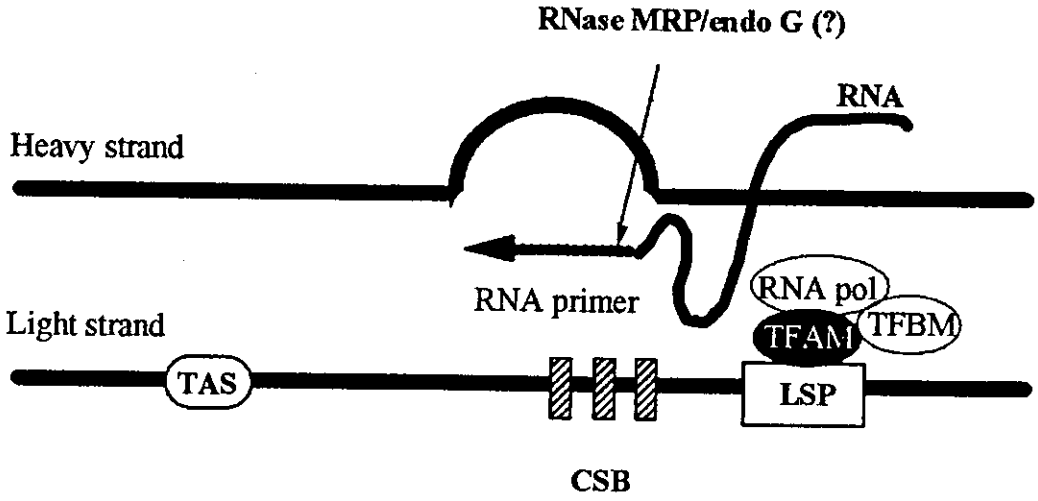

FIGURE 1. Transcription from LSP. TFAM binds to LSP and then recruits mitochondrial RNA polymerase and mitochondrial transcription factor B (TFBM). The C-terminal is responsible for binding to TFBM. TFBM confers the promoter specificity to the TFAM-dependent transcription. Some transcripts originating from LSP make stable RNA-DNA heteroduplexes in the conserved sequence block (CSB). The RNA strand of the heteroduplex is processed by an RNase and serves as a primer for DNA synthesis by DNA polymerase.

review, we focus on the essential roles of TFAM in the maintenance of mtDNA integrity.

TFAM AS A TRANSCRIPTION FACTOR

TFAM was purified from mitochondria of human HeLa cells using transcription activation as a marker[3] and then cloned.[2] TFAM is a member of an HMGB subfamily in high-mobility group (HMG) protein families. TFAM has two HMG boxes and a C-terminal tail composed of 25 amino acids. Each HMG box has DNA-binding activity. Although TFAM has a higher affinity to the two promoters on mtDNA, light- and heavy-strand promoters (LSP and HSP, respectively), it also has nonspecific DNA-binding activity irrespective of DNA sequence. The C-terminal tail is required for the promoter-specific transcription *in vitro*. Abf2p, a counterpart of TFAM in *Saccharomyces cerevisiae*, also has two HMG boxes but not the C-terminal tail.[4] In fact, Abf2p does not activate the transcription *in vitro*[5] or is not required *in vivo* for the expression of mtDNA genes (i.e., transcription). The current general model for the human mitochondrial transcription is outlined in FIGURE 1. TFAM binds to LSP and then recruits mitochondrial RNA polymerase and mitochondrial transcription factor B (TFBM).[6] The C-terminal is responsible for binding to TFBM.[7] Without TFBM, TFAM activates promoter-independent nonspecific transcription in the presence of RNA polymerase. TFBM confers the promoter specificity to the TFAM-dependent transcription. However, it is still unknown how TFBM enhances the transcription from LSP or, in other words, how TFBM recognizes the promoter.

MODULATION OF REPLICATION

Some transcripts originating from LSP make stable RNA-DNA heteroduplexes in the conserved sequence block (CSB).[8,9] The RNA strand of the heteroduplex is processed by an RNase and serves as a primer for DNA synthesis by DNA polymerase. The majority of the nascent DNA-strand synthesis starting from this region terminates prematurely at np 16105, about 50 nucleotides downstream of the terminating association sequence (TAS), resulting in the formation of the triple-stranded structure, D-loop. The D-loop is composed of the parental light and heavy strands, as well as the prematurely terminated nascent DNA strands, called D-loop strands or 7S DNA.[10] These D-loop strands have exactly the same free 5′ ends as those of the nascent strands that exceed the termination point and therefore are considered true nascent strands leading to complete replication.[11] In fact, the premature termination is well correlated inversely with the replication rate,[12] suggesting that the DNA synthesis starting from this region may contribute to the replication of mtDNA. Thus, the transcription is coupled with the replication according to the classic strand asymmetric replication model, and TFAM therefore is also essential for the replication of mtDNA. Recently, multiple replication origins for the heavy strand were reported in other than the D-loop region. In this model, called strand-coupled replication, the role of TFAM in the replication is unclear.[13,14]

In addition to the possible straightforward contribution via transcription, TFAM may modulate the mtDNA replication through destabilizing the D-loop structure. As described above, a certain portion of mtDNA molecules steadily take on the D-loop structure. 1-Methyl-4-phenyl-pyridinium ion (MPP^+), a parkinsonism-causing toxin, inhibits selectively the replication of mtDNA at least in part by destabilizing the D-loop.[15–18] Similarly, TFAM at a level normally existing in mitochondria also destabilizes the triple-stranded structure and releases the D-loop strand.[19] Mitochondrial single-stranded DNA-binding protein (SSB) protects the D-loop from this destabilization caused by TFAM. The D-loop structure may be maintained under a balance of the two proteins *in vivo*.[19]

NUCLEOID STRUCTURE OF mtDNA

During the course of examining the D-loop destabilizing effect, we have found that human TFAM is much more abundant than previously thought. TFAM exists about 1,000 molecules per one mtDNA molecule.[19] This amount is almost enough to cover the entire region of mtDNA, given that TFAM occupies about 20 nucleotides. A similar amount of TFAM exists in *Xenopus leavis* oocytes.[20] It has been considered that mtDNA of higher eukaryotes is somewhat naked except for the D-loop region.[21–24] However, in lower eukaryotes, there is good evidence that mtDNA is packaged and forms a large mtDNA-protein complex resembling a nuclear DNA nucleosome.[25,26] Such a nucleosome-like structure of mtDNA is called nucleoid. Recent reports of our and other groups support that mtDNA of higher eukaryotes also takes on the nucleoid structure. Considering that (i) HMG proteins have generally an ability to package DNA, (ii) human TFAM molecules are indeed mostly associated with the mtDNA-proteins complexes, and (iii) human TFAM is abundant, it

is very likely that human TFAM makes a major contribution in the formation of the nucleoid structure as a main component.[27,28]

Homozygous gene disruption of Tfam is lethal in mouse and chicken cells.[29,30] The heterozygous disruption decreases the amount of TFAM by about 50%.[29,30] Expression of exogenous TFAM increases the amount of mtDNA in a finely correlated manner to the total amount of TFAM in mouse,[31] chicken,[30] and human cells (unpublished data). When the TFAM expression was suppressed by RNA interference, the amount of mtDNA gradually decreased with the daily decrease in the TFAM amount after the treatment. The decrease in mtDNA was strongly correlated with the decrease in TFAM (unpublished data). These observations of our and other groups suggest that the TFAM amount is a major determinant of the mtDNA amount. Probably, only mtDNA in the nucleoid structure can be stably maintained in mitochondria, and TFAM may be a dose-limiting factor for the number of nucleoids.

DAMAGE AND REPAIR

mtDNA is much more vulnerable than nuclear DNA.[32,33] First, mtDNA is under much stronger oxidative stress than nuclear DNA.[34,35] Mitochondria normally account for ~90% of oxygen consumption in mammalian cells. It is considered that 1–5% of the mitochondrially consumed oxygen is converted to reactive oxygen species (ROS) due to electron leaks from the respiratory chain.[36–38] mtDNA is associated with inner membranes where the ROS-generating respiratory chain exists. Second, mtDNA is subject to chemical damage much more strongly than nuclear DNA.[39] Mitochondria have a membrane potential with matrix-side negative by which ATP synthesis is driven. Because of this membrane potential, lipophilic cations tend to accumulate in mitochondria. Mitochondria can take up lipophilic cations from the cytosol and concentrate these cations inside up to 1000-fold.[40] Unfortunately, many toxic xenobiotics as well as medically beneficial drugs are also lipophilic and have positive charges. Thus, higher mutation rates in mtDNA than in nuclear DNA are expected. In fact, mutation rates of human mtDNA are reported to be several hundredfold higher than nuclear gene mutation rates.[41]

The oxidative damage of mtDNA is evidenced, for example, by a fact that 8-oxoguanine (8-oxoG), an oxidatively modified guanine base, accumulates more and increases more rapidly in mtDNA than in nuclear DNA.[34] TFAM binds more strongly to DNA containing 8-oxoG than to normal DNA. The binding of TFAM to a C:8-oxoG pair is stronger than that of hOGG1,[42] a human functional counterpart of a bacterial MutM that binds to C:8-oxoG and excises the 8-oxoG. Similarly, the binding of TFAM to an A:8-oxoG pair is stronger than that of hMYH,[42] a human homologue of a bacterial MutY that binds to A:8-oxoG and excises the A. Hence, it is naturally expected that TFAM affects the repair of oxidatively damaged DNA by those enzymes.

Human TFAM shows higher affinity to DNA with cisplatin adducts.[42] Cisplatin is an anticancer drug that produces inter- and intrastrand DNA cross-linking. Interestingly, many cisplatin-resistant cell lines overexpress TFAM than do their parental cells,[43] suggesting that TFAM may play a role in protecting mtDNA from modification by cisplatin or in enhancing the repair of cisplatin-modified mtDNA. p53, which is a tumor suppressor and associates preferentially with damaged DNA, is transferred

to mitochondria upon death signals.[44,45] p53 physically interacts with TFAM in mitochondria.[43] The interaction enhances the binding of TFAM to cisplatin-modified DNA, but conversely inhibits the binding of TFAM to DNA containing an A:8-oxoG pair.[43] These alterations in properties of TFAM-binding could affect the repair or maintenance of mtDNA and possibly modulate the p53-mediated apoptosis.

BRANCHED STRUCTURE

HMG proteins preferentially bind to branched structures including four-way junctions, typical recombination intermediates.[46] Abf2p promotes or stabilizes Holliday recombination junction intermediates in rho+ mtDNA in *Saccharomyces cerevisiae*.[47] Human TFAM has about a 10-fold higher affinity to a synthesized four-way junction than to a corresponding linear form of DNA.[48] It has long been believed that recombination reactions do not occur, or very rarely occur, in mammalian mitochondria because recombined mtDNA molecules were hardly detected in mitochondria artificially harboring hetroplasmic mtDNAs. However, fairly good recombination activity is detected in rat mitochondrial lysates.[49] Furthermore, prominent recombination intermediates are detected in human heart mtDNA.[50] The presence of intramolecular recombination is suggested based on the fact that partially duplicated mtDNA molecules produced the wild-type mtDNA during cultivation by releasing the partially duplicated parts.[51] Although a physiological role of the intramolecular recombination of mtDNA is unclear at present, the strong binding of TFAM to branched structures may no doubt affect such recombination events. The triple-stranded D-loop in a strand asymmetric replication model and the replication Y-folk suggested in a strand-coupled replication model are also typical branched structures. In this aspect, TFAM should have effects on the proceeding of DNA synthesis.

OXIDATIVE STRESS ON HEART

As described previously, mtDNA is physiologically more vulnerable than in nuclear DNA. In addition, mtDNA is feasibly more damaged by pathological insults as well. For example, when isolated rat cardiomyocytes are treated with 50 μM H_2O_2, the amount of intact 16 kbp mtDNA is decreased by 50% in 15 min.[52] In other words, one-half of mtDNA molecules are rapidly degraded by the 15-min H_2O_2 exposure. In a partial myocardial infarction model of mouse, the non-ischemic region works harder to compensate the loss of function of infarcted myocardium. This working overload causes oxidative stress leading to mitochondrial dysfunction. Under these conditions, the activities of complexes I, III, and IV—all of which contain mtDNA-encoded subunits—all declined, but the activity of complex II which does not contain any mtDNA-encoded subunits was maintained,[53] suggesting that mtDNA is a primary target of ROS. This mitochondrial dysfunction followed by cardiomyopathy was largely lessened by pretreatment with antioxidants. Most importantly, this cardiac dysfunction was almost completely prevented by the overexpression of TFAM, strongly suggesting that TFAM has a protective effect on mtDNA from the oxidative attack and indicating how the integrity of mtDNA is important for survival (unpublished data).

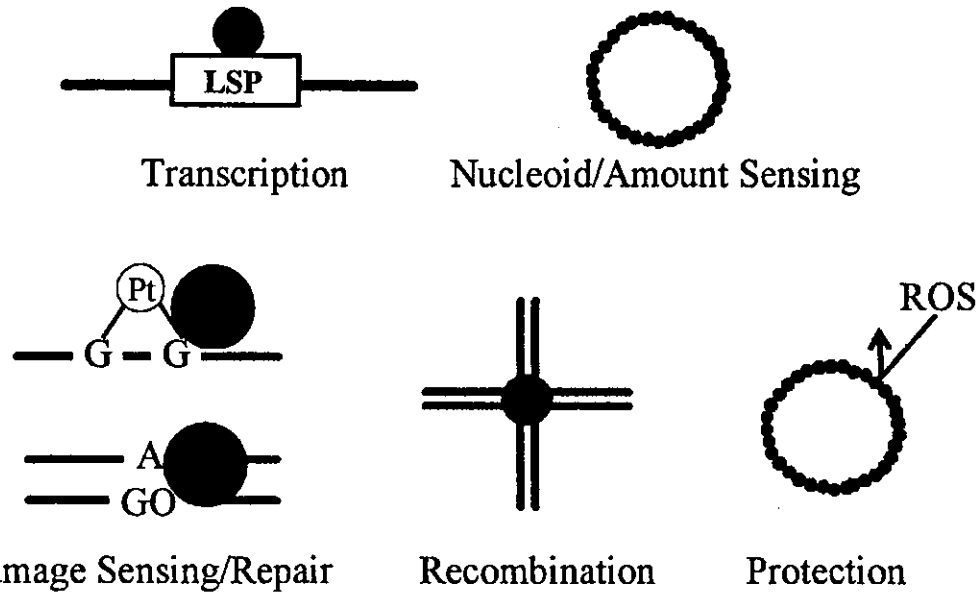

FIGURE 2. Multiple roles of TFAM in maintenance of mtDNA. Circular ring patterns represent TFAM. Pt, cisplatin; GO, 8-oxo-guanine; ROS, reactive oxygen species.

In conclusion, mtDNA is essential for individuals to live normally. This crucial genome, however, is very fragile. TFAM plays multiple and critical roles in the maintenance of mtDNA (FIG. 2).

ACKNOWLEDGMENTS

This work was supported, in part, by the Uehara Memorial Foundation, the Naito Foundation, and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture of Japan.

REFERENCES

1. KANG, D. *et al.* 1998. Introduction. *In* Mitochondrial DNA Mutations in Aging, Disease and Cancer. K.K. Singh, Ed.: 1–15. Springer-Verlag and R.G. Landes Company. Austin, TX.
2. PARISI, M.A. & D.A. CLAYTON. 1991. Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. Science **252:** 965–969.
3. FISHER, R.P. & D.A. CLAYTON. 1988. Purification and characterization of human mitochondrial transcription factor 1. Mol. Cell. Biol. **8:** 3496–3509.
4. DIFFLEY, J.F. & B. STILLMAN. 1991. A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. Proc. Natl. Acad. Sci. USA **88:** 7864–7868.
5. DAIRAGHI, D.J. *et al.* 1995. Addition of a 29 residue carboxyl-terminal tail converts a simple HMG box-containing protein into a transcriptional activator. J. Mol. Biol. **249:** 11–28.
6. FALKENBERG, M. *et al.* 2002. Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. Nat. Genet. **31:** 289–294.

7. McCULLOCH, V. & G.S. SHADEL. 2003. Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. Mol. Cell. Biol. **23:** 5816–5824.
8. LEE, D.Y. & D.A. CLAYTON. 1996. Properties of a primer RNA-DNA hybrid at the mouse mitochondrial DNA leading-strand origin of replication. J. Biol. Chem. **271:** 24262–24269.
9. OHSATO, T. *et al.* 1999. R-Loop in the replication origin of human mitochondrial DNA is resolved by RecG, a Holliday junction-specific hHelicase. Biochem. Biophys. Res. Commun. **255:** 1–5.
10. SHADEL, G.S. & D.A. CLAYTON. 1997. Mitochondrial DNA maintenance in vertebrates. Annu. Rev. Biochem. **66:** 409–435.
11. KANG, D. *et al.* 1997. In vivo determination of replication origins of human mitochondrial DNA by ligation-mediated polymerase chain reaction. J. Biol. Chem. **272:** 15275–15279.
12. KAI, Y. *et al.* 1999. Mitochondrial DNA replication in human T lymphocytes is regulated primarily at the H-strand termination site. Biochim. Biophys. Acta **1446:** 126–134.
13. BOWMAKER, M. *et al.* 2003. Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. J. Biol. Chem. **278:** 50961–50969.
14. HOLT, I.J. *et al.* 2000. Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. Cell **100:** 515–524.
15. MIYAKO, K. *et al.* 1999. 1-Methyl-4-phenylpyridinium ion (MPP+) selectively inhibits the replication of mitochondrial DNA. Eur. J. Biochem. **259:** 412–418.
16. MIYAKO, K. *et al.* 1997. The content of intracellular mitochondrial DNA is decreased by 1-methyl-4-phenylpyridinium ion (MPP+). J. Biol. Chem. **272:** 9605–9608.
17. UMEDA, S. *et al.* 2000. The D-loop structure of human mitochondrial DNA is destabilized directly by 1-methyl-4-phenylpyridinium ion (MPP+), a parkinsonism-causing toxin. Eur. J. Biochem. **267:** 200–206.
18. IWAASA, M. *et al.* 2002. 1-Methyl-4-phenylpyridinium ion (MPP+), a toxin that can cause parkinsonism, alters branched structures of DNA. J. Neurochem. **82:** 30–37.
19. TAKAMATSU, C. *et al.* 2002. Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. EMBO Rep. **3:** 451–456.
20. SHEN, E.L. & D.F. BOGENHAGEN. 2001. Developmentally-regulated packaging of mitochondrial DNA by the HMG-box protein mtTFA during Xenopus oogenesis. Nucleic Acids Res. **29:** 2822–2828.
21. ALBRING, M. *et al.* 1977. Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication. Proc. Natl. Acad. Sci. USA **74:** 1348–1352.
22. CARON, F. *et al.* 1979. Characterization of a histone-like protein extracted from yeast mitochondria. Proc. Natl. Acad. Sci. USA **76:** 4265–4269.
23. POTTER, D.A. *et al.* 1980. DNA-protein interactions in the *Drosophila melanogaster* mitochondrial genome as deduced from trimethylpsoralen crosslinking patterns. Proc. Natl. Acad. Sci. USA **77:** 4118–4122.
24. DEFRANCESCO, L. & G. ATTARDI. 1981. In situ photochemical crosslinking of HeLa cell mitochondrial DNA by a psoralen derivative reveals a protected region near the origin of replication. Nucleic Acids Res. **9:** 6017–6030.
25. Miyakawa, I. *et al.* 1987. Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. J. Cell Sci. **88:** 431–439.
26. SASAKI, N. *et al.* 2003. Glom is a novel mitochondrial DNA packaging protein in Physarum polycephalum and causes intense chromatin condensation without suppressing DNA functions. Mol. Biol. Cell **14:** 4758–4769.
27. KANKI, T. *et al.* 2004. Mitochondrial nucleoid and transcription factor A. Ann. N.Y. Acad. Sci. **1011:** 61–68.
28. ALAM, T.I. *et al.* 2003. Human mitochondrial DNA is packaged with TFAM. Nucleic Acids Res. **31:** 1640–1645.
29. LARSSON, N.G. *et al.* 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nat. Genet. **18:** 231–236.

30. MATSUSHIMA, Y. *et al.* 2003. Functional domains of chicken mitochondrial transcription factor A for the maintenance of mitochondrial DNA copy number in lymphoma cell line DT40. J. Biol. Chem. **278:** 31149–31158.
31. EKSTRAND, M.I. *et al.* 2004. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. Hum. Mol. Genet. **13:** 935–944.
32. KANG, D. & N. HAMASAKI. 2003. Mitochondrial oxidative stress and mitochondrial DNA. Clin. Chem. Lab. Med. **41:** 1281–1288.
33. KANG, D. & N. HAMASAKI. 2002. Maintenance of mitochondrial DNA integrity: repair and degradation. Curr. Genet. **41:** 311–322.
34. BECKMAN, K.B. & B.N. AMES. 1996. Detection and quantification of oxidative adducts of mitochondrial DNA. Methods Enzymol. **264:** 442–453.
35. AMES, B.N. *et al.* 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA **90:** 7915–7922.
36. KANG, D. *et al.* 1983. Kinetics of superoxide formation by respiratory chain NADH-dehydrogenase of bovine heart mitochondria. J. Biochem. **94:** 1301–1306.
37. ETO, Y. *et al.* 1992. Succinate-dependent lipid peroxidation and its prevention by reduced ubiquinone in beef heart submitochondrial particles. Arch. Biochem. Biophys. **295:** 101–106.
38. PAPA, S. 1996. Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications. Biochim. Biophys. Acta **1276:** 87–105.
39. BANDY, B. & A.J. DAVISON. 1990. Mitochondrial mutations may increase oxidative stress: implication for carcinogenesis and aging? Free Radical Biol. Med. **8:** 523–539.
40. SINGER, T.P. & R.R. RAMSAY. 1990. Mechanism of the neurotoxicity of MPTP. An update. FEBS Lett. **274:** 1–8.
41. KHRAPKO, K. *et al.* 1997. Mitochondrial mutational spectra in human cells and tissues. Proc. Natl. Acad. Sci. USA **94:** 13798–13803.
42. YOSHIDA, Y. *et al.* 2002. Human mitochondrial transcription factor A binds preferentially to oxidatively damaged DNA. Biochem. Biophys. Res. Commun. **295:** 945–951.
43. YOSHIDA, Y. *et al.* 2003. p53 physically interacts with mitochondrial transcription factor A and differentially regulates binding to damaged DNA. Cancer Res. **63:** 3729–3734.
44. MARCHENKO, N.D. *et al.* 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J. Biol. Chem. **275:** 16202–16212.
45. SANSOME, C. *et al.* 2001. Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells. FEBS Lett. **488:** 110–115.
46. P-OHLER, J.R. *et al.* 1998. HMG box proteins bind to four-way DNA junctions in their open conformation. EMBO J. **17:** 817–826.
47. MACALPINE, D.M. *et al.* 1998. The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates in vivo. Proc. Natl. Acad. Sci. USA **95:** 6739–6743.
48. OHNO, T. *et al.* 2000. Binding of human mitochondrial transcription factor A, an HMG box protein, to a four-way DNA junction. Biochem. Biophys. Res. Commun. **271:** 492–498.
49. THYAGARAJAN, B. *et al.* 1996. Mammalian mitochondria possess homologous DNA recombination activity. J. Biol. Chem. **271:** 27536–27543.
50. KAJANDER, O.A. *et al.* 2001. Prominent mitochondrial DNA recombination intermediates in human heart muscle. EMBO Rep. **2:** 1007–1012.
51. TANG, Y. *et al.* 2000. Maintenance of human rearranged mitochondrial DNAs in long-term cultured transmitochondrial cell lines. Mol. Biol. Cell **11:** 2349–2358.
52. SUEMATSU, N. *et al.* 2003. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation **107:** 1418–1423.
53. IDE, T. *et al.* 2001. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts following myocardial infarction. Circ. Res. **88:** 529–535.

Thrombosis Research (2005) 115, 351–358

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REGULAR ARTICLE

Characterization of two novel mutations of the antithrombin gene observed in Japanese thrombophilic patients

Masako Kurihara, Kumiko Watanabe, Sumiko Inoue, Yui Wada, Miyuki Ono, Machiko Wakiyama, Hiroko Iida, Sachiko Kinoshita, Naotaka Hamasaki*

Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Hospital, 3-1-1, Maidashi, Hgashi-Ku, Fukuoka 812-8582, Japan

Received 15 April 2004; received in revised form 20 July 2004; accepted 2 August 2004
Available online 3 October 2004

KEYWORDS
Structure and function of antithrombin;
Gene analysis;
Deep vein thrombosis, 20210A, factor V Leiden

Abstract We investigated the molecular basis of reduced functional levels of antithrombin (AT) in two individuals suffering from thromboembolic events. In each case direct sequencing of amplified DNA revealed 13,260–13,262 del in one patient and 2511C>A in the other patient, predicting a heterozygous E381del and P16H, respectively. Both patients had no 20210A allele and factor V Leiden mutation. To understand the molecular mechanism responsible for antithrombin deficiency, stable expression experiments were performed using HEK293 cells transfected with the expression vector containing the wild-type or the mutated recombinant cDNA. In these experiments, the media levels of the two mutated antithrombins were the same as that of wild type, but the specific activity of the E381del mutant decreased significantly compared with that of wild type. These results showed that the E381del mutation was responsible for type II deficiency, whereas the other mutation, P16H, did not produce any definite abnormality which could contribute to antithrombin deficiency.

Abbreviations: AT, antithrombin; PS, protein S; PC, protein C; PT, prothrombin time; APTT, activated partial thromboplastin time; FDP, fibrin degradation product; TAT, thrombinantithrombin complex; PIC, plasmin–plasmin inhibitor complex; HEK293, human embryo kidney 293 cells.

* Corresponding author. Tel.: +81 92 642 5748; fax: +81 92 642 5772.
E-mail address: hamasaki@cclm.med.kyushu-u.ac.jp (N. Hamasaki).

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doi:10.1016/j.thromres.2004.08.025

Antithrombin (AT) is a 58-kDa plasma glycoprotein which is mainly synthesized in hepatocytes and circulates at a concentration of approximately 112–140 mg/l with a half-life of 2 to 3 days [1,2]. It is composed of 432 amino acid residues with four Asn-linked glycosylation sites [3,4]. AT belongs to the serine proteinase inhibitor (serpin) superfamily and plays a critical role in the regulation of the blood coagulation system by inhibiting the final two proteinases of factor Xa and thrombin [2,5–7]. Inhibition of proteinases by AT involves interaction between the active site of the proteinase and the reactive center loop of the inhibitor, which leads to an inactive and stable proteinase–inhibitor complex [8]. The reactive center of AT is held on an exposed peptide loop that extends from 15 residues (P15) on the amino-terminal side of the P1 residue (Arg393) to five residues (P5′) on the carboxy-terminal side [6–9]. After thrombin cleaves the reactive site of Arg393–Ser394, AT undergoes a dramatic conformational change when the cognate proteinases interact directly with the reactive center loop [6–11]. The reactive center loop is incorporated as strand four in the central β-sheet A when the loop interacts with the proteinases, and induces the massive conformational change of AT [8,12]. Inhibition of thrombin by AT is enhanced at least 1000-fold in the presence of heparin [2,13]. The human AT gene is mapped to chromosome 1q23.1–23.9 and comprises seven exons and six introns spanning a total of 13.5 kb of genomic DNA [14].

Since the existence of AT deficiency was first reported in 1965 [15], there has been an increasing line of evidence that a molecular anomaly of AT is an integral risk factor for thrombosis [16–22]. Our laboratory has been directing efforts to identify the causal factors that generate thrombosis by performing a systematic haemostatic investigation [23]. With respect to the AT anomaly, to date, all the coding exons and intron–exon boundaries of the AT gene were analyzed in 6 out of 22 patients having reduced AT activity. Abnormalities of AT gene were detected in two out of six thrombotic patients, with one a deletion mutation and the other a missense mutation. In the present study, we report on the identification and characterization of two novel mutations of the AT gene.

Materials and methods

Patients

Patient 1: A 51-year-old male patient had acute arterial obstruction from a right common iliac artery to a right thigh artery. He had undergone an operation on his right leg to remove the blood clots and a plasty in the right thigh arteries. The patient had a medical history of deep vein thrombosis in both legs at the age of 38. However, there was no suggestion of a family history of thrombosis.

Patient 2: The patient was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. A filling defect and deterioration of blood flow in the right femoral vein were observed by venography. She recovered from the deep vein thrombosis by heparin/warfarin treatment for a month. She has been suffering from myeloma for 10 years and impaired function of the liver due to chronic hepatitis B. There was no suggestion of family history of thrombosis. We were unable to study the hematological profile of her family.

Subjects

The subjects consisted of the proband and his daughter in patient 1 and the proband in patient 2. In addition, 50 healthy individuals recruited from the employees of our institution were subjected to this study as normal controls. Prior to the trial, written informed consent was individually obtained from all of them by the attending physicians following full explanation of the aim of the research and guarantee of privacy.

Plasma

Peripheral blood samples collected in 3.13% sodium citrate were centrifuged at 1500×*g* for 20 min, and the resulting supernatant fraction was used to perform clot-based tests. Aliquots of supernatant fractions divided into smaller portions were stored at −80 °C for future use.

Haemostatic examination

The haemostatic profile involved measurements of AT, protein S (PS), protein C (PC), fibrinogen, plasminogen, α2-plasmin inhibitor, heparin cofactor II, lupus anticoagulant, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombotest, fibrin degradation product (FDP), thrombin–antithrombin complex (TAT), and plasmin–plasmin inhibitor complex (PIC) [24]. In addition, protein levels of AT and progressive AT activity were also assessed. AT activity was assayed by chromogenic substrate as heparin-dependent inhibition of bovine thrombin (heparin cofactor activity) using Testzym AT III 2 kit (Daiichi Kagaku, Tokyo, Japan). The reference interval ranged from 80% to 130%. AT

activity independent of heparin (progressive AT activity) was determined by chromogenic substrate after precipitation of fibrinogen by incubating at 56 °C for 15 min as previously described [23].

DNA extraction

Genomic DNA was extracted from peripheral lymphocytes collected from the patients, their relatives, and the healthy individuals using an automated DNA extraction device (NA-1000, KURABO, Osaka, Japan).

Polymerase chain reaction (PCR)

Early studies suggested that there were only six exons but subsequent analysis revealed a 1-kb intron within exon 3 [14]. Although we follow the old nomenclature of AT gene as consisting of exons 1–6, the primers cover all the exons from 1 to 7. Genomic regions of exons 1–6 of the AT gene were each amplified using appropriate primers in a reaction mixture containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM 4dNTPs, 0.2–0.4 μM primer-set, 0.8–4 ng/μl template DNA, and 0.025 U/μl Extaq (Takara Shuzo, Otsu, Japan). A reaction cycle consisting of sequential incubations for denaturation at 94 °C for 1 min, for annealing at 56–61 °C for 1 min, and for extension at 72 °C for 1 min was repeated twice with denaturation at 94 °C for an additional 4 min being included in the first cycle. Additionally, a reaction cycle consisting of sequential incubations for denaturation at 94 °C for 30 s, annealing at 56–61 °C for 30 s, and extension at 72 °C for 30 s was repeated 30 times, followed by incubation at 72 °C for 10 min. These reactions were performed in an automated device (Gene Amp PCR system 9600R, Roche Diagnostic Systems, Basel, Switzerland). Detection of the G20210A mutation in the prothrombin gene and factor V Leiden was performed as described by Finan et al. [25].

DNA sequencing

The PCR products derived from exons/introns of the AT gene were purified through a Micro Spin™ S-300HR column (Amersham Pharmacia Biotech, Bucks, UK) and processed for pretreatment using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The reaction products were purified through a Centri-Step Spin column (Perkin Elmer Applied Biosystems) and subjected to direct sequencing in an automated sequencer (ABI PRISM 377 sequencer, Perkin Elmer Applied Biosystems). To confirm the presence of mutated AT gene in patient 1, the PCR product of exon 6 was subcloned with the original TA cloning kit (Invitrogen, Carlsbad, CA, USA). For DNA sequencing, the insert DNA was amplified by the colony PCR method. The colony PCR products were then used for sequencing analysis.

Mutagenesis of AT cDNA

The full-length human AT cDNA was prepared from a human liver cDNA library (Uni-ZapRXR Library, Stratagene, CA, USA) by PCR using a mutagenic primer set (sense sequence of 5′-TGTCGACGAT-TAGCGGCCATGTATTC-3′ and antisense sequence of 5′-AACCCG GGAAGAGGTGCAAAG-3′, mutagenic C, C and CCC are underlined), by which new *Sal*1 and *Sma*1 sites are produced in the amino-terminal and carboxyl-terminal regions of the complete AT coding sequence, respectively. The PCR product was sequenced to check it had a proper sequence. An expression vector for the wild-type AT was constructed as follows by inserting the full-length AT cDNA into pC1neo Mammalian Expression Vector (Promega, WI, USA). The 1466-bp PCR product was restricted with *Sal*1 and *Sma*1 and then ligated to a 5466-bp *Sal*1–*Sma*1 restriction fragment of pC1neo Mammalian Expression Vector with T4 DNA ligase. The wild-type AT cDNA-vector construct was transformed into Epricurian Coli XL1-Blue supercompetent cells (Invitrogen). The sequences of DNA from the resulting colonies were verified to be correct by sequence analysis. Mutations were generated by the overlap extension method [26] using the wild-type AT cDNA-vector as a template. The final mutated PCR fragments were also inserted into pC1neo Mammalian Expression Vector as described above and the mutation was confirmed by sequencing of the resulting vector.

Stable expression of recombinant AT

Human embryo kidney 293 (HEK293) cells (Health Science Research Resources Bank, Osaka, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, USA) supplemented with 10% fetal calf serum (Gibco BRL), penicillin and streptomycin. After 11 μg of expression vector DNA and 3 μl/DNA μg of liposome reagent (Trans FastTM Transfection Reagent, Promega) were combined and incubated for 15 min at room temperature, the mixture was added to HEK293 cells

(3×105 per 60-mm dish). After 24 h, the medium was replaced with DMEM containing serum and antibiotics. After an additional 48 h, the transformed cells were selected in medium to which 1 mg/ml G418 (Gibco BRL) was added. The medium was replaced every 3 days. When the resistant cells were grown at about 50% confluency, the concentration of G418 in the medium was reduced to 0.6 mg/ml. After 2–3 weeks, the resistant cells were grown at 80–100% confluency and the medium was replaced with serum-free DMEM. After 48 h, the media were harvested and centrifuged at 1500×*g* for 5 min and the resulting supernatant fraction was stored in aliquots at −80 °C for future use.

Measurement of the activity and the antigen of recombinant AT in the culture medium

Assays of AT activity in the culture media were performed by an amidolytic assay using Testzym AT III 2 kit (Daiichi Kagaku). Assays of AT antigen level in the culture media were performed by Western blotting analysis. The supernatant of the culture media and purified plasma AT (Sigma, Missouri, USA) as a standard were electrophoresed on a 10% polyacrylamide gel for 60 min at 25 mA, and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was then immersed in the blocking buffer containing 50 mM Tris–HCl, 100 mM NaCl, 0.05% Tween 20 (pH 7.4) and 5% skim milk for 1 h, and incubated with goat anti-human AT antibody (1:6000) (Enzyme Research Laboratories, Indiana, USA) in the TBS–Tween buffer at 4 °C for 18 h. The membranes were then washed three times for 10 min each with the TBS–Tween buffer, and incubated for 1 h at room temperature with HRP anti-goat immunoglobulin (1:6000) (Amersham Pharmacia Biotech). The membranes were subsequently washed three times for 10 min each with the TBS–Tween buffer. Immunoreactive bands were visualized with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), and measured using a luminoimage-analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan).

Results

Haemostatic examination

Patient 1: AT activity in the presence or absence of heparin in blood samples collected from the patient was nearly half of the normal level at 48% and 49%, respectively. The protein level was within the normal range at 117% (Table 1). All other analytes were within the normal range (Table 1). AT activity of the patient's daughter was within the normal range (data not shown).

Patient 2: AT activity in the presence of heparin and protein level of AT were nearly half of the normal level at 64% and 52%, respectively (Table 1). All other analytes were within the normal range with exception of the FDP and D-dimer, which were 0.0153 and 0.0062 g/l, respectively (Table 1).

Nucleotide sequence of the AT gene

Genomic DNA was extracted from peripheral blood cells of individuals. Genomic regions of exons 1–6 and their exon/intron junctions of the AT gene were amplified using appropriate primers and sequenced.

Patient 1: In direct sequence analysis of the AT gene of patient 1, additional aberrant peaks were observed from nt 13,260 in exon 6, suggesting heterozygous deletion or insertion mutation (Fig. 1). As a consequence of subcloning, deletion of AAG from nucleotide position 13,260 to 13,262, the mutation of which produces a deletion of Glu381, was detected in 7 out of 14 subclones. The remaining subclones were normal implying that the patient was

Table 1 Laboratory examination of the patient

	Reference interval	Patient 1	Patient 2
PT (%)	>70	51	72
APTT (s)	24.0–38.0	24.7	38.6
Fbg (g/l)	2.0–4.0	2.49	4.26
TBT (%)	>60	47	90
HPT (%)	60–120	55	87
PLG (%)	85–145	127	113
a2PI (%)	80–130	96	77
LAC	(−)	(−)	(−)
AT (%)	80–120	48	64
PS (%)	59–128	83	93
PC (%)	75–131	67	72
FDP (g/l)	0.0–0.005	0.0034	0.0153
PIC (g/l)	0.0–0.008	0.0003	0.001
D-D (g/l)	0.0–0.005	NT	0.0062
AT activity (%) Heparin (−)		49	NT
AT activity (%) Heparin (+)	80–120	48	64
AT antigen (%)	80–130	117	52

PT: prothrombin time; APTT: activated partial thromboplastin time; Fbg: fibrinogen; TBT: thrombotest; HPT: hepaplastintest; PLG: plasminogen; α2PI: α2 plasmin inhibitor; LAC: lups anticoagulants; AT: antithrombin; PS: protein S; PC: protein C; FDP: fibrinogen and fibrin degradation product; PIC: plasmin-α2PI complex; D-D: D-dimer; NT: not tested.

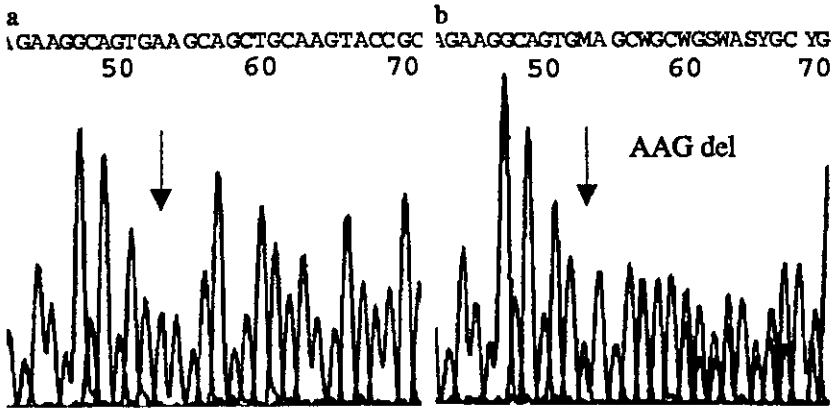

Figure 1 Nucleotide sequences of antithrombin exon 6 from patient 1. A deletion of AAG was observed in the patient's exon 6. (a) Normal control; (b) patient.

heterozygous for the deletion mutation. In his daughter, no mutations were detected in the AT gene.

Patient 2: Direct sequencing of the amplified exon 2 of patient 2 showed a cytosine to adenine transversion mutation at nucleotide position 2511 that converts proline-16 to histidine (P16H). The patient was heterozygous for the mutant (Fig. 2).

We analyzed genomic DNA from 50 healthy individuals for exon 2 and exon 6 of AT molecule to exclude the possibility of the detected mutations being polymorphisms (data not shown).

Secretion of wild-type and mutant ATs in stably transfected HEK293 cells

Wild-type and mutant ATs were expressed in cultured HEK293 cells to confirm whether the identified deletion mutation or amino acid substitution causes an AT deficiency. The AT activities and antigen levels in the culture supernatants were examined by an amidolytic assay and Western blotting analysis, respectively, and expressed as the concentrations of AT where purified plasma AT was used as a standard.

As shown in Fig. 3, both AT mutants of E381del and P16H were secreted normally from the transfected HEK293 cells to media as in the case of wild-type AT. The specific activities of wild-type and P16H mutant were 0.99±0.22 units/ng (mean±S.D., n=5) and 0.90±0.40 units/ng (mean±S.D., n=5), respectively, while the activity of E381del mutant was below a detectable level (Table 2).

Discussion

AT circulates in blood in an inactive form and becomes active upon association with glycosaminoglycans such as heparin and heparan sulphate which interact with the helix D region of AT [27,28]. The unusual long N-terminus region and the carbo-

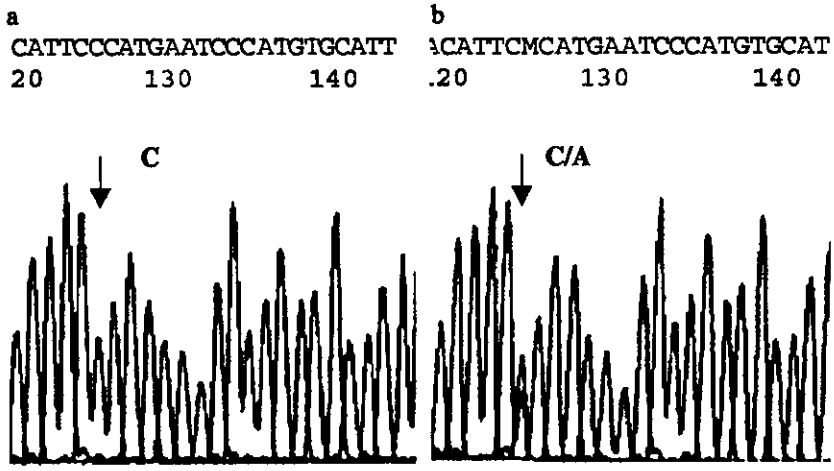

Figure 2 Nucleotide sequences of antithrombin exon 2 from patient 2. A substitution of C to A was observed in the patient's exon 2. (a) Normal control; (b) patient.

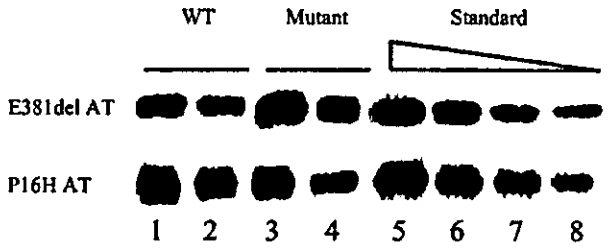

Figure 3 Immunoblot analysis of antithrombin in culture supernatant from E381del mutant (patient 1) under Reducing Conditions. Five microliters of culture supernatant (1:0–1:2 dilution) and standard plasma AT (1.8–14.3 ng of AT) was separated by SDS-PAGE (10% acrylamide) and the gels were electroblotted onto PVDF membranes. Antithrombin was stained with goat anti-human-antithrombin antibody (see Materials and methods). 1: wild; 2: wild (1:2 dilution); 3: mutant, 4: mutant (1:2 dilution); 5–8: standard plasma AT (14.3, 7.3, 3.7, 1.8 ng of AT, respectively).

hydrate attached at Asn135 are specific in AT among the serpin superfamily and affect the heparin binding to AT [8]. The effect of heparin binding to the helix D region is to expel the sheet-inserted residue P15 (Gly379) and P14 (Ser380) from β-sheet A, so that the whole reactive center loop is exposed [29,30]. The allosteric effect between the hinge region (Gly379–Glu381) and the heparin binding region is essential for the heparin activation [31].

The AT deficiency is roughly divided into type I and type II deficiencies. Type I deficiency ("Classical deficiency") has reduced levels of immunologically and functionally determined AT. Type II deficiency has been applied to the cases in which the functionally determined AT is reduced. Type II deficiency is further divided into the heparin binding site (HBS)-type in which heparin binding is abnormal; reactive site (RS)-type in which the reactive center loop is abnormal; and pleiotropic effect (PE)-type in which the influence is pleiotropic [32]. Patient 1 in this study was type II deficiency showing a normal immunological (protein) level and a reduced AT activity (Table 1), and the E381del mutation was detected by base sequencing (Fig. 1). Patient 2 was type I with reduced AT level and reduced activity in plasma (), and base sequencing detected a mutation at P16H on the N-terminal (Fig. 2).

Expression experiments of these AT mutants transfected into HEK293 cells indicated that both of the mutant molecules were secreted normally into culture media (Fig. 3). However, the E381 del mutation had no AT activity, while the P16H mutant had the same specific activity as that of wild type (Table 2). Glutamic acid at 381 (P13) is in the hinge region (P15–P8; Gly379–Thr386) of the reactive center loop and is highly conserved to the same extent as P12 (Ala382) in the serpin superfamily [33,34]. The hinge region and the reactive center loop play pivotal roles for the structural/function relationship of AT [6,8,29,30] and A382T at P12 mutation is known as AT Hamilton or Glasgow II [35–37]. A glutamic acid (Glu381) in the reactive center loop at position P13 has a central role for the allosteric activation of AT by heparin binding [38]. The crystal structure shows that Glu381 contacts stabilize the activated conformation [38]. The loop in almost all the serpins is formed by 17 residues [33] and the tight conservation of the length of serpin reactive center loops is striking when compared with the wide variance in the length of surface loops for other protein families. The inhibitory activity of AT to factor Xa depended upon the length of the N-terminal portion of the reactive center loop, and the deletion of one or two residues lowered the inhibitory activity of AT as well as PAI-1 and PAI-2 [39]. The deletion of residues in the reactive center loop converts the serpin into a substrate [39]. Considering these evidence, we would safely conclude that E381 del mutation had lost the AT activity because the reactive center loop was shortened by deleting glutamic acid at 381.

In the case of P16H mutant of Patient 2, the AT level secreted into the culture media and the specific activity were similar to those of the wild-type (Fig. 3 and Table 2), suggesting that both the protein processing for secretion and the inhibitory activity were normal. Since conservation of proline at position 16 is low in the serpin superfamily [33], its involvement in the retention of the stereostructure of AT and proteinase-inhibitory activity may be low. Incubation of P16H mutant at 40°C did not demonstrate the thermal instability compared to the wild type (data not shown). How do we then explain the fact that Patient 2 exhibited the phenotype of type I deficiency (see Table 2)? In this study, we sequenced all seven exons and the exon–intron boundaries of AT gene by PCR (see Materials and methods). Our method applied in this study, how-

Table 2 AT specific activities in normal pooled plasma and in media secreted from HEK293 cells transfected with wild-type and mutants AT genes

Type of AT genes	Specific activity [arbitrary unit] (units/ng)
Wild-type	0.99±0.22 (n=5)
E381-del mutant	undectectable (n=3)
P16H mutant	0.90±0.40 (n=5)
Normal pooled plasma	1.35±0.33 (n=3)

ever, is not a valid method for detecting partial gene deletion or rearrangement, indicating that we could not exclude a possibility of gene deletion or rearrangement. Patient 2 was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. She has been suffering from myeloma for 10 years and also suffering from liver dysfunction due to hepatitis B. Her deep vein thrombosis in the right leg was induced by infectious arthritis in the same leg. She responded to heparin/warfarin treatment during the course of a month, indicating that the deep vein thrombosis was relatively mild. The P16H missense mutation was not due to polymorphism (see Results). Kondo et al. analyzed the molecular deficiency mechanism of heparin cofactor II, and reported a patient whose heparin cofactor II mutant was secreted normally into the culture media of transfected HEK293 cells, although the heparin cofactor II level was decreased in the pateint's plasma [40]. Although the P16H mutant did not show the thermal instability at 40°C, it might be possible that the half-life is shortened due to mutation in some reasons, thereby increasing elimination from the circulation. Regarding the genesis of the decreased plasma AT level in Patient 2, either the P16H mutant may have been secreted into the circulation, but was rapidly degraded, or the mutation may not have been directly involved in the reduction of AT in Patient 2. At the present time, however, we are unable to offer a reasonable explanation that she exhibited the phenotype of type I AT deficiency. It could be due to a complication of myeloma, liver dysfunction and arthritis.

References

[1] Murano G, Williams L, Miller-Andersson M, Aronson DL, King C. Some properties of antithrombin-III and its concentration in human plasma. *Thromb Res* 1980;**18**:259–62.

[2] Mammen EF. Antithrombin: its physiological importance and role in DIC. *Semin Thromb Hemost* 1998;**24**:19–25.

[3] Franzen L-E, Svensson S, Larm O. Structual studies on the carbohydrate portion of human antithrombin. *J Biol Chem* 1980;**255**:5090–3.

[4] Prochownik EV, Markham AF, Orkin SH. Isolation of a cDNA clone for human antithrombin. *J Biol Chem* 1983;**258**: 8389–94.

[5] Huntington JA, Read RJ, Carrell RW. Structure of a serpin–protease complex shows inhibition by deformation. *Nature* 2000;**407**:923–6.

[6] Stein PE, Carrell RW. What do dysfunctional serpin tell us about molecular mobility and diseases? *Struct Biol* 1995;**2**:96–113.

[7] Wright HT, Scarsdale JN. Structual basis for serpin inhibitor activity. *Proteins* 1995;**22**:210–25.

[8] Whisstock J, Skinner R, Lesk AM. An atlas of serpin conformations. *Trends Biochem Sci* 1998;**23**:63–7.

[9] Stratikos E, Gettins PG. Formation of the covalent serpin–proteinase complex involves translocation of the proteinase by more than 70A and full insertion of the reactive center loop into beta-sheet A. *Proc Natl Acad Sci U S A* 1999;**96**:4808–13.

[10] Bjork I, Danielsson A, Fenton JW, Jornvall H. The site in human antithrombin for functional proteolytic cleavage by human thrombin. *FEBS Lett* 1981;**126**:257–60.

[11] Bjork I, Jackson CM, Jornvall H, Lavine KK, Nordling K, Salsgive WJ. The active site of antithrombin. Release of the same proteolytically cleaved form of the inhibitor from complexes with factor χa, factor χa, and thrombin. *J Biol Chem* 1982;**257**:2406–11.

[12] Loebermann H, Tokuoka R, Deisenhofer J, Huber R. Human alpha 1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J Mol Biol* 1984;**177**:531–57.

[13] Rosenberg RD, Damus PS. The purification and mechanism of action antithrombinheparin cofactor. *J Biol Chem* 1973;**248**:6490–505.

[14] Olds RJ, Lane DA, Chowdhury V, De Stefano V, Leone SL, Thein SL. Complete nucleotide sequence of the antithrombin gene: evidence for homologous recombination causing thrombophilia. *Biochemistry* 1993;**32** 4216–24.

[15] Egeberg O. Inherited antithrombin deficiency causing thrombophilia. *Thromb Diath Haemorrh* 1965;**13**: 516–30.

[16] Sas G, Blasko G, Banhegyi D. Abnormal antithrombin III (Antithrombin III "Budapest") as a cause of familial thrombophilia. *Thromb Diath Haemorrh* 1974;**32**:105–15.

[17] Thaler E, Lechner K. Antithrombin III deficiency and thromboembolism. *Clin Haematol* 1981;**10**:369–90.

[18] Finazzi G, Tran TH, Barbui T, Duckert B. Purification of antithrombin 'Vicenza': a molecule with normal heparin affinity and impaired reactivity to thrombin. *Br J Haematol* 1985;**59**:259–63.

[19] Hirsh J, Piovella F, Pini M. Congenital antithrombin III deficiency: incidence and clinical features. *Am J Med* 1989;**87**:34–8.

[20] Caso R, Lane DA, Thompson EA, Olds RJ, Thein SL, Panico M, et al. Antithrombin Vicenza, Ala 384 to Pro (GCA to CCA) mutation, transforming the inhibitor into a substrate. *Br J Haematol* 1991;**77**:87–92.

[21] Demers C, Ginsberg JS, Hirsh J, Henderson P, Blajchman MA. Thrombosis in antithrombin III-deficient persons: report of a large kindred and Literature review. *Ann Intern Med* 1992;**116**:754–61.

[22] Aiach M, Gandrille S, Emmerich J. A review of mutations causing deficiencies of antithrombin, protein C, protein S. *Thromb Haemost* 1995;**74**:81–9.

[23] Tsuda H, Hattori S, Tanabe S, Iida H, Nakahara M, Nishioka S, et al. Screening for aetiology of thrombophilia: a high prevalence of protein S abnormality. *Ann Clin Biochem* 1999;**36**:423–32.

[24] Iida H, Nakahara M, Komori K, Fujise M, Wakiyama M, Urata M, et al. Failure in the detection of aberrant mRNA from the heterozygotic splice site mutant allele for protein S in a patient with protein S deficiency. *Thromb Res* 2001;**102**:187–96.

[25] Finan RR, Tamim H, Ameen G, Sharida HE, Rashid M, Almawi WY. Prevalence of factor V G1691A (factor V-Leiden) and prothrombin G20210A gene mutations in a recurrent miscarriage population. *Am J Hematol* 2002; **71**:300–5.

[26] Ho S, Hunt H, Horton R, Pullen J, Pease L. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989;77:51–9.

[27] Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. *Proc Natl Acad Sci U S A* 1997;94: 14683–8.

[28] Pike RN, Potempa J, Skinner R, Fitton HL, McGraw WT, Travis J, et al. Heparin dependent modification of the reactive center arginine of antithrombin and cosequent increase in heparin binding affinity. *J Biol Chem* 1997;272:19652–5.

[29] Huntington JA, Olson ST, Fan B, Gettins PG. Mechanism of heparin activation of antithrombin. Evidence for reactive center loop preinsertion with expulsion upon heparin binding. *Biochemistry* 1996;35:8495–503.

[30] McCoy AJ, Pei XY, Skinner R, Abrahams JP, Carrell RW. Structure of betaantithrombin and the effect of glycosylation on antithrombin's heparin affinity and activity. *J Mol Biol* 2003;326:823–33.

[31] Huntington JA, McCoy A, Belzar KJ, Pei XY, Gettins PG, Carrell RW. The conformational activation of antithrombin. A 2.85-Å structure of a fluorescein derivative reveals an electrostatic link between the hinge and heparin binding regions. *J Biol Chem* 2000;275:15377–83.

[32] Lane DA, Bayston T, Olds RJ, Fitches AC, Cooper DN, Millar DS, et al. Antithrombin mutation database: 2nd update. For the plasma coagulation inhibitors subcommittee of the Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis. *Thromb Haemost* 1997;77:197–211.

[33] Huber R, Carrell RW. Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins. *Biochemistry* 1989;28:8951–66.

[34] Skriver K, Wikoff WR, Patston PA, Pataton PA, Tausk F, Schapira M, et al. Substrate properties of C1 inhibitor Ma (alanine 434–glutamic acid). Genetic and structural evidence suggesting that the P12-region contains critical determinants of serineprotease inhibitor/substrate status. *J Biol Chem* 1991;266:9216–21.

[35] Devraj-Kizuk R, Chui DHK, Prochownik EV, Carter CJ, Ofosu MA, Blajchman MA. Antithrombin III Hamilton: a gene with a point mutation (guanine to adenine) in codon 382 causing impaired serine protease reactivity. *Blood* 1988;72:1518–23.

[36] Austin RC, Rachubinski RA, Ofosu FA, Blajchman MA. Antithrombin-III-Hamilton, Ala 382 to Thr: an Antithrombin-IIIvariant that acts a substrate but not an inhibitor of alpha-thrombin and Factor χa. *Blood* 1991; 77:2185–9.

[37] Ireland H, Lane DA, Thompson E, Walker ID, Blench I, Morris HR, et al. Antithrombin Glasgow II: alanine 382 to threonine mutation in the serpin P12 position, resulting in a substrate reaction with thrombin. *Br J Haematol* 1991;79:70–4.

[38] Johnson DJ, Huntington JA. The influence of hinge region residue Glu-381 on antithrombin allostery and metastability. *J Biol Chem* 2004;279:4913–21.

[39] Zhou A, Carrell RW, Huntington JA. The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop. *J Biol Chem* 2001;276: 27541–7.

[40] Kondo S, Tokunaga F, Kario K, Matsuo T, Koide T. Molecular and cellular basis for type 1 heparin cofactor IIdeficiency (Heparin CofactorIIAwaji). *Blood* 1996;87: 1006–12.

Circ J 2005; **69:** 1–6

Relation of Serum Total Cholesterol and Other Factors to Risk of Cerebral Infarction in Japanese Men With Hypercholesterolemia

—— The Kyushu Lipid Intervention Study ——

Mikio Iwashita, BS; Yasuyuki Matsushita, BS*,†; Jun Sasaki, MD‡;
Kikuo Arakawa, MD§; Suminori Kono, MD
for the Kyushu Lipid Intervention Study (KLIS) Group

Background Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT), and few studies have examined the risk factors in the elderly.

Methods and Results Clinical and behavioral risk factors for cerebral infarction were examined in 4,349 Japanese men aged 45–74 years with a serum total cholesterol (TC) concentration of 220 mg/dl or greater who participated in the Kyushu Lipid Intervention Study. A total of 81 men developed definite cerebral infarction in a 5-year follow-up period. The Cox proportional hazards model was used with serum TC at baseline and during the follow-up, serum high-density lipoprotein-cholesterol (HDL-C), HT, diabetes mellitus (DM), and other factors as covariates. Serum TC during the follow-up, not at baseline, was positively associated with cerebral infarction, showing a stronger association in the elderly (≥65 years old) than in the middle-aged (<65 years old). Statin use was related to a moderate decrease in the risk of cerebral infarction when follow-up TC was not considered, but the decrease was almost nullified after adjustment for follow-up TC. A low concentration of serum HDL-C, diabetes mellitus, hypertension, and angina pectoris were each related to an increased risk. No clear association was observed for body mass index, smoking or alcohol use.

Conclusions Lowering cholesterol is important in the prevention of cerebral infarction in men with moderate hypercholesterolemia. A low concentration of HDL-C, DM, and HT are independent predictors of cerebral infarction. (*Circ J* 2005; **69:** 1–6)

Key Words: Cerebral infarction; Diabetes mellitus; High-density lipoprotein-cholesterol; Hypercholesterolemia; Japanese men

Stroke is a leading cause of death and disability in industrialized countries and of the 2 major types of stroke, cerebral infarction predominates, although hemorrhagic stroke remains common in Asian populations[1] Risk factors for cerebral infarction have not been well clarified, except for hypertension (HT)[2] Findings regarding the relation between serum total cholesterol (TC) or low-density lipoprotein-cholesterol (LDL-C) and cerebral infarction are inconsistent in observational studies[3–16] whereas cholesterol-lowering trials have shown a decrease in the risk of cerebral infarction among patients assigned to statin treatment[17,18] The role of serum high-density lipoprotein-cholesterol (HDL-C) is receiving particular interest in the epidemiology of cerebral infarction. Low concentrations of serum HDL-C have been fairly consistently associated with an increased risk of cerebral infarction[4–9,14,19] Several[5,7,8,12,15,19] but not all[3,6] prospective studies reported that diabetes mellitus (DM) was associated with an increased risk of cerebral infarction. In the study reported here, we examined the relation of serum TC and HDL-C and other factors to the risk of cerebral infarction using data from the Kyushu Lipid Intervention Study (KLIS), a primary prevention trial of coronary heart disease (CHD) events and cerebral infarction in Japanese men with moderately elevated concentrations of serum TC[20–23] Furthermore, because few studies have investigated the risk factors for cerebral infarction in elderly persons[6,15,19] we examined the association with these factors in middle-aged and elderly men separately.

(Received May 19, 2004; revised manuscript received September 17, 2004; accepted October 13, 2004)

*Department of Preventive Medicine, Faculty of Medical Sciences, Kyushu University, Fukuoka, *Department of Clinical Pharmacology and Biostatistics, Sankyo Company Limited, †Department of Management Sciences, Faculty of Engineering, Tokyo University of Science, Tokyo, ‡International University of Health and Welfare Graduate School of Public Health Medicine and §The Second Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka, Japan*

Members of the KLIS Group are listed in the Appendix 1.

Mailing address: Suminori Kono, MD, Department of Preventive Medicine, Faculty of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: skono@phealth.med.kyushu-u.ac.jp

Methods

Details of the study design, patient characteristics at baseline, and primary results of the KLIS have been described previously[20–23] In brief, a total of 5,640 men aged 45–74 years with serum TC concentration of 220 mg/dl or greater were enrolled by 902 physicians in Kyushu District during the period between May 1990 and September 1993.

Table 1 Characteristics of the Study Subjects by Statin Use

	Mean (SD) or proportion		p-value for difference*
	Statin (−)	Statin (+)	
No. of subjects	1,637	2,712	–
Age (years)	58.1 (8.2)	58.0 (7.9)	0.78
Baseline total cholesterol (mg/dl)	244 (17.9)	258 (25.8)	<0.0001
Follow-up total cholesterol (mg/dl)	225 (25.1)	219 (26.7)	<0.0001
Baseline HDL-cholesterol (mg/dl)	50 (12.0)	49 (12.0)	0.05
Body mass index (kg/m²)	23.9 (2.8)	24.2 (2.7)	0.008
Angina pectoris (%)	8.2	10.5	0.01
Hypertension (%)	42.6	44.5	0.24
Diabetes mellitus (%)	24.9	22.8	0.11
Prior use of hypolipidemics (%)	7.6	14.3	<0.0001
Current smoking (%)	40.0	37.5	0.10
Daily alcohol use (%)†	41.7	39.6	0.16

Values are mean (SD) unless otherwise specified.
**Comparison of means was based on t-test, and the chi-square test was used for proportions.*
†Drinking alcohol 5 days per week or more frequently.

All the patients gave consent to participate in the study. Ineligible for the study were a history of myocardial infarction, coronary bypass surgery, coronary angioplasty, cerebral hemorrhage, or cerebral infarction; serum HDL-C concentration of 80 mg/dl or greater; and a life-limiting morbid condition 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 that instruction[20] The patients were followed up until the end of 1997 for the occurrence of coronary events and cerebral infarction. The study was approved by the ethical committee of the principal investigator's affiliated institution.

Subjects

The present analysis included 4,349 of the 5,640 enrolled men; 1,291 were excluded for the following reasons: (1) withdrawal of consent (n=147), (2) no institutional contract (n=616; this category represented the lack of a written agreement between a participating hospital or clinic and a sponsoring pharmaceutical company, which became necessary in the course of the study because of the introduction of a new regulation for clinical trials in Japan), (3) found to be ineligible during follow-up (n=97), and (4) missing data (n=431).

Laboratory and Clinical Data

Serum concentrations of TC, HDL-C, triglycerides (TG), and other clinical and biochemical variables were determined at baseline and subsequently in the follow-up. Laboratory measurements were done at different laboratories, but each 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 periodical follow-up measurements at 3 months, 6 months, and every year thereafter; the number of measurements ranged from 1 to 10 with a median of 6. Serum LDL-C was not used in the present study because the Friedewald method[24] was not applicable for 7% of the men who even at baseline had serum TG concentrations of 400 mg/dl or greater (n=282) or who had missing data (n=5). HDL-C was not measured in 46 men during the follow-up.

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

Endpoints

Cerebral infarction was the secondary endpoint and coronary events were the primary endpoint. Definite cerebral infarction was diagnosed when typical symptoms and signs were accompanied with diagnostic findings on brain imaging or cerebral angiography. Diagnosis based only on clinical signs and symptoms was regarded as a suspected case[20] These endpoints were determined by the Endpoint and Adverse Effect Committee on the basis of periodical reports from the study physicians and, if necessary, by supplementary inquiry. From January to May 1998, an ad hoc survey was carried out to ascertain the occurrence of coronary events and cerebral infarction up to the end of 1997. Vital status was unknown for 36 men, and cerebral infarction and coronary events were not ascertained for 97 men. A total of 81 definite cases and 10 suspected cases of cerebral infarction were identified in an average observation period of 5.05 years. One definite case and 2 suspected cases of cerebral infarction occurred subsequent to a coronary event. None developed coronary events after cerebral infarction. Only definite cases of cerebral infarction were used in the present study, and the follow-up period continued until the event of a definite cerebral infarction regardless of coronary events.

Statistical Analysis

The Cox proportional hazards model was used to examine the relation of clinical and behavioral factors to the risk of cerebral infarction. The principal model included indicator variables for age (5-year class), baseline serum TC (<240, 240–259, ≥260 mg/dl), follow-up TC (<220, 220–239, ≥240 mg/dl), serum HDL-C (<40, 40–59, ≥ 60 mg/dl), BMI (<22.5, 22.5–24.9, ≥25.0 kg/m²), AP, HT, DM, prior

use of lipid-lowering drugs, current smoking (0, 1–19, ≥20 cigarettes per day), and alcohol use (0, 1–4, ≥5 days per week). The association with statin use was examined using the model with and without follow-up TC. Adjusted relative risk and 95% confidence intervals (CI) were obtained from a regression coefficient and standard error for the corresponding indicator variable. Statistical significance of the interaction was assessed by the likelihood ratio test. Statistical significance was declared when the 95%CI did not include unity or when the two-sided p-value was less than 0.05. Statistical computations were done with the SAS software version 8.2 (SAS Institute, Inc, Carry, NC, USA).

Results

The mean age of the study subjects was 58.0 years, and the mean concentrations of baseline and follow-up serum TC were 253 mg/dl and 221 mg/dl, respectively. The characteristics of the study subjects are summarized by statin use in Table 1. Serum TC concentrations were higher at baseline and lower during the follow-up among men taking statins than in those not taking the medication. The differences were highly significant. The number of cerebral infarctions was 47 (1.7%) in men taking statins and 34 (2.1%) in those who were not.

Although baseline TC was not associated with the risk of cerebral infarction, the follow-up TC concentration was strongly, positively associated with cerebral infarction (Table 2). A nearly 4-fold increase in the risk was observed for men with a follow-up serum TC ≥240 mg/dl compared with those with a concentration <220 mg/dl. High concentrations of serum HDL-C (≥60 mg/dl) was associated with a lower risk of cerebral infarction, although the decrease was not statistically significant (p=0.08). Diabetes mellitus was associated with a statistically significant increase in the risk of cerebral infarction. Men with AP or HT also showed a moderate increase in the risk, and the increased risk associated with HT was statistically significant. No clear association was observed for BMI. A statistically nonsignificant decrease in the risk was observed for each of the intermediate categories of smoking and alcohol use.

Statin use was associated with a statistically non-significant, moderate decrease in the risk of cerebral infarction without adjustment for follow-up TC; adjusted relative risk for statin use was 0.73 (95%CI 0.46–1.16) when follow-up TC was replaced with statin use in the model used for Table 2. When both statin use and follow-up TC were included in the model, the adjusted relative risk for statin use was 0.91 (95%CI 0.57–1.47), and adjusted relative risks for the follow-up TC concentrations of <220, 220–239, and ≥240 mg/dl were 1.00 (referent), 1.69 (95%CI 0.97–2.97), and 3.76 (95%CI 2.15–6.56), respectively. Further, the association between follow-up TC and cerebral infarction was examined by statin use. In that analysis, follow-up TC was used as continuous variable to avoid unstable estimation because of the smaller number of cases. Adjusted relative risks for an increase of 10 mg/dl in TC were 1.31 (95%CI 1.14–1.51) in non-statin users and 1.11 (95%CI 0.99–1.25) in statin users. The increased risk associated with follow-up TC seemed to be attenuated in the latter group, but the interaction was not statistically significant (p=0.16).

When separate analyses were done for men aged less than 65 years and those aged 65 years or older (Table 3), the increased risk of cerebral infarction associated with elevated concentrations of serum TC during the follow-up was slightly more evident in the elderly men than in the middle-aged men. A decreased risk associated with high concentrations of HDL-C was more apparent in the middle-aged men, but neither of the decreases in risk for the 2 age groups was statistically significant. Diabetes mellitus and AP were each associated with a statistically significant increase in the risk of cerebral infarction in the elderly only. Hypertension was statistically non-significantly associated with an increased risk in both middle-aged and elderly men. As regards alcohol use, a statistically significant decrease in the risk was observed for the category of 1–4 days per week in elderly men only.

Table 2 Adjusted Relative Risks of Cerebral Infarction According to Selected Risk Factors

Variable	*No. of men*	*No. of cases*	*Adjusted RR (95%CI)**
Baseline TC (mg/dl)			
<240	*1,463*	*21*	*1.00 (referent)*
240–259	*1,538*	*38*	*1.42 (0.83–2.46)*
≥260	*1,348*	*22*	*0.78 (0.42–1.46)*
Follow-up TC (mg/dl)			
<220	*2,097*	*29*	*1.00 (referent)*
220–239	*1,338*	*23*	*1.72 (0.99–3.00)*
≥240	*914*	*29*	*3.86 (2.23–6.62)*
HDL-cholesterol (mg/dl)			
<40	*970*	*22*	*1.00 (referent)*
40–59	*2,485*	*49*	*0.94 (0.56–1.57)*
≥60	*894*	*10*	*0.50 (0.23–1.09)*
Body mass index (kg/m²)			
<22.5	*1,197*	*22*	*1.00 (referent)*
22.5–24.9	*1,609*	*31*	*1.15 (0.66–2.01)*
≥25.0	*1,543*	*28*	*1.16 (0.65–2.07)*
Angina pectoris			
None	*3,930*	*66*	*1.00 (referent)*
(+)	*419*	*15*	*1.74 (0.98–3.08)*
Diabetes mellitus			
None	*3,325*	*54*	*1.00 (referent)*
(+)	*1,024*	*27*	*1.81 (1.13–2.89)*
Hypertension			
None	*2,445*	*30*	*1.00 (referent)*
(+)	*1,904*	*51*	*1.65 (1.04–2.63)*
Cigarettes per day			
0	*2,679*	*60*	*1.00 (referent)*
1–19	*474*	*6*	*0.54 (0.23–1.26)*
≥20	*1,196*	*15*	*0.82 (0.46–1.46)*
Alcohol use (days/week)			
0	*1,683*	*44*	*1.00 (referent)*
1–4	*910*	*10*	*0.50 (0.25–1.01)*
≥5	*1,756*	*27*	*0.85 (0.52–1.41)*

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

Discussion

Methodological problems need to be clarified before interpreting the present findings. Because the patients were non-randomly allocated to pravastatin treatment, cardiovascular risk factors were generally more prevalent among statin users, as shown in Table 1. Although statistical adjustment was done for these factors, residual confounding effects possibly remained. Statin use was strongly associated with lower concentrations of follow-up TC, and it may be difficult to conclude which is more importantly related to the risk of cerebral infarction on statistical grounds. All the patients were treated for hypercholesterolemia regard-

Table 3 Adjusted Relative Risk of Cerebral Infarction According to Selected Risk Factors in Men Aged Less Than 65 Years and Older Men

Variable	<65 years (n=3,115)		≥65 years (n=1,070)	
	No. of cases	Adjusted RR (95%CI)*	No. of cases	Adjusted RR (95% CI)*
Baseline TC (mg/dl)				
<240	*7*	*1.00 (referent)*	*14*	*1.00 (referent)*
240–259	*16*	*1.91 (0.78–4.70)*	*22*	*1.15 (0.57–2.31)*
≥260	*15*	*1.65 (0.64–4.26)*	*7*	*0.38 (0.15–0.98)*
Follow-up TC (mg/dl)				
<220	*13*	*1.00 (referent)*	*16*	*1.00 (referent)*
220–239	*12*	*1.40 (0.63–3.12)*	*11*	*1.91 (0.87–4.19)*
≥240	*13*	*2.45 (1.07–5.60)*	*16*	*5.26 (2.53–10.95)*
HDL-cholesterol (mg/dl)				
<40	*12*	*1.00 (referent)*	*10*	*1.00 (referent)*
40–59	*23*	*0.70 (0.35–1.43)*	*26*	*1.33 (0.63–2.82)*
≥60	*3*	*0.33 (0.09–1.18)*	*7*	*0.70 (0.25–1.94)*
Body mass index (kg/m²)				
<22.5	*7*	*1.00 (referent)*	*15*	*1.00 (referent)*
22.5–24.9	*14*	*1.26 (0.51–3.15)*	*17*	*1.20 (0.59–2.46)*
≥25.0	*11*	*1.45 (0.59–3.56)*	*11*	*1.01 (0.45–2.27)*
Angina pectoris				
None	*34*	*1.00 (referent)*	*32*	*1.00 (referent)*
(+)	*4*	*0.99 (0.35–2.82)*	*11*	*2.70 (1.32–5.53)*
Diabetes mellitus				
None	*26*	*1.00 (referent)*	*28*	*1.00 (referent)*
(+)	*12*	*1.39 (0.70–2.77)*	*15*	*2.42 (1.25–4.68)*
Hypertension				
None	*16*	*1.00 (referent)*	*14*	*1.00 (referent)*
(+)	*22*	*1.50 (0.77–2.90)*	*29*	*1.89 (0.98–3.67)*
Cigarettes per day				
0	*27*	*1.00 (referent)*	*33*	*1.00 (referent)*
1–19	*2*	*0.45 (0.11–1.92)*	*4*	*0.59 (0.21–1.72)*
≥20	*9*	*0.83 (0.38–1.81)*	*6*	*0.83 (0.33–2.04)*
Alcohol use (days/week)				
0	*17*	*1.00 (referent)*	*27*	*1.00 (referent)*
1–4	*7*	*0.79 (0.32–1.92)*	*3*	*0.28 (0.08–0.94)*
≥5	*14*	*0.83 (0.40–1.73)*	*13*	*0.94 (0.47–1.90)*

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

less of statin use, and cardiovascular risk factors ascertained at baseline may have changed in varying degrees. The relation of clinical risk factors at baseline to the risk of cerebral infarction may have been attenuated because comorbid conditions such as HT and DM were probably well treated during the follow-up[25] Finally, results from the subgroup analysis should be interpreted cautiously. In the analysis by statin use or by age group, the estimated relative risks were more subject to random fluctuation because of the smaller number of cases.

The present study demonstrated an evident, positive association between the serum TC concentration in the follow-up period, but not at baseline, and cerebral infarction. Statin use was associated with a moderate, statistically non-significant decrease in the risk of cerebral infarction when follow-up TC was not taken into consideration. The magnitude of the decrease in the risk associated with statin use was the same as reported for pravastatin use previously in the KLIS[21] However, the decreased risk associated with statin use was almost nullified after adjustment for follow-up TC. The positive association with follow-up TC remained after adjustment for statin use. These findings indicate that lowering cholesterol itself is important in the prevention of cerebral infarction among men with moderate hypercholesterolemia.

It remains a matter of controversy whether the reduced risk of stroke or ischemic stroke associated with use of statins can be ascribed to the cholesterol-lowering effect of statins or to other properties[26,27] Statins are known to ameliorate endothelial dysfunction, stabilize atherosclerotic plaques, and modify inflammatory responses and thrombus formation[28] The observation that statin treatment confers a reduced risk of ischemic stroke among hypertensive patients with average or below-average cholesterol concentrations suggests a role of the nonlipid-lowering effects of statins[29] On the other hand, a meta-analysis of randomized controlled trials indicated that the beneficial effect on stroke incidence was seen only when the final cholesterol concentration was <232 mg/dl (6.0 mmol/L), suggesting the importance of lowering cholesterol[30] Another meta-analysis of 7 prospective observational studies showed a statistically significant decrease of 15% in the risk of thromboembolic stroke for a 1.0 mmol/L decrease in LDL-C[31] In this regard, the interaction between statin use and follow-up TC is of particular interest. In the present study, the positive relation between follow-up TC and cerebral infarction seemed weaker among statin users. The findings may be interpreted as suggestive of a protective effect of statins other than their cholesterol-lowering effect, but it is also possible that uncontrolled risk factors other than elevated concentrations of serum TC may be major determinants of the risk of cerebral infarction among statin users because they are generally at a higher risk of cardiovascular diseases on entry. A larger study is needed to clarify the relation between

achieved concentrations of TC or LDL-C and cerebral infarction among patients under treatment with different classes of cholesterol-lowering drugs.

The positive association between serum TC in the follow-up and cerebral infarction seemed to be stronger in the elderly men than in the middle-aged men in the present study. Not many studies have addressed the relation between cholesterol and cerebral infarction in the elderly. In a prospective study of those aged 60 years or older[6] serum TC was unrelated to the risk of cerebral infarction. In a clinical trial of the elderly in Europe[32] statin use did not result in a decrease in the incidence of stroke, not specifically of cerebral infarction, but there was a substantial decrease in coronary events. On the other hand, in Japanese men in Hawaii[15] a statistically significant increase in the risk of ischemic stroke was observed for the highest vs lowest quartile of serum TC in men aged 60–74 years, but not in those aged 51–59 years. The seemingly differential association with serum TC according to age group in the present study could be a random fluctuation as discussed earlier, but the findings are consistent with the progression of atherosclerosis; that is, atherosclerosis in the cerebral arteries occurs later in life than coronary atherosclerosis[15]

Although HDL-C has been emphasized as a predictor of coronary heart disease in Western countries[33] and in Japan[34] it is only very recently that low concentrations of serum HDL-C were found to be associated with an increased risk of cerebral infarction[4–9,14,19] Our findings on HDL-C add to evidence for a protective association between HDL-C and cerebral infarction. The non-HDL-C concentration may be more useful than that of TC in predicting the risk of cardiovascular diseases[35] When the non-HDL-C concentrations at base line and during the follow-up, categorized at each quartile, were included in the model used for Table 2 instead of the baseline and follow-up TC concentrations and HDL-C at baseline, the relation between non-HDL-C concentration during the follow-up and the risk of cerebral infarction was not as strong as observed for follow-up TC; adjusted relative risk for the highest (≥188 mg/dl) vs lowest (<153 mg/dl) quartile was 3.16 (95%CI 1.62–6.16). The role of serum TG in the development of atherosclerotic diseases is also of recent interest because of its relevance to metabolic syndrome[33,35] In the present study, however, the baseline concentrations of serum TG were unrelated to the risk of cerebral infarction; when serum TG were additionally included in the model used for Table 2, adjusted relative risks for triglycerides <150, 150–199, and ≥200 mg/dl were 1.00 (referent), 1.17 (95%CI 0.68–2.04), and 0.95 (95%CI 0.56–1.63), respectively.

Diabetes mellitus, HT and AP were found to be related to an increased risk of cerebral infarction, especially among the elderly men. Further confirmation is needed regarding possible differential relations according to age group. The present findings regarding smoking and alcohol use were difficult to interpret because past smokers and past drinkers were not distinguished from lifelong nonsmokers and nondrinkers. Occasional alcohol use was related to a substantial decrease in the risk of cerebral infarction among elderly men. In general, no material association between alcohol use and cerebral infarction has been found in prospective studies[3,7,10–12] Exceptionally, alcohol use was associated with a decreased risk of atherosclerotic stroke in a study of elderly persons[19] Further studies are warranted in view of much evidence that moderate alcohol consumption confers protection against atherosclerosis[36,37]

In summary, in men undergoing treatment for moderately elevated concentrations of serum TC, higher concentrations during the treatment, but not at baseline, were strongly related to an increased risk of cerebral infarction and the decreased risk associated with statin use was almost nullified when follow-up TC was taken into account. Lowering cholesterol itself, rather than the choice of cholesterol-lowering regimen, is important in the prevention of cerebral infarction in patients with hypercholesterolemia.

Acknowledgments

The KLIS was funded by a grant from Sankyo, Co Ltd (Tokyo). Participating physicians are listed in references 20 and 21.

References

1. Sudlow CLM, Warlow CP for the International Stroke Incidence Collaboration. Comparative studies of the incidence of stroke and its pathological types: Results from an international collaboration. *Stroke* 1997; **28:** 491–499.
2. The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997; **157:** 2413–2446.
3. Harmsen P, Rosengren A, Tsipogianni A, Wilhelmsen L. Risk factors for stroke in middle-aged men in Göteborg, Sweden. *Stroke* 1990; **21:** 223–229.
4. Wannamethee SG, Shaper AG, Ebrahim S. HDL-cholesterol, total cholesterol, and the risk of stroke in middle-aged British men. *Stroke* 2000; **31:** 1882–1888.
5. Tanne D, Yaari S, Goldbourt U. High-density lipoprotein cholesterol and risk of ischemic stroke mortality: A 21-year follow up of 8586 men from the Israeli Ischemic Heart Disease Study. *Stroke* 1997; **28:** 83–87.
6. Simons LA, McCallum J, Friedlander Y, Simons J. Risk factors for ischemic stroke: Dubbo Study of the elderly. *Stroke* 1998; **29:** 1341–1346.
7. Leppälä JM, Virtamo J, Fogelholm R, Albanes D, Heinonen OP. Different risk factors for different stroke subtypes: Association of blood pressure, cholesterol, and antioxidants. *Stroke* 1999; **30:** 2535–2540.
8. Shahar E, Chambless LE, Rosamond WD, Boland LL, Ballantyne CM, McGovern PG, et al. Plasma lipid profile and incident ischemic stroke: The Atherosclerosis Risk in Communities (ARIC) Study. *Stroke* 2003; **34:** 623–631.
9. Soyama Y, Miura K, Morikawa Y, Nishijo M, Nakanishi Y, Naruse Y, et al. High-density lipoprotein cholesterol and risk of stroke in Japanese men and women: The Oyabe Study. *Stroke* 2003; **34:** 863–868.
10. Tanaka H, Ueda Y, Hayashi M, Date C, Baba T, Yamashita H, et al. Risk factors for cerebral hemorrhage and cerebral infarction in a Japanese rural community. *Stroke* 1982; **13:** 62–73.
11. Nakayama T, Date C, Yokoyama T, Yoshiike N, Yamaguchi M, Tanaka H. A 15.5-year follow-up study of stroke in a Japanese provincial city: The Shibata Study. *Stroke* 1997; **28:** 45–52.
12. Tanizaki Y, Kiyohara Y, Kato I, Iwamoto H, Nakayama K, Shinohara N, et al. Incidence and risk factors for subtype of cerebral infarction in a general population: The Hisayama Study. *Stroke* 2000; **31:** 2616–2622.
13. Iso H, Jacobs DR Jr, Wentworth D, Neaton JD, Cohen JD for the MRFIT Research Group. Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the Multiple Risk Factor Intervention Trial. *N Engl J Med* 1989; **320:** 904–910.
14. Lindenstrøm E, Boysen G, Nyboe J. Influence of total cholesterol, high density lipoprotein cholesterol, and triglycerides on risk of cerebrovascular disease: The Copenhagen city heart study. *BMJ* 1994; **309:** 11–15.
15. Benfante R, Yano K, Hwang LJ, Curb JD, Kagan A, Ross W, et al. Elevated serum cholesterol is a risk factor for both coronary heart disease and thromboembolic stroke in Hawaiian Japanese men: Implications of shard risk. *Stroke* 1994; **25:** 814–820.
16. Eastern Stroke and Coronary Heart Disease Collaborative Research Group. Blood pressure, cholesterol, and stroke in eastern Asia. *Lancet* 1998; **352:** 1801–1807.
17. White HD, Simes RJ, Anderson NE, Hankey GJ, Watson JDG, Hunt D, et al. Pravastatin therapy and the risk of stroke. *N Engl J Med* 2000; **343:** 317–326.