concentration during follow-up and diabetes mellitus are important predictors of CHD events in Japanese men with moderately elevated serum TC. (*Circ J* 2004; **68:** 405–409)

Key Words: Coronary heart disease; Diabetes mellitus; Japanese men; Serum total cholesterol

Elevated concentrations of serum total cholesterol (TC) or low-density lipoprotein (LDL) cholesterol are related to an increased risk of coronary heart disease (CHD), not only in Western populations[1] but also in Asian populations.[2–5] Further, primary and secondary prevention trials have demonstrated that lowering cholesterol results in a substantial decrease in the risk of CHD events.[6–10] However, the value of lowering cholesterol in the elderly has been a matter of controversy. Several,[11–13] but not all,[14–16] prospective studies suggested a diminished or null association between serum TC and CHD risk in elderly persons. Recently in Europe, pravastatin use was shown to be beneficial in the prevention of CHD in those aged 70 years or older who had a history of, or risk factors for, vascular disease.[17] Very few studies have addressed the relation of serum TC or LDL cholesterol and other risk factors to CHD in the elderly in Japan. A case–control study showed an increased risk of nonfatal myocardial infarction associated with hypercholesterolemia in middle-aged Japanese, but not in the elderly.[4] In a small trial of elderly Japanese men and women,[18] a high dose of pravastatin resulted in a greater decrease in the combined events of cardiovascular diseases including stroke, as compared with a low dose of pravastatin. In the study reported here, we examined the relation of serum TC and other risk factors to the risk of CHD events in middle-aged and elderly Japanese men separately, using the data compiled in the Kyushu Lipid Intervention Study (KLIS), a primary prevention trial of CHD events and cerebral infarction in Japanese men with moderately elevated concentrations of serum TC.[19–21]

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Methods

Details of the study design and primary results of the KLIS have been described previously.[19–21] In brief, a total of 5,640 men aged 45–74 years with serum TC of 220 mg/dl or greater were enrolled by 902 physicians in the Kyushu District during the period between May 1990 and September 1993. The following subjects were ineligible: those with a history of myocardial infarction, coronary

Table 1 Adjusted Relative Risks of Coronary Heart Disease Events According to Selected Factors*

Variable	*No. of men*	*No. of cases*	*Adjusted RR (95% CI)*
Baseline TC (mg/dl)			
<240	*1,463*	*29*	*1.00 (referent)*
240–259	*1,538*	*47*	*1.38 (0.86–2.22)*
≥260	*1,348*	*47*	*1.49 (0.89–2.49)*
Follow-up TC (mg/dl)			
<220	*2,097*	*50*	*1.00 (referent)*
220–239	*1,338*	*34*	*1.18 (0.75–1.84)*
≥240	*914*	*39*	*2.07 (1.31–3.29)*
HDL cholesterol (mg/dl)			
<40	*970*	*26*	*1.00 (referent)*
40–59	*2,485*	*81*	*1.27 (0.82–1.99)*
≥60	*894*	*16*	*0.74 (0.39–1.39)*
Body mass index (kg/m²)			
<22.5	*1,197*	*37*	*1.00 (referent)*
22.5–24.9	*1,609*	*39*	*0.84 (0.53–1.33)*
≥25.0	*1,543*	*47*	*1.06 (0.67–1.66)*
Angina pectoris			
None	*3,930*	*94*	*1.00 (referent)*
(+)	*419*	*29*	*2.62 (1.71–4.01)*
Diabetes mellitus			
None	*3,325*	*77*	*1.00 (referent)*
(+)	*1,024*	*46*	*2.02 (1.39–2.92)*
Hypertension			
None	*2,445*	*58*	*1.00 (referent)*
(+)	*1,904*	*65*	*1.32 (0.91–1.90)*
Cigarettes per day			
0	*2,679*	*76*	*1.00 (referent)*
1–19	*474*	*18*	*1.26 (0.75–2.13)*
≥20	*1,196*	*29*	*1.04 (0.67–1.62)*
Alcohol use (days/week)			
0	*1,683*	*56*	*1.00 (referent)*
1–4	*910*	*26*	*0.90 (0.56–1.45)*
≥5	*1,756*	*41*	*0.80 (0.53–1.22)*

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, statin use in the follow-up, and listed variables.*

bypass surgery, coronary angioplasty, or stroke; those with serum high-density lipoprotein (HDL) cholesterol of 80 mg/dl or greater; and those having life-limiting morbid conditions such as severe renal or hepatic disease. Each physician was instructed to randomly allocate patients to either pravastatin treatment or conventional treatment as specified in a sealed envelope, but participating physicians did not necessarily follow the instruction of the random assignment.[19] Pravastatin was prescribed at a dosage of 10–20 mg/day, and the conventional treatment included dietary and/or exercise therapy and medication with hypolipidemic drugs other than probucol, bezafibrate, and statins. The patients were followed up until the end of 1997. Methodological issues relevant to the present study are described below.

Subjects

The present analysis included 4,349 of the 5,640 enrolled men; 1,291 were excluded for the following reasons: withdrawal of consent (n=147), no institutional contract (n=616), delayed discovery of ineligibility (n=97), and missing values for covariates (n=431). The category of 'no institutional contract' resulted from the introduction during the course of the study of a new regulation for clinical trials, which required a written agreement of contract between a participating institution and a sponsoring pharmaceutical company.

Laboratory and Clinical Data

Serum TC, HDL cholesterol, triglycerides, and other clinical and biochemical variables were determined at baseline and again after follow-up. Laboratory measurements were done at different laboratories, but each study physician was requested to use the same laboratory throughout the study period. Average serum TC concentrations during the follow-up were determined on the basis of periodic measurements at 3 months, 6 months, and every year thereafter. Serum HDL cholesterol was not measured during the follow-up for no less than 10% of the study subjects, and only baseline HDL cholesterol was used in the present study.

Hypertension was defined as present if a patient had systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or if he was under medication for hypertension. Subjects were defined as having diabetes mellitus if they had either a fasting plasma glucose ≥140 mg/dl or hemoglobin A1c ≥6.5% or if they were under medication for diabetes mellitus. The presence of angina pectoris and prior use of hypolipidemic drugs were based on the report of study physicians. Statin use was defined if any statin drugs were prescribed during the follow-up period. Body mass index (kg/m²) was used as an index of obesity. Current habits of smoking and alcohol drinking were ascertained, and the number of cigarettes smoked per day and frequency of alcohol drinking per week were determined.

Table 2 Adjusted Relative Risks of Coronary Heart Disease Events According to Selected Factors in Men Aged Less Than 65 Years and Older Men*

Variable	<65 years (n=3,115) No. of cases	<65 years (n=3,115) Adjusted RR (95% CI)	≥65 years (n=1,070) No. of cases	≥65 years (n=1,070) Adjusted RR (95% CI)
Baseline TC (mg/dl)				
<240	18	1.00 (referent)	11	1.00 (referent)
240–259	27	1.34 (0.72–2.47)	20	1.51 (0.71–3.23)
≥260	32	1.69 (0.88–3.26)	15	1.26 (0.54–2.94)
Follow-up TC (mg/dl)				
<220	34	1.00 (referent)	16	1.00 (referent)
220–239	17	0.72 (0.40–1.31)	17	2.45 (1.20–4.98)
≥240	26	1.59 (0.90–2.81)	13	3.20 (1.44–7.09)
HDL cholesterol (mg/dl)				
<40	18	1.00 (referent)	8	1.00 (referent)
40–59	50	1.04 (0.60–1.78)	31	1.78 (0.80–3.93)
≥60	9	0.69 (0.30–1.56)	7	0.91 (0.32–2.60)
Body mass index (kg/m²)				
<22.5	22	1.00 (referent)	15	1.00 (referent)
22.5–24.9	23	0.65 (0.36–1.17)	16	1.11 (0.54–2.28)
≥25.0	32	0.85 (0.49–1.49)	15	1.45 (0.68–3.11)
Angina pectoris				
None	61	1.00 (referent)	33	1.00 (referent)
(+)	16	2.81 (1.60–4.95)	13	2.35 (1.22–4.54)
Diabetes mellitus				
None	43	1.00 (referent)	34	1.00 (referent)
(+)	34	2.36 (1.49–3.72)	12	1.46 (0.74–2.87)
Hypertension				
None	39	1.00 (referent)	19	1.00 (referent)
(+)	38	1.32 (0.83–2.10)	27	1.36 (0.73–2.52)
Cigarettes per day				
0	49	1.00 (referent)	27	1.00 (referent)
1–19	8	0.90 (0.42–1.91)	10	1.94 (0.91–4.13)
≥20	20	0.90 (0.53–1.54)	9	1.32 (0.60–2.89)
Alcohol use (days/week)				
0	30	1.00 (referent)	26	1.00 (referent)
1–4	18	1.02 (0.56–1.84)	8	0.67 (0.30–1.52)
≥5	29	0.85 (0.50–1.44)	12	0.71 (0.35–1.44)

RR, relative risk; CI, confidence interval; TC, total cholesterol.
**Based on Cox proportional hazards model controlling for age (5-year class), prior use of cholesterol-lowering drugs, statin use in the follow-up, and listed variables.*

Follow-up

The end-point was a composite of CHD events comprising fatal and nonfatal myocardial infarction, coronary artery surgery, coronary angioplasty, CHD death, and sudden and unexpected death. The diagnostic criteria for these conditions were in accordance with those used in the Lipid Research Clinic Study.[22] Only definite cases of CHD events were used in the present study. These end-points were determined by the End-point and Adverse Effect Committee on the basis of periodic reports from the study physicians and the follow-up survey carried out from January to May in 1998.

As of the end of 1997, vital status was unknown for 36 men, and CHD events were not ascertained for 97 men. The average observation period was 5.03 years. There were 123 CHD events, including 66 cases of myocardial infarction, 11 cases of coronary bypass surgery, 24 cases of coronary angioplasty, 7 cardiac deaths, and 15 sudden deaths.

Statistical Analysis

The Cox proportional hazards model was used to examine the relation of clinical and behavioral factors to the risk of CHD events. The model included indicator variables for age (5-year class), use of statins, baseline serum TC (<240, 240–259, and ≥260 mg/dl), follow-up serum TC (<220, 220–239, and ≥240 mg/dl), baseline serum HDL cholesterol (<40, 40–59, and ≥60 mg/dl), body mass index (<22.5, 22.5–24.9, and ≥25.0 kg/m²), angina pectoris, hypertension, diabetes mellitus, prior use of lipid-lowering drugs, current smoking (0, 1–19, and ≥20 cigarettes per day), and current alcohol use (0, 1–4, and ≥5 days per week). Adjusted relative risk (RR) and 95% confidence interval (CI) were obtained from a regression coefficient and standard error for the corresponding indicator variable. Statistical significance was declared when the 95% CI did not include unity. Statistical computations were done by the SAS software version 8.2 (SAS Institute, Inc, Cary, NC, USA).

Results

The mean age of the study subjects at baseline was 58.0 years (SD 8.0). The average concentrations of baseline serum TC, follow-up TC, and baseline HDL cholesterol were 253 (SD 24.1), 221 (SD 26.2), and 49 (SD 12.0) mg/dl, respectively. The Pearson's correlation coefficient between baseline and follow-up TC was 0.39. The prevalence of diabetes mellitus was 23.5%, and that of hypertension was 43.8%. Men with angina pectoris and those who were overweight (body mass index ≥25) accounted for 9.6% and 35.5%, respectively. Proportions of smokers and men drinking alcohol once per week or more frequently were 38.4% and 40.4%, respectively.

Table 1 shows the relation of selected risk factors to CHD events in all subjects. Although the baseline serum TC was statistically nonsignificantly associated with a modest increase in the risk of CHD events, a statistically significant, 2-fold increase in the risk was observed for men with the highest concentrations of follow-up TC as compared with those with the lowest concentrations. A small, statistically nonsignificant decrease in the risk was observed for men with the highest concentrations of HDL cholesterol. Angina pectoris and diabetes mellitus were each associated with a statistically significant increase in the risk of CHD events. Hypertension was associated with a slightly increased risk of CHD events whereas body mass index showed no association with CHD events. Alcohol use was associated with a modest, statistically nonsignificant decrease in the risk, and cigarette smoking was unrelated to CHD events.

Table 2 presents results from the analysis stratified by age class. An increased risk of CHD events associated with elevated concentrations of follow-up TC was more marked in elderly men than in middle-aged men. In the latter group, the increase was modest, and statistically nonsignificant. On the other hand, a decrease in the risk associated with high concentrations of HDL cholesterol was almost limited to middle-aged men, although the decrease was not statistically significant. An increased risk associated with diabetes mellitus was also much greater in middle-aged men. A protective association with alcohol use was seemingly more evident in the elderly. Associations of angina pectoris and hypertension with CHD events did not differ in the 2 age groups.

Discussion

The present study showed that the serum TC concentration during the follow-up period, not at baseline, was related to an increased risk of CHD events in elderly men especially. This apparent difference in the association with TC between middle-aged and elderly men may be ascribed to random fluctuation in the sub-group analysis. Nonetheless, the present findings add to evidence that elevated concentrations of serum TC are an important risk factor for CHD in the elderly as well.

In a recent case–control study in Japan,[4] hypercholesterolemia, which was defined as serum TC ≥220 mg/dl or use of cholesterol-lowering drugs, was associated with an increased risk of nonfatal myocardial infarction in those aged less than 65 years but not in older persons. Several prospective studies showed that elevated concentrations of TC was unrelated or more weakly related to the risk of CHD in older persons.[11–13] The null or weak association between TC and CHD in older people was ascribed to comorbidity and frailty associated with low cholesterol in a prospective study.[16] The finding in a randomized trial that statin use resulted in a measurable decrease in CHD events in older men and women strengthens the importance of hypercholesterolemia in the occurrence of CHD among elderly persons.[17]

An inverse association between HDL cholesterol and CHD events was not substantial in the present study. High concentrations of serum HDL cholesterol are generally related to a decreased risk of CHD independently of TC or LDL cholesterol,[23] although the protective association is not a universal observation.[24,25] Exclusion of men with HDL cholesterol of 80 mg/dl or greater may partly explain the lack of a clear, protective association with HDL cholesterol in the present study. It may be more relevant that HDL cholesterol at baseline rather than during the follow-up was used. In the Japan Lipid Intervention Trial (J-LIT),[3] a cohort study of men and women under simvastatin treatment for hypercholesterolemia, high concentrations of HDL cholesterol during the treatment were associated with an evident decrease in the risk of CHD events.

The increased risk of CHD events associated with diabetes mellitus is consistent with the observation in the J-LIT,[3] as well as with the current knowedge.[23] However, the increased risk associated with diabetes mellitus was confined to middle-aged men. Insulin resistance is often accompanied by low HDL cholesterol, and part of the protective association between HDL cholesterol and CHD may be a reflection of the relation between insulin resistance and CHD.[23,26] In this regard, the apparently stronger, positive association with diabetes mellitus in middle-aged men is compatible with a decreased risk associated with high concentrations of HDL cholesterol observed almost exclusively in this age group. However, these different associations with diabetes mellitus and HDL cholesterol according to age groups need confirmation because the confidence intervals of the RRs estimated for diabetes mellitus and the highest concentrations of HDL cholesterol in the 2 age groups overlapped well.

Hypertension was not appreciably associated with CHD events in either middle-aged or elderly men, which is not surprising. Hypertension has been well recognized as a risk factor for cardiovascular diseases, and effective medication for the condition is used in routine clinical practice. Patients under treatment for hypercholesterolemia were probably well treated for co-existing hypertension. A population survey in Japan showed that hypertensives on drug were more frequent in men with hypercholesterolemia under medication than in those without.[27] However, hypertension ascertained at baseline was associated with a statistically significant, 2-fold increase in the risk of CHD events in the J-LIT.[3]

The present findings regarding alcohol use were in line with a protective association between alcohol use and CHD.[28] Cigarette smoking was not materially associated with CHD events. In addition to the fact that past smokers were not distinguished from lifelong nonsmokers, it is possible that smoker patients under treatment for hypercholesterolemia may have been more likely to quit smoking or to reduce the amount of cigarettes.

The present study examined risk factors for CHD events in men undergoing treatment for moderately elevated concentrations of TC, and the findings are not generalized to all Japanese men. The reported magnitudes in the risk of CHD events associated with risk factors were probably underestimated because of treatment for comorbid conditions and a change in lifestyle during the treatment. However, the present findings have practical implications in the management of patients with hypercholesterolemia. Diabetes mellitus, as well as the serum TC concentration under treatment, was found to be highly predictive of CHD events, and more importantly diabetes mellitus was not as appropriately treated as hypertension.

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Appendix 1

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