chaperone proteins, which increase folding capacity of the ER and prevent protein aggregation (Mori 2000; Liu and Kaufman 2003); (iii) removing misfolded proteins from the ER through retrograde transport coupled to their degradation by 26S proteasome, called ER-associated degradation (ERAD) (Travers et al. 2000).

Herp (homocysteine-induced ER protein) is a novel 54 kDa protein recently described as a stress-response protein localized in the ER membrane (Kokame et al. 2000). Under ER-stress, Herp is the most highly inducible protein (Yamamoto et al. 2004). Unlike other stress-induced ER chaperones, Herp is an integral membrane protein whose N- and C- terminals both face the cytoplasm (Kokame et al. 2000; Sai et al. 2002). Its N-terminus has an ubiquitin-like domain (ULD) (Kokame et al. 2000), which is probably involved in Herp degradation via the ubiquitin–proteasome pathway (Sai et al. 2003). Cellular functions of Herp are gradually being discovered. It was suggested that during ER stress, Herp plays a neuroprotective role in brain by stabilizing ER Ca^{2+} homeostasis and maintaining mitochondrial function (Chan et al. 2004). Herp was also proposed to improve ER folding capacity, decrease ER protein load (Hori et al. 2004), and participate in the ERAD (Yamamoto et al. 2004; Schroder and Kaufman 2005). Herp was also proposed to prevent apoptosis triggered by ER stress (Chan et al. 2004; Hori et al. 2004). Furthermore, Herp was reported to interact with presenilins and, through this mechanism, to increase production of amyloid-b (Ab) (Sai et al. 2002), which is considered to be cytotoxic in sporadic inclusion-body myositis (s-IBM) muscle and in Alzheimer disease (AD) brain (Askanas and Engel 2001, 2003; Selkoe 2001; Cuello 2005).

s-IBM, the most common progressive degenerative muscle disease of persons age 50 years and older, is of unknown etiology and pathogenesis (Askanas and Engel 2001, 2002). It leads to severe disability and there is no successful treatment (Askanas and Engel 2001, 2003). The main light-microscopic features of s-IBM muscle biopsies include: (i) vacuolated muscle fibers, (ii) intramuscle-fiber multiprotein aggregates, and (iii) various degrees of mononuclear-cell inflammation (Askanas and Engel 2001, 2003). An intriguing feature is the similarity of the s-IBM muscle-fiber phenotype to that of AD brain, including accumulation of Ab, phosphorylated tau, and several other Alzheimer characteristic proteins (Askanas and Engel 2001, 2003). Two major types of intracellular inclusion-bodies in s-IBM muscle contain either Ab or phosphorylated tau (Askanas and Engel 2001, 2002). Both types of inclusions are congophilic, indicating that they contain proteins in alternate conformation (unfolded or misfolded) that are assembled into the b-pleated sheet configuration of amyloid (Askanas and Engel 2001, 2003). The cytoplasmic inclusion-bodies are present mainly in vacuole-free regions of vacuolated muscle fibers, and in muscle fibers not vacuolated at the level of the inclusion-bodies.

Ab and phosphorylated-tau inclusions contain several other accumulated proteins, some of which are present in both (Askanas and Engel 2001, 2003). Some, such as a- synuclein, have, similarly to Ab and tau, a propensity to unfold, misfold, and form b-pleated-sheet amyloid (Borden 1998).

We have recently demonstrated in s-IBM muscle fibers that several ER chaperones are accumulated in the form of aggregates and their total expression is increased, suggesting that ER stress and UPR play a role in the s-IBM pathogenesis (Vattemi et al. 2004). Those ER chaperones physically associate with Ab, suggesting that they may play a role in Ab folding and/or attempted disposal of Ab in s-IBM muscle fibers (Vattemi et al. 2004). Our most recent study demonstrated significant proteasomal inhibition and aggresome formation in s-IBM fibers (Fratta et al. 2005). Those studies further support our previous proposal that unfolding and misfolding of proteins play a role in formation of the multiprotein-aggregates (inclusions) within s-IBM muscle fibers (Askanas and Engel 2002, 2003).

In the present study, we asked whether Herp might play a role in the s-IBM pathogenesis. We examined Herp expression in s-IBM muscle fibers on the mRNA and protein levels, and Herp light- and electronmicroscopic immunolocalization, including its co-immunolocalization with Ab, ER chaperone BiP/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein 78), 20S b2 proteasome subunit, and ubiquitin. To explore the mechanisms involved in regulating Herp in human muscle fibers, we utilized ER stress-induced and proteasome-inhibited cultured human muscle fibers.

Experimental procedures

Muscle biopsies

Immunocytochemical studies were performed on 10-1m-thick unfixed sections of fresh-frozen diagnostic muscle biopsies obtained (with informed consent) from 34 patients with these diagnoses: 13 s-IBM; four polymyositis; one dermatomyositis; two morphologically non-specific myopathy; two mitochondrial myopathy; one peripheral neuropathy; two amyotrophic lateral sclerosis; two oculopharyngeal muscular dystrophy, one non-IBM vacuolar myopathy, and six normal muscle. Diagnoses were based on clinical and laboratory investigations, including our routinely performed 18-reaction diagnostic histochemistry of the biopsies. All s-IBM biopsies had muscle fibers with vacuoles on Engel trichrome staining (Engel and Cunningham 1963), 15–21 nm paired helical filaments by SMI-31 immunoreactivity (Askanas et al. 1996a) and by electronmicroscopy, and Congo-red positivity using fluorescence enhancement (Askanas et al. 1993b).

Light- microscopic immunocytochemistry

Fluorescence immunocytochemistry was performed as described (Askanas et al. 1993a, 1996a, 2000; Vattemi et al. 2003, 2004)

using a well-characterized polyclonal antibody against Herp, diluted 1 : 100 (Kokame et al. 2000). Double-immunofluorescence utilized an antibody against Herp combined with one of the following: (i) mouse monoclonal antibody 6E10 (Signet, Dedham, MA, USA), diluted 1 : 100, which morphologically recognizes Ab in both AD brain (Kim et al. 1990) and s-IBM muscle (Askanas et al. 2000); (ii) mouse monoclonal antibody recognizing BiP/GRP78 (BD Transduction Laboratories, San Diego, CA, USA) diluted 1 : 20; (iii) mouse monoclonal antibody recognizing 20S proteasomal subunit b2 (Affinity Research Products Ltd, UK) diluted 1 : 50; (iv) mouse monoclonal antibody recognizing ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 50.

To block non-specific binding of antibody to Fc receptors, sections were pre-incubated with normal goat or rabbit serum diluted 1 : 10, as described (Askanas et al. 1993a, 2000). Controls for staining specificity were (i) omission of the primary antibody or (ii) its replacement with non-immune sera or irrelevant antibody.

Immuno-electronmicroscopy

Double-label gold immuno-electronmicroscopy was performed on 10-1m unfixed frozen biopsy sections adhered to the bottom of 35-mm Petri dishes, as detailed (Askanas et al. 1993a, 1996a, 2000). In brief, a primary antibody against Herp was used in combination with an antibody against Ab, 20S proteasome, or BiP/GRP78. After incubation with the appropriate secondary antibodies conjugated to 5 nm and 15 nm gold particles, sections were processed for electronmicroscopy as described (Askanas et al. 1993a, 1996a, 2000).

Immunoblotting

Muscle biopsies of seven s-IBM and seven age-matched control patients were immunoblotted, as recently detailed (Vattemi et al. 2003, 2004). Briefly, 10-1m-thick frozen sections were collected in ristocetin-induced platelet agglutination (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 0.1% sodium dodecyl sulfate) containing phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). All samples were rapidly homogenized on ice. Protein concentration was measured using the Bradford method (Vattemi et al. 2004): 20 lg of protein were loaded onto 10% NuPAGE gels (Invitrogen, Carlsbad, CA, USA), electrophoresed, transferred to nitrocellulose membranes, and immuno-probed with antibodies against Herp. After incubation in the appropriate secondary antibodies, blots were developed using an enhanced chemiluminescence system (ECL) (Amersham Biosciences, Piscataway, NJ, USA). Protein loading was evaluated by the actin band visualized with a mouse monoclonal antibody (Santa Cruz Biotechnology). Quantification of the immunoreactivity was performed by densitometric analysis using NIH Image J 1.310 software.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA from 10-1m-thick frozen sections of s-IBM and control muscle biopsies was isolated using an RNA isolation kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. RNA samples were treated with Dnase I (Amplification Grade, Invitrogen) and 1 lg of RNA was submitted for cDNA synthesis using an oligo-dT primer (Invitrogen) and the Omniscript RT kit (Qiagen, Valencia, CA, USA). Then 1/10 of the RT reaction was amplified using multiplex PCR in a total volume of 20 lL, with Platinum Taq DNA Polymerase (Invitrogen), utilizing previously described primers for Herp (Kokame et al. 2000) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lehmann et al. 2002) as an internal control. The optimized conditions for Herp and GAPDH amplification were 2 min at 94•C followed by 28 cycles of amplification (94•C for 30 s, 60•C for 30 s, 72•C for 90 s), and the final incubation was 10 min at 72•C. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The conditions of the reactions were experimentally checked to ensure that signals were in the linear range of the PCR. Identity of the products was confirmed by sequencing.

Statistical analysis

The statistical significance of differences between groups was determined by Student's t-test. The level of significance was set at $p < 0.05$. Data are presented as means ± SEM for all groups.

Cultured human muscle fibers

Primary cultures of normal human muscle were established from satellite cells of portions of diagnostic muscle biopsies from patients who, after all tests were performed, were considered free of muscle disease (Askanas and Engel 1992; Askanas et al. 1996b). Experiments were performed on seven culture sets, each established from a different muscle biopsy. All experimental conditions were studied on sister cultures in the same culture sets. Three-week-old cultured muscle fibers were treated for 24 h with either (i) one of two well-known ER stress inducers, N-glycosylation inhibitor tunicamycin (4 lg/mL) or an inhibitor of ER-calcium-ATPase thapsigargin (300 nM) (Back et al. 2005; Lee 2005) (both from Sigma Co, St. Louis, MO, USA) or (ii) epoxomicin (1 lM) (Biomol Research Laboratories, Plymouth Meeting, PA, USA), a specific and irreversible proteasome inhibitor (Meng et al. 1999). After treatment, control and experimental cultures were fixed for immunostainings, harvested for immunoblot studies, or used for RNA isolation.

Light-microscopic immunocytochemistry

Single and double immunofluorescence on paraformaldehyde-fixed cultures was performed as described (Askanas et al. 1996b, 1997; Fratta et al. 2005).

Immunoblotting

Cultured cells were harvested in ristocetin-induced platelet agglutination (RIPA) buffer containing phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche Diagnostic). The immunoblotting procedure was performed as described above for muscle biopsies.

Reverse transcription–polymerase chain reaction and northern blots

Total RNA from control and experimental cultures was extracted using RNA-bee reagent (Tel Tech, Friendwood, TX, USA). After treatment with Dnase I, 150 ng of RNA was used for one-step synthesis of cDNA and the multiplex PCR reaction (Qiagen). The optimized conditions were 30 min at 50•C, 15 min at 95•C

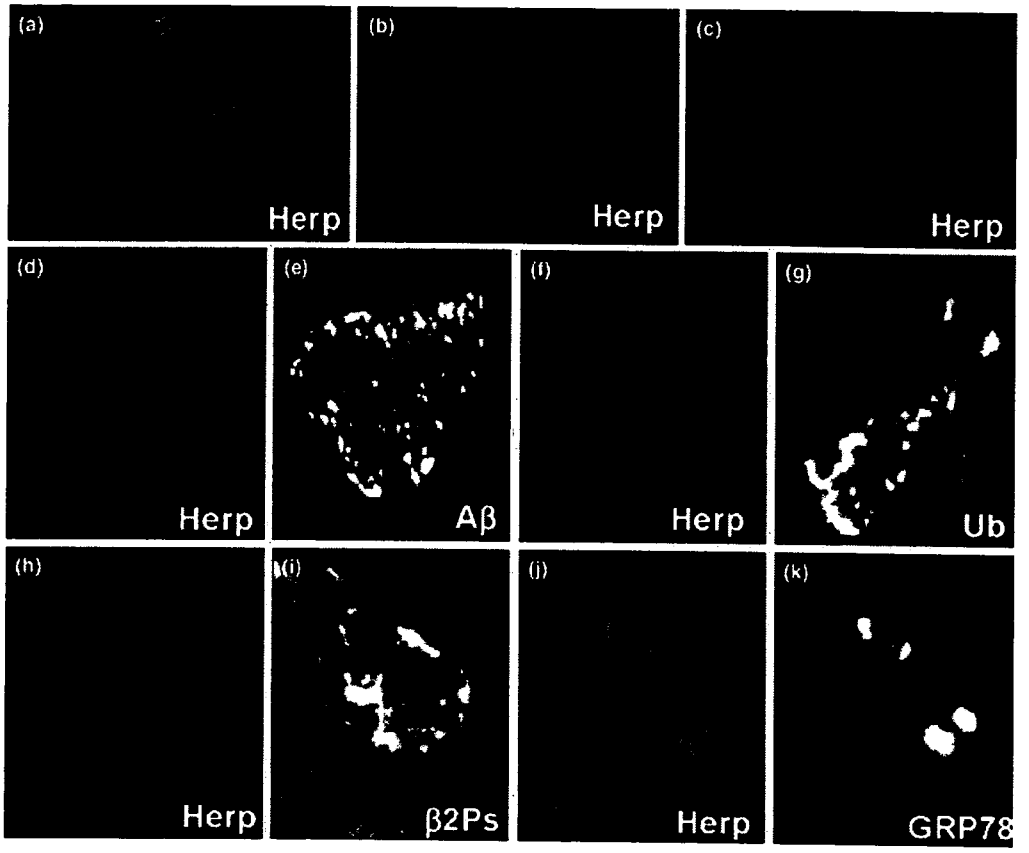

Fig. 1 Immunofluorescence within sporadic inclusion-body myositis muscle fibers. (a–c) Single-label immunofluorescence illustrates immunoreactivity of homocysteine-induced endoplasmic reticulum protein (Herp) in the form of various-sized aggregates. Double-label immunofluorescence illustrates that Herp immunoreactive inclusions (d, f, h, j) co-localized with amyloid-b (Ab) (e), ubiquitin (g), b2 20S proteasome subunit (i), and Bip/GRP78 ER chaperone (k). Magnification: a–c, f–k, · 1100; d, e · 700. BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; b2Ps, b2 20S proteasome subunit; Ub, ubiquitin.

followed by 28 cycles of amplification (94•C for 30 s, 60•C for 30 s, 72•C for 90 s), and the final incubation of 10 min at 72•C.

For northern blots, aliquots of RNA (10 lg) isolated from experimental and control cultures were denatured and subjected to electrophoresis in 1% agarose gel containing 0.41 M formaldehyde. After electropheretic fractionation, RNA was transferred overnight to a positively charged nylon membrane by capillary blotting and fixed by UV light. A Herp-specific probe was generated by PCR and fluorescein-labelled (Kokame et al. 2000). Hybridization and detection procedures using anti-fluorescein-AP conjugate were performed as described (Kokame et al. 2000).

Statistical analysis

The statistical analysis was as described above.

Results

Muscle biopsies

Light-microscopic immunocytochemistry

In all s-IBM muscle biopsies, 70–80% of the vacuolated muscle fibers contained, mainly in their non-vacuolated cytoplasm, numerous various-sized aggregates immunoreactive with antibodies against Herp (Figs 1a–c). Approximately 25% of non-vacuolated muscle fibers also contained similar aggregates. By double immunofluorescence, Herp immunoreactive aggregates co-localized with Ab (Figs 1d and e), ubiquitin (Figs 1f and g), b2 proteasome subunit (Figs 1h and i), and BiP/GRP78 (Figs 1j and k). None of the control non-s-IBM, diseased or normal, human muscle biopsies had muscle fibers containing aggregates immunoreactive with anti-Herp antibody. Eliminating the primary antibodies or replacing them with non-relevant antibodies resulted in non-staining.

Gold-immuno-electronmicroscopy

Immunoreactive Herp was associated with Ab, 20S b2 proteasome subunit, and BiP/GRP78 within the same structures, namely the 6–10 nm fibrils and amorphous and floccular material (Fig. 2). Herp was not associated with paired helical filaments, which are known to contain phosphorylated tau in IBM muscle fibers (Mirabella et al. 1996).

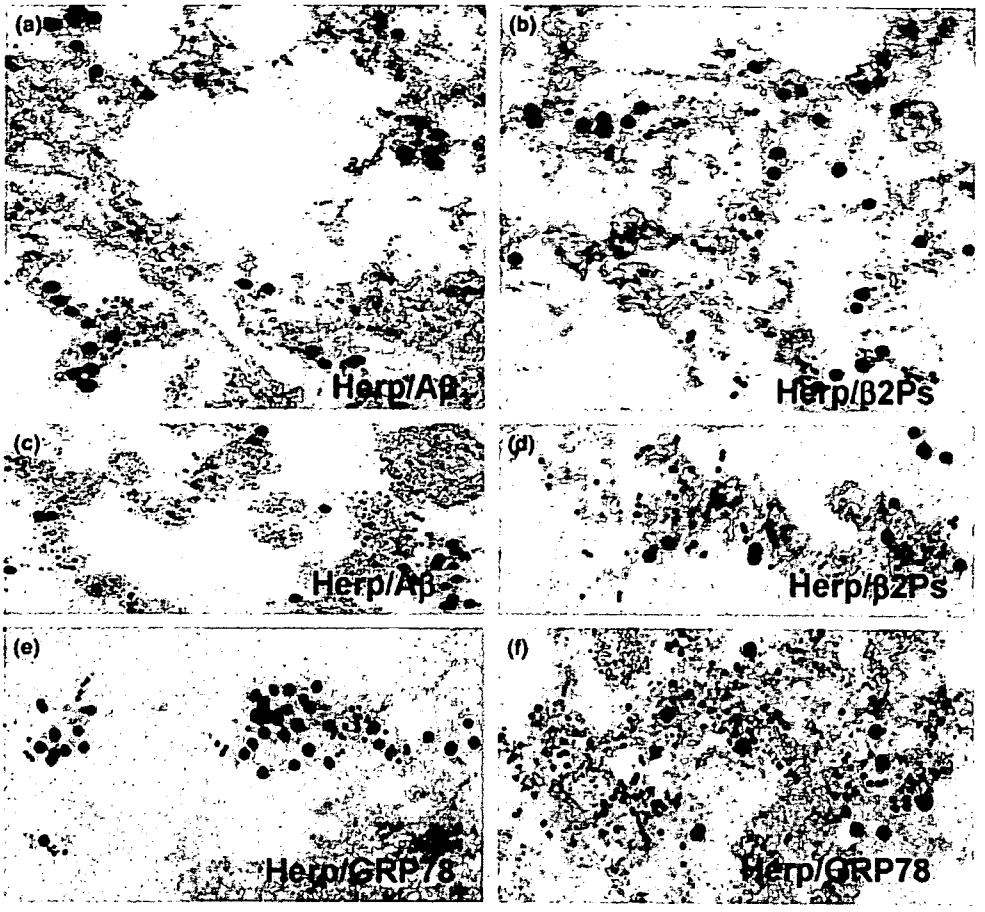

Fig. 2 Double-label gold-immuno-electronmicroscopy in sporadic inclusion-body myositis muscle fibers. This illustrates that both homocysteine-induced endoplasmic reticulum protein (Herp) (5 nm gold particles) and amyloid-b (Ab) (15 nm gold particles) are associated with 6–10 nm fibrils (a) and floccular material (c). Similarly, Herp (5 nm gold) and b2 20S proteasome subunit (15 nm gold) (b, d) and Herp (5 nm gold) and BiP/GRP78 (15 nm gold) (e, f) are associated with the same 6–10 nm fibrils and floccular material. Magnification: a, c–f, · 51 000; b, · 60 000. BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein 78; b2Ps, b2 20S proteasome subunit; Ub, ubiquitin.

Immunoblots

In normal and s-IBM muscle, Herp migrated as a single 54 kDa band (Fig. 3a), and its expression was much stronger in s-IBM muscle as compared to controls. Omission of the primary antibody resulted in no bands being present (Fig. 3b). Figure 3(c) provides analysis of the data derived from densitometric scans (obtained from seven samples), normalized to b-actin and expressed in arbitrary units. These data indicate that in s-IBM muscle biopsies Herp protein was increased eight-fold ($p < 0.05$) as compared to controls.

RNA expression by reverse transcription–polymerase chain reaction

As determined by RT–PCR analysis, Herp mRNA was prominently increased in s-IBM as compared to controls (Figs 3d and e).

Cultured human muscle fibers

Light-microscopic immunocytochemistry

In control fibers, Herp immunoreactivity was only weak and diffuse (Fig. 4a). ER stress-induced cultured human muscle fibers had strong and diffuse Herp immunoreactivity (Figs 4b and c). In contrast, approximately 80% of the epoxomicin-treated muscle fibers had the Herp protein immunoreactivity in the form of large aggregates (Figs 4d, e, and g), which co-localized with ubiquitin immunoreactivity (Figs 4f and h).

Immunoblots

Treatment with ER-stress inducers or with epoxomicin prominently increased the Herp protein level as illustrated by two representative culture sets (Fig. 5a). Figure 5(b) provides densitometric analysis of the data from seven

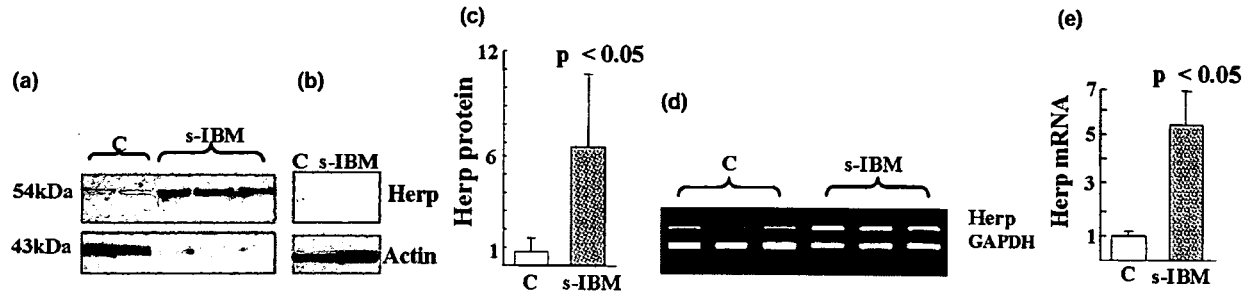

Fig. 3 Immunoblots and RT–PCR in control and sporadic inclusion-body myositis (s-IBM) samples. (a) Immunoblots of muscle homogenates of normal-control (C) and s-IBM muscle biopsies demonstrate much stronger expression of homocysteine-induced endoplasmic reticulum protein (Herp) in s-IBM. The actin bands indicate protein loading in each sample. (b) Omission of the primary anti-Herp antibody resulted in no bands. (c) Densitometric analysis of the blots performed using NIH Image J 1.310 indicates that in s-IBM muscle biopsies Herp protein was eight-fold increased as compared to controls. Data are indicated as mean ± SEM. Significance was determined by t-test. The level of significance was set at $p < 0.05$. (d) Representative agarose gel electrophoresis of products corresponding to Herp and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amplified by the Multiplex PCR method. (e) Densitometric analysis of the PCR bands, expressed in arbitrary units, indicates that Herp mRNA is increased in s-IBM muscle fibers. The GAPDH mRNA was used to normalize corresponding Herp results.

experimental sets, normalized to b-actin. Thapsigargin, the strongest Herp-inducer, increased Herp about 15-fold ($p < 0.01$). Tunicamycin and epoxomicin increased Herp six-fold ($p < 0.01$) and 10-fold ($p < 0.05$), respectively (Fig. 5b). In some thapsigargin, tunicamycin, or epoxomicin treated cultures, additional bands lower than 54 kDa were occasionally observed (not shown). This suggests, as shown previously in PC12 cells (Chan et al. 2004), that under certain experimental conditions, some cleavage of Herp can also occur in cultured muscle fibers.

Reverse transcription–polymerase chain reaction and northern blotting analysis

To investigate the molecular basis of Herp protein increase, semiquantitative multiplex RT–PCR analysis was performed. Those studies indicated in each experiment that thapsigargin and tunicamycin increased Herp mRNA, whereas epoxomicin appeared to slightly decrease it (Figs 5c and d). The above data were confirmed by northern blots (Fig. 5e).

Discussion

We investigated expression of Herp in normal and diseased human muscle, and studied the mechanisms of Herp regulation in cultured human muscle fibers. We report that in s-IBM muscle fibers, Herp was accumulated in various-sized multifocal aggregates, wherein it co-localized with Ab, ER chaperone BiP/GRP78, and 20S b2 proteasome subunit, by both light- and electronmicroscopic immunocytochemistry.

In addition, Herp protein and its mRNA were prominently increased in s-IBM muscle fibers. Even though normal human muscle fibers had, on immunoblots, a definite band of Herp, neither in them nor in several non-s-IBM diseased human muscle biopsies was Herp discerned immunocytochemically by light-microscopy. Previous studies have shown Herp mRNA expression in various human organs, including skeletal muscle (Kokame et al. 2000), but they did not study Herp protein.

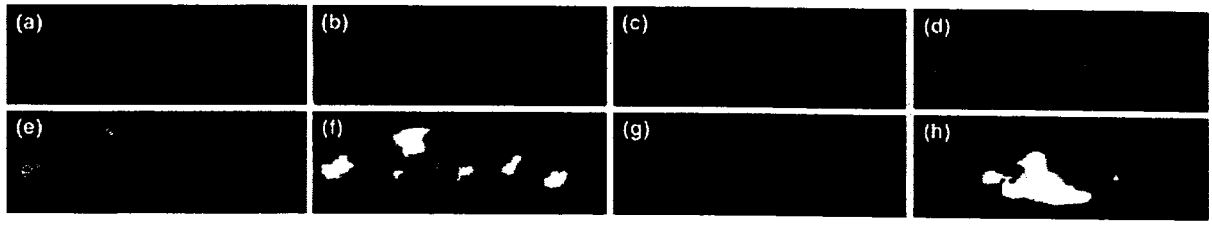

Fig. 4 Immunofluorescence in cultured human muscle fibers. Single-label immunofluorescence of homocysteine-induced endoplasmic reticulum protein (Herp) (a–d) shows very weak, barely detectable immunoreactivity in a control (a) cultured muscle fiber. Strong and diffuse immunoreactivity was present in cultures treated with endoplasmic reticulum (ER) stress inducers thapsigargin (b) and tunicamycin (c). In cultures treated with the proteasome inhibitor, epoxomicin, Herp immunoreactivity was in the form of large aggregates (d). Double-label immunofluorescence illustrates that in epoxomicin-treated cultures Herp immunoreactive aggregates (e, g) were also immunoreactive with an antibody against ubiquitin (f, h). Magnification: all · 1400.

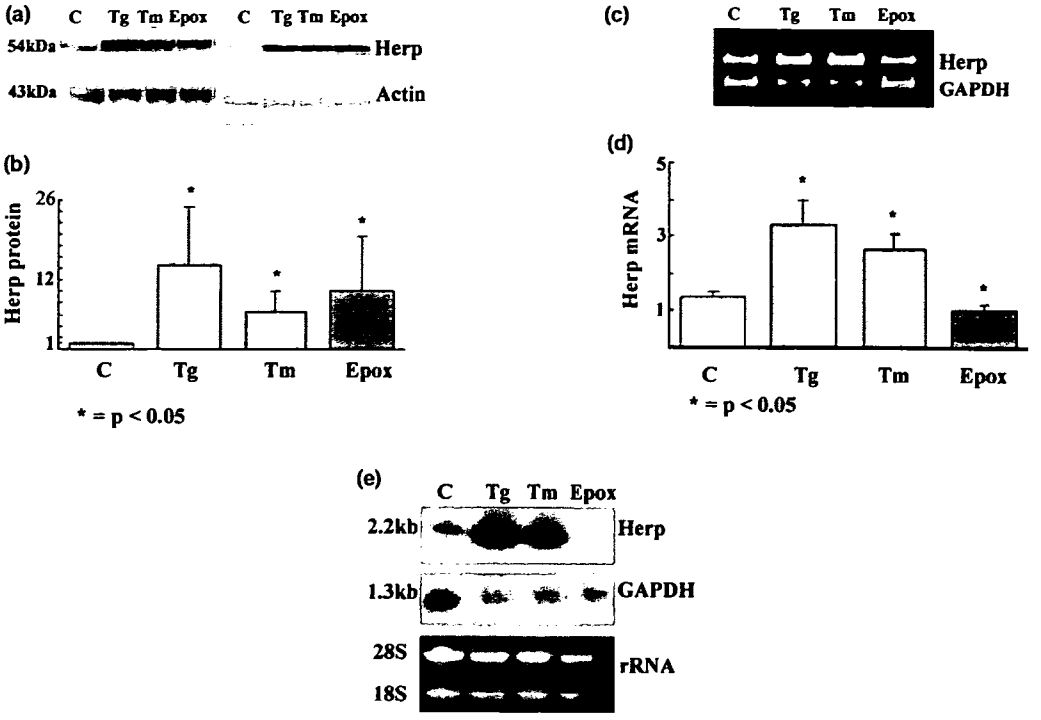

Fig. 5 Immunoblots, RT–PCR, and northern blotting in cultured human muscle fibers. (a) Immunoblots of two representative culture sets show the increase of homocysteine-induced endoplasmic reticulum protein (Herp) in thapsigargin (Tg), tunicamycin (Tm), and epoxomicin (Epox) treated cultured human muscle fibers as compared to controls (C). (b) Densitometric analysis of blots in (a), analyzed using NIH Image J 1.310, shows that thapsigargin, tunicamycin, and epoxomicin prominently increased Herp protein. Data were obtained from seven independent experimental sets of cultured human muscle, and are presented as the mean ± SEM of fold increase. Significance was determined by t-test. The level of significance was set at $p < 0.05$. (c) Representative agarose gel electrophoresis of products corresponding to Herp and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amplified by Multiplex PCR method. (d) Densitometric analysis of the PCR bands expressed in arbitrary units. The GAPDH mRNA was used to normalize corresponding Herp results. Those data show that thapsigargin and tunicamycin increased Herp mRNA, whereas epoxomicin appears to slightly decrease Herp mRNA. (e) Northern blotting of Herp mRNA in control (C), thapsigargin (Tg), tunicamycin (Tm), and epoxomicin (Epox) treated cultured human muscle fibers shows results similar to those obtained by PCR. GAPDH illustrates RNA loading, which corresponds to the 28S and 18S RNA visualized by staining of the agarose gel with ethidium bromide.

Our studies, based on experimentally modified cultured human muscle, demonstrated two distinct mechanisms of Herp increase in normal human muscle, and they seem directly relevant to s-IBM muscle fibers. Specifically, two ER-stress inducers, tunicamycin and thapsigargin, each increased Herp transcription and translation, which resulted in very strong and diffuse immunoreactivity of its protein. However, proteasome inhibition by epoxomicin increased the amount of Herp total protein without increasing its transcription, and produced striking aggregations of Herp, which, similarly to the s-IBM muscle biopsies, were associated with ubiquitin. As ER-stress and proteasome inhibition are present in s-IBM muscle fibers, we propose that those two mechanisms contribute to the demonstrated Herp abnormalities in s-IBM muscle fibers.

Even though Herp is considered the most inducible protein during ER-stress (Yamamoto et al. 2004), its exact relationship to the ER-stress is not well understood. Accumulation of unfolded/misfolded proteins in the ER induces ER-stress, which activates three pathways of the UPR, the role of which is to mitigate the ER-stress. One pathway involves preferential translation of transcription-factor ATF4 (Shen et al. 2004; Schroder and Kaufman 2005). The Herp promoter contains ER-stress-responsive elements, including a cis-acting element recognized by ATF4 (Ma and Hendershot 2004; Yamamoto et al. 2004). Our preliminary data (not shown) indicate that ATF4 is increased in s-IBM muscle fibers, suggesting that ATF4 may be involved in overexpression of Herp. Whether other transcription factors, such as ATF6 or X-box-binding protein 1 (XBP-1), participate in Herp induction in s-IBM muscle fibers and in our culture model is not known. Thus, the exact pathways that contribute to Herp activation in s-IBM muscle fibers remain to be studied.

The half-life of Herp is considered to be very short (2.5 h) (Sai et al. 2003). Its ubiquitin-like domain (ULD) is responsible for its rapid degradation via the 26S proteasome (Sai et al. 2003). Because 26S proteasome activity is

inhibited in s-IBM muscle fibers (Fratta et al. 2005), this inhibition may contribute to the total increase of Herp protein, as well as its accumulation in the form of ubiquitin-associated aggregates.

We have also shown other proteins accumulated and aggregated in s-IBM muscle fibers and in our proteasome-inhibited cultures of human muscle fibers (Fratta et al. 2005). As Herp was proposed to be linked to both UPR and ERAD (Yamamoto et al. 2004; Schroder and Kaufman 2005), its co-localization with BiP/GRP78, 20S proteasome, and Ab in s-IBM muscle fibers might suggest that Herp in s-IBM is involved in attempted proper folding of proteins and in the pathway of proteasomal removal of improperly folded ones.

Herp has been previously shown to influence processing of amyloid-b precursor protein (AbPP) and Ab production through its binding to presenilin 1 (Sai et al. 2002). Presenilin 1 is increased in s-IBM muscle fibers (Askanas et al. 1998), but our present studies did not address the question of whether Herp participates in AbPP processing and Ab production in s-IBM muscle fibers or in our cultured human muscle fibers.

A role of Herp in preventing ER-stress-induced apoptotic cell death in non-muscle cells (Chan et al. 2004; Hori et al. 2004) is of interest regarding s-IBM muscle fibers. s-IBM fibers do not exhibit features of apoptosis (Behrens et al. 1997; Askanas and Engel 2001), despite intracellular existence of several factors, such as increased Ab, oxidative stress, and mitochondrial abnormalities (Askanas and Engel 2001, 2003) known to induce apoptosis in other cells (Loo et al. 1993; Simonian and Coyle 1996; Parone et al. 2002). We have previously proposed that the multinucleated muscle fibers do not undergo classic apoptosis (Broccolini et al. 1999; Askanas and Engel 2001). In s-IBM muscle fibers, survival motor neuron (SMN) factor and IAP-like protein were previously proposed to play an anti-apoptotic role (Broccolini et al. 2000; Li and Dalakas 2000; Askanas and Engel 2001). Based on our current data, it is possible that Herp might also contribute to the anti-apoptotic milieu in s-IBM muscle fibers, as was shown in relation to other cells (Chan et al. 2004; Hori et al. 2004).

Conclusion

Our novel findings related to the increase of Herp and its abnormal multifocal accumulation in s-IBM muscle fibers are reported. We demonstrate, for the first time, that two different mechanisms are involved in Herp regulation and accumulation in human muscle fibers. We suggest that Herp, amelioratively, might be facilitating the unfolded protein response and attempting to enhance the ER-associated protein degradation of malfolded proteins in s-IBM muscle fibers. Nevertheless, despite the existence in the s-IBM muscle fibers of Herp and components of the unfolded protein response that are putatively protective, s-IBM is a severely progressive degenerative disease, probably because the factors causing progressive degeneration are more influential than those having a putative protective influence. Accordingly, developing methods that would further up-regulate the protective factors might lead to novel therapeutic avenues.

Acknowledgements

Supported by grants from the National Institutes of Health (AG16768 Merit Award), the Myositis Association, the Muscular Dystrophy Association (to VA), and the Helen Lewis Research Fund. Maggie Baburyan provided excellent technical assistance in electronmicroscopy.

References

Askanas V. and Engel W. K. (1992) Cultured normal and genetically abnormal human muscle, in The Handbook of Clinical Neurology, Myopathies, Vol. 18 (Rowland L. P. and Di Mauro S., eds), pp. 85–116. North Holland, Amsterdam.

Askanas V. and Engel W. K. (2001) Inclusion-body myositis: newest concepts of pathogenesis and relation to aging and Alzheimer disease. J. Neuropathol. Exp. Neurol. 60, 1–14.

Askanas V. and Engel W. K. (2002) Inclusion-body myositis and myopathies: different etiologies, possibly similar pathogenic mechanisms. Curr. Opin. Neurol. 15, 525–531.

Askanas V. and Engel W. K. (2003) Proposed pathogenetic cascade of inclusion-body myositis: importance of amyloid-beta, misfolded proteins, predisposing genes, and aging. Curr. Opin. Rheumatol. 15, 737–744.

Askanas V., Alvarez R. B. and Engel W. K. (1993a) Beta-amyloid precursor epitopes in muscle fibers of inclusion body myositis. Ann. Neurol. 34, 551–560.

Askanas V., Engel W. K. and Alvarez R. B. (1993b) Enhanced detection of congo-red-positive amyloid deposits in muscle fibers of inclusion body myositis and brain of Alzheimer's disease using fluorescence technique. Neurology 43, 1265–1267.

Askanas V., Alvarez R. B., Mirabella M. and Engel W. K. (1996a) Use of anti-neurofilament antibody to identify paired-helical filaments in inclusion-body myositis. Ann. Neurol. 39, 389–391.

Askanas V., McFerrin J., Baque S., Alvarez R. B., Sarkozi E. and Engel W. K. (1996b) Transfer of beta-amyloid precursor protein gene using adenovirus vector causes mitochondrial abnormalities in cultured normal human muscle. Proc. Natl Acad. Sci. USA 93, 1314–1319.

Askanas V., McFerrin J., Alvarez R. B., Baque S. and Engel W. K. (1997) Beta APP gene transfer into cultured human muscle induces inclusion-body myositis aspects. Neuroreport 8, 2155–2158.

Askanas V., Engel W. K., Yang C. C., Alvarez R. B., Lee V. M. and Wisniewski T. (1998) Light and electron microscopic immunolocalization of presenilin 1 in abnormal muscle fibers of patients with sporadic inclusion-body myositis and autosomal-recessive inclusion-body myopathy. Am. J. Pathol. 152, 889–895.

Askanas V., Engel W. K., Alvarez R. B., McFerrin J. and Broccolini A. (2000) Novel immunolocalization of alpha-synuclein in human muscle of inclusion-body myositis, regenerating and necrotic muscle fibers, and at neuromuscular junctions. J. Neuropathol. Exp. Neurol. 59, 592–598.

Back S. H., Schrèoder M., Lee K., Zhang K. and Kaufman R. J. (2005) ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. Methods 35, 395–416.

Behrens L., Bender A., Johnson M. A. and Hohlfeld R. (1997) Cytotoxic mechanisms in inflammatory myopathies. Co-expression of Fas and protective Bcl-2 in muscle fibres and inflammatory cells. Brain 120, 929–938.

Borden K. L. (1998) Structure/function in neuroprotection and apoptosis. Ann. Neurol. 44, S65–S71.

Broccolini A., Engel W. K. and Askanas V. (1999) Localization of survival motor neuron protein in human apoptotic-like and regenerating muscle fibers, and neuromuscular junctions. Neuroreport 10, 1637–1641.

Broccolini A., Engel W. K., Alvarez R. B. and Askanas V. (2000) Paired helical filaments of inclusion-body myositis muscle contain RNA and survival motor neuron protein. Am. J. Pathol 156, 1151–1155.

Chan S. L., Fu W., Zhang P., Cheng A., Lee J., Kokame K. and Mattson M. P. (2004) Herp stabilizes neuronal Ca2+ homeostasis and mitochondrial function during endoplasmic reticulum stress. J. Biol. Chem. 279, 28 733–28 743.

Cuello A. C. (2005) Intracellular and extracellular Abeta, a tale of two neuropathologies. Brain Pathol. 15, 66–71.

Ellgaard L. and Helenius A. (2001) ER quality control: towards an understanding at the molecular level. Curr. Opin. Cell Biol. 13, 431–437.

Engel W. K. and Cunningham G. G. (1963) Rapid examination of muscle tissue and improved trichrome method for fresh-frozen biopsy sections. Neurology 13, 919–923.

Fratta P., Engel W. K., McFerrin J., Davies K. J., Lin S. W. and Askanas V. (2005) Proteasome inhibition and aggresome formation in sporadic inclusion-body myositis and in amyloid-{beta} precursor protein-overexpressing cultured human muscle fibers. Am. J. Pathol. 167, 517–526.

Hori O., Ichinoda F., Yamaguchi A. et al. (2004) Role of Herp in the endoplasmic reticulum stress response. Genes Cells 9, 457–469.

Kaufman R. J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 13, 1211–1233.

Kim K. S., Wen G. Y., Bancher C., Chen J., Sapienza V., Hong H. and Wisniewski H. M. (1990) Detection and quantitation of amyloid B-peptide with 2 monoclonal antibodies. Neurosci. Res. Commun. 7, 113–122.

Kokame K., Agarwala K. L., Kato H. and Miyata T. (2000) Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. J. Biol. Chem. 275, 32 846–32 853.

Lee A. S. (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35, 373–381.

Lehmann M. H., Weber J., Gastmann O. and Sigusch H. H. (2002) Pseudogene-free amplification of human GAPDH cDNA. Biotechniques 33, 769–770.

Li M. and Dalakas M. C. (2000) Expression of human IAP-like protein in skeletal muscle: a possible explanation for the rare incidence of muscle fiber apoptosis in T-cell mediated inflammatory myopathies. J. Neuroimmunol. 106, 1–5.

Liu C. Y. and Kaufman R. J. (2003) The unfolded protein response. J. Cell Sci. 116, 1861–1862.

Loo D. T., Copani A., Pike C. J., Whittemore E. R., Walencewicz A. J. and Cotman C. W. (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc. Natl Acad. Sci. USA 90, 7951–7955.

Ma Y. and Hendershot L. M. (2004) Herp is dually regulated by both the endoplasmic reticulum stress-specific branch of the unfolded protein response and a branch that is shared with other cellular stress pathways. J. Biol. Chem. 279, 13 792–13 799.

Meng L., Mohan R., Kwok B. H., Elofsson M., Sin N. and Crews C. M. (1999) Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc. Natl Acad. Sci. USA 96, 10 403–10 408.

Mirabella M., Alvarez R. B., Bilak M., Engel W. K. and Askanas V. (1996) Difference in expression of phosphorylated tau epitopes between sporadic inclusion-body myositis and hereditary inclusion-body myopathy. J. Neuropathol. Exp. Neurol. 55, 774–786.

Mori K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101, 451–454.

Parone P. A., James D. and Martinou J. C. (2002) Mitochondria: regulating the inevitable. Biochimie 84, 105–111.

Rutkowski D. T. and Kaufman R. J. (2004) A trip to the ER: coping with stress. Trends Cell Biol. 14, 20–28.

Sai X., Kawamura Y., Kokame K. et al. (2002) Endoplasmic reticulum stress-inducible protein, Herp, enhances presenilin-mediated generation of amyloid beta-protein. J. Biol. Chem. 277, 12 915–12 920.

Sai X., Kokame K., Shiraishi H., Kawamura Y., Miyata T., Yanagisawa K. and Komano H. (2003) The ubiquitin-like domain of Herp is involved in Herp degradation, but not necessary for its enhancement of amyloid beta-protein generation. FEBS Lett. 553, 151–156.

Schroder M. and Kaufman R. J. (2005) The mammalian unfolded protein response. Annu. Rev. Biochem. 74, 739–789.

Selkoe D. J. (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 81, 741–766.

Shen X., Zhang K. and Kaufman R. J. (2004) The unfolded protein response-a stress signaling pathway of the endoplasmic reticulum. J. Chem. Neuroanat. 28, 79–92.

Simonian N. A. and Coyle J. T. (1996) Oxidative stress in neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol. 36, 83–106.

Travers K. J., Patil C. K., Wodicka L., Lockhart D. J., Weissman J. S. and Walter P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101, 249–258.

Vattemi G., Engel W. K., McFerrin J. and Askanas V. (2003) Cystatin C colocalizes with amyloid-beta and coimmunoprecipitates with amyloid-beta precursor protein in sporadic inclusion-body myositis muscles. J. Neurochem. 85, 1539–1546.

Vattemi G., Engel W. K., McFerrin J. and Askanas V. (2004) Endoplasmic reticulum stress and unfolded protein response in inclusion body myositis muscle. Am. J. Pathol. 164, 1–7.

Yamamoto K., Yoshida H., Kokame K., Kaufman R. J. and Mori K. (2004) Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. J. Biochem. (Tokyo) 136, 343–350.

Adiponectin Acts as an Endogenous Antithrombotic Factor

Hisashi Kato, Hirokazu Kashiwagi, Masamichi Shiraga, Seiji Tadokoro, Tsuyoshi Kamae, Hidetoshi Ujiie, Shigenori Honda, Shigeki Miyata, Yoshinobu Ijiri, Junichiro Yamamoto, Norikazu Maeda, Tohru Funahashi, Yoshiyuki Kurata, Iichiro Shimomura, Yoshiaki Tomiyama, Yuzuru Kanakura

Objective—Obesity is a common risk factor in insulin resistance and cardiovascular diseases. Although hypoadiponectinemia is associated with obesity-related metabolic and vascular diseases, the role of adiponectin in thrombosis remains elusive.

Methods and Results—We investigated platelet thrombus formation in adiponectin knockout (APN-KO) male mice (8 to 12 weeks old) fed on a normal diet. There was no significant difference in platelet counts or coagulation parameters between wild-type (WT) and APN-KO mice. However, APN-KO mice showed an accelerated thrombus formation on carotid arterial injury with a He-Ne laser (total thrombus volume: $13.36\pm4.25\times10^7$ arbitrary units for APN-KO and $6.74\pm2.87\times10^7$ arbitrary units for WT; n=10; $P<0.01$). Adenovirus-mediated supplementation of adiponectin attenuated the enhanced thrombus formation. In vitro thrombus formation on a type I collagen at a shear rate of 250 s^{-1}, as well as platelet aggregation induced by low concentrations of agonists, was enhanced in APN-KO mice, and recombinant adiponectin inhibited the enhanced platelet aggregation. In WT mice, adenovirus-mediated overexpression of adiponectin additionally attenuated thrombus formation.

Conclusion—Adiponectin deficiency leads to enhanced thrombus formation and platelet aggregation. The present study reveals a new role of adiponectin as an endogenous antithrombotic factor. (***Arterioscler Thromb Vasc Biol.* 2006;26:224-230.**)

Key Words: acute coronary syndromes ■ obesity ■ platelets ■ thrombosis

Obesity is associated with insulin resistance, accelerated atherothrombosis, and cardiovascular diseases.[1,2] Recent studies have revealed that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules, known as adipocytokines, including tumor necrosis factor (TNF) α, leptin, resistin, and plasminogen activator inhibitor type-1.[2–4] Dysregulated production of adipocytokines participates in the development of obesity-related metabolic and vascular diseases.[2–4]

Adiponectin is an adipocytokine identified in the human adipose tissue cDNA library, and Acrp30/AdipoQ is the mouse counterpart of adiponectin (reviewed in reference[5]). Adiponectin, of which mRNA is exclusively expressed in adipose tissue, is a protein of 244 amino acids consisting of 2 structurally distinct domains, an N-terminal collagen-like domain and a C-terminal complement C1q-like globular domain. Adiponectin is abundantly present in plasma (5 to 30 μg/mL), and its plasma concentration is inversely related to the body mass index.[5] Plasma adiponectin levels decrease in obesity, type 2 diabetes, and patients with coronary artery disease (CAD).[5–9] Indeed, adiponectin (APN) knockout (KO) mice showed severe diet–induced insulin resistance.[10] In cultured cells, we have demonstrated that human recombinant adiponectin inhibited the expression of adhesion molecules on endothelial cells, the transformation of macrophages to foam cells, and TNF-α production from macrophages.[5,11] Furthermore, APN-KO mice showed severe neointimal thickening in mechanically injured arteries.[12] Adenovirus-mediated supplementation of adiponectin attenuated the development of atherosclerosis in apolipoprotein E–deficient mice as well as postinjury neointimal thickening in APN-KO mice.[12,13] These data suggest the antiatherogenic properties of adiponectin, and, hence, hypoadiponectinemia may be associated with a higher incidence of vascular diseases in obese subjects. Although it is also possible that an altered hemostatic balance may contribute to the pathogenesis of acute cardiovascular events in such patients, the roles of adiponectin in hemostasis and thrombosis remains elusive.

Original received August 4, 2005; final version accepted October 24, 2005.

From the Departments of Hematology and Oncology (H. Kato, H. Kashiwagi, M.S., S.T., T.K., H.U., Y.T., Y.Ka.) and Internal Medicine and Molecular Science (N.M., T.F., I.S.), Graduate School of Medicine, Osaka University, Suita; National Cardiovascular Center Research Institute (S.H.), Suita, Osaka; Division of Transfusion Medicine (S.M.), National Cardiovascular Center, Suita, Osaka; Department of Nutrition Management (Y.I.), Faculty of Health Science, Hyogo University, Kakogawa, Hyogo; Laboratory of Physiology, Faculty of Nutrition (J.Y.) and High Technology Research Centre (J.Y.), Kobe Gakuin University, Kobe; and Department of Blood Transfusion (Y.Ku.), Osaka University Hospital, Suita, Japan.

Correspondence to Yoshiaki Tomiyama, Osaka University, Department of Hematology and Oncology, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail yoshi@hp-blood.med.osaka-u.ac.jp

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000194076.84568.81

Here we have provided the first evidence that adiponectin affects thrombus formation, and, hence, hypoadiponectinemia may directly contribute to acute coronary syndrome. Our data indicate a new role of adiponectin as an antithrombotic factor.

Methods

Mice

APN-KO male mice (8 to 12 weeks old) were generated as described previously.[10,12] We analyzed mice backcrossed to C57BL/6 for 5 generations.[10,12]

Preparation of Mouse Platelets and Measurement of Coagulation Parameters

Mouse platelet-rich plasma (PRP) was obtained as described previously.[14] Coagulation parameters were measured by SRL Inc.

Platelet Aggregation Study, Adhesion Study, and Flow Cytometry

Platelet aggregation and platelet adhesion study was performed as described previously.[14] Integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion of wild-type (WT) and APN-KO platelets were detected by phycoerythrin-conjugated JON/A monoclonal antibody (mAb), which binds specifically to mouse-activated $\alpha_{IIb}\beta_3$ (Emfret Analytics) and FITC-conjugated anti-P-selectin mAb (Becton Dickinson), respectively.[14]

Assessment of Atherosclerosis and Bleeding Time Measurement

Assessment of atherosclerosis was performed as described previously.[15] The tail of anesthetized mice (nembutal 65 mg/kg; 8 to 12 weeks old) was transected 5 mm from the tip and then immersed in 0.9% isotonic saline at 37°C. The point until complete cessation of bleeding was defined as the bleeding time.

He-Ne Laser–Induced Thrombosis

The observation of real-time thrombus formation in the mouse carotid artery was performed as described previously.[15] Anesthetized mice (nembutal 65 mg/kg) were placed onto a microscope stage, and the left carotid artery (450 to 500 μm in diameter) was gently exposed. Evans blue dye (20 mg/kg) was injected into the left femoral artery via an indwelt tube, and then the center of the exposed carotid artery was irradiated with a laser beam (200 μm in diameter at the focal plane) from a He-Ne laser (Model NEO-50MS; Nihon Kagaku Engineering Co, Ltd). Thrombus formation was recorded on a videotape through a microscope with an attached CCD camera for 10 minutes. The images were transferred to a computer every 4 s, and the thrombus size was analyzed using Image-J software (National Institutes of Health). We calculated thrombus size by multiplying each area value and its grayscale value together. We then regarded the total size values for an individual thrombus obtained every 4 s during a 10-minute observation period as the total thrombus volume and expressed them in arbitrary units.

Flow Chamber and Perfusion Studies

The real-time observation of mural thrombogenesis on a type I collagen-coated surface under a shear rate of 250 s^{-1} was performed as described previously.[16] Briefly, whole blood obtained from anesthetized mice was anticoagulated with argatroban, and then platelets in the whole blood were labeled by mepacrine. Type I collagen-coated glass cover slips were placed in a parallel plate flow chamber (rectangular type; flow path of 1.9-mm width, 31-mm length, and 0.1-mm height). The chamber was assembled and mounted on an epifluorescence microscope (Axiovert S100 inverted microscope, Carl Zeiss Inc) with the computer-controlled z-motor (Ludl Electronic Products Lts). Whole blood was aspirated through the chamber, and the entire platelet thrombus formation process was observed in real time and recorded with a video recorder.

Preparation of Adenovirus and Recombinant Adiponectin

Adenovirus producing the full-length mouse adiponectin was prepared as described previously.[10] Plaque-forming units (1×10^8) of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-βgal) were injected into the tail vein. Experiments were performed on the fifth day after viral injection. The plasma concentrations of adiponectin were measured by a sandwich ELISA. Mouse and human recombinant proteins of adiponectin were prepared as described previously.[11,17]

RT-PCR

Total cellular RNA of platelets from WT or APN-KO mice was obtained, and contaminated genomic DNA was removed using a QuantiTect Reverse-Transcription kit (QIAGEN). One microgram of total RNA was used as a template for RT-PCR as described previously.[18] For the amplification of transcripts of mouse adiponectin receptors AdipoR1 and AdipoR2, the following primers were used: mouse AdipoR1 5′-ACGTTGGAGAGTCATCCCGTAT-3′ (sense) and 5′-CTCTGTGTGGATGCGGAAGAT-3′ (antisense) and mouse AdipoR2 5′-TGCGCACACATTTCAGTCTCCT-3′ (sense) and 5′-TTCTATGATCCCCAAAAGTGTGC-3′ (antisense).[19,20] For human platelet isolation, PRP obtained from 50 mL of whole blood was passed through a leukocyte removal filter as described previously.[21] This procedure removed >99.9% of the contaminated leukocytes.[21] For human AdipoR1 and AdipoR2, the following primers were used: human AdipoR1 5′-CTTCTACTGCTCCCCACAGC-3′ (sense) and 5′-GACAAAGCCCTCAGCGATAG-3′ (antisense) human AdipoR2 5′-GGACCGAGCAAAAGACTCAG-3′ (sense) and 5′-CACCCAGAGGCTGCTACTTC-3′ (antisense). In addition, total cellular RNA obtained from a megakaryocytic cell line, CMK, and that from a human monocytic cell line, THP-1 (positive control)[22] was examined in parallel. RT-PCR samples omitting reverse transcriptase were used as negative controls.

Statistical Analysis

Results were expressed as mean±SD. Differences between groups were examined for statistical significance using Student t test.

Results

Characteristics of Adiponectin-Deficient Mice and Assessment of Atherosclerotic Lesions

The basal profiles of APN-KO male mice have been previously described.[10,12] To exclude the effects of diet on APN-KO mice, we used APN-KO male mice (8 to 12 weeks old) fed on a normal diet in this study. There were no differences in platelet counts, PT, APTT, and plasma fibrinogen concentrations (Table I, available online at http://atvb.ahajournals.org). Histological analyses revealed that neither Oil Red O staining of the inner surface of whole aorta nor elastin-van Gieson staining of transverse sections of carotid arteries showed any apparent atherosclerotic lesions in WT or APN-KO mice (data not shown).

Bleeding Time in APN-KO Mice

To examine the effects of adiponectin deficiency on thrombosis and hemostasis, we studied bleeding time in APN-KO mice. The bleeding time in APN-KO mice was slightly but significantly shorter (96.9±34.9 s; n=30; $P<0.05$) than that in WT mice (130.9±52.1 s; n=30).

Enhanced Thrombus Formation in APN-KO Mice and Adiponectin Adenovirus Ameliorates the Thrombogenic Tendency

We next examined the effect of adiponectin deficiency on thrombus formation using the He-Ne laser-induced carotid

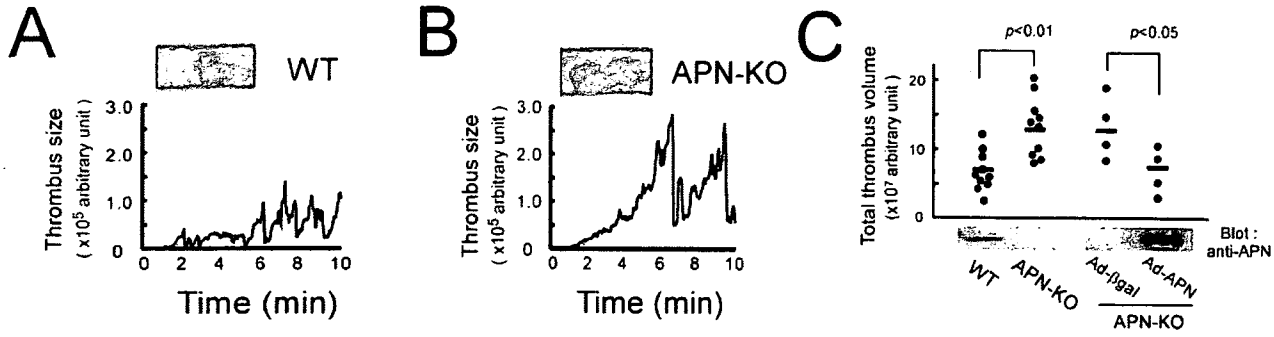

Figure 1. He-Ne laser-induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) WT or (B) APN-KO mice is shown. (C) The total thrombus volume was significantly larger in APN-KO mice (n=10; $P<0.01$). In another set of experiments, administration of adenovirus-producing mouse adiponectin (Ad-APN) significantly attenuated the total thrombus volume, as compared with control adenovirus (Ad-βgal)-infected APN-KO mice (n=4; $P<0.05$). Plasma adiponectin levels detected in immunoblots are shown in the lower panel.

artery thrombus model. Endothelial injury of the carotid artery was induced by the interaction of Evans blue dye with irradiation from the He-Ne laser. In WT mice, thrombus formation started 61.0±25.0 s after the initiation of He-Ne laser irradiation (n=10). When the thrombi reached a certain size, they frequently ruptured and detached themselves from the wall because of increased shear stress. Thus, thrombus formation in this in vivo model showed a cyclic fluctuation, and complete occlusion was not observed (Figure 1). During a 10-minute observation period, the cycles of thrombus formation were 8.5±2.3 in WT mice. In APN-KO mice, there was no significant difference in the initiation time for thrombus formation (54.8±8.9 s; n=10; $P=0.46$). However, the cycles of thrombus formation during the 10-minute observation period were significantly fewer (5.4±2.0; n=10; $P<0.01$) in APN-KO mice. The thrombi in APN-KO mice grew larger and appeared to be stable and more resistant to the increased shear stress. Accordingly, the total thrombus volume was significantly larger in APN-KO mice ($6.74\pm2.87\times10^7$ arbitrary units in WT mice and $13.36\pm4.25\times10^7$ arbitrary units in APN-KO mice; n=10; $P<0.01$).

To confirm that adiponectin deficiency is responsible for the enhanced thrombus formation in APN-KO mice, we injected Ad-βgal or Ad-APN into APN-KO mice. On the fifth day after adenoviral injection, we confirmed the elevated plasma adiponectin level in Ad-APN-infected APN-KO mice in an ELISA assay (48.7±6.8 μg/mL; n=4), as well as in an immunoblot assay. In the carotid artery thrombus model, the total thrombus volume in Ad-βgal-infected APN-KO was $12.94\pm4.67\times10^7$ arbitrary units, which was compatible with that of APN-KO mice shown in Figure 1. In contrast, Ad-APN infection significantly decreased the total thrombus volume in APN-KO mice ($6.23\pm3.09\times10^7$ arbitrary units; n=4; $P<0.05$). These results indicate that adiponectin deficiency is responsible for the thrombogenic tendency in vivo.

Platelet-Thrombus Formation on Immobilized Collagen Under Flow Conditions

Because endothelial function may affect in vivo thrombus formation, we next performed in vitro mural thrombus formation on a type I collagen-coated surface under flow conditions. Figure 2 shows thrombus formation during a

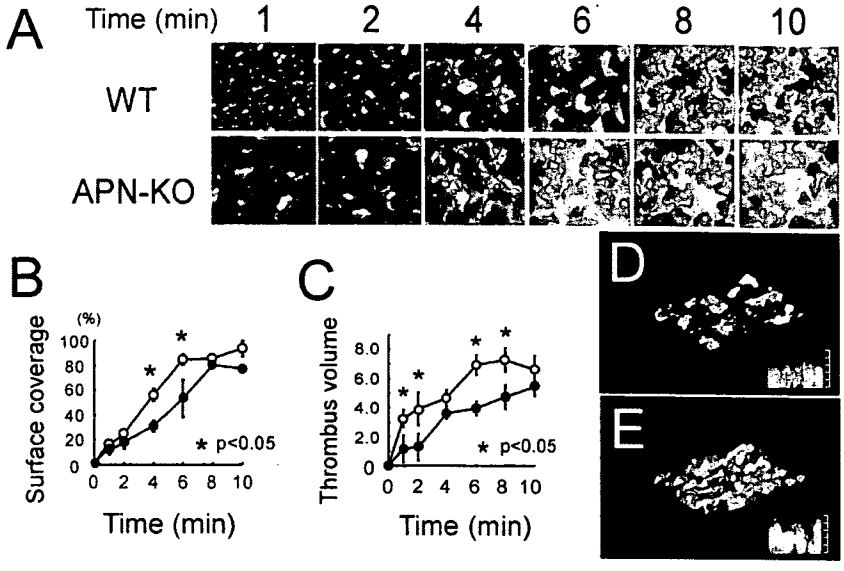

Figure 2. Thrombogenesis on a type I collagen-coated surface under flow conditions. (A) Mepacrine-labeled whole blood obtained from WT (top) or APN-KO mice (bottom) was perfused on a type I collagen-coated surface at a shear rate of 250 s^{-1}. (B) Platelet surface coverage (%) and (C) thrombus volume are shown at indicated time points. (●, WT; ○, APN-KO; *$P<0.05$). Shown are representative 3D images of thrombus formation at 6-minute perfusion in whole blood obtained from (D) WT and (E) APN-KO mice. Each inserted figure shows thrombus height.

10-minute perfusion of mouse whole blood anticoagulated with thrombin inhibitor at a low shear rate (250 s^{-1}). In whole blood obtained from WT mice, the thrombus fully covered the collagen-coated surface after 8 to 10 minutes of perfusion. In contrast, the thrombus grew more rapidly and fully covered the surface at 6 minutes in APN-KO mice. At 1 and 2 minutes of perfusion, there was no apparent difference in the initial platelet adhesion to the collagen surface between WT and APN-KO mice, whereas the platelet aggregate formation was significantly enhanced in APN-KO, even at 1 minute. We additionally examined the possibility that adiponectin might inhibit platelet adhesion onto collagen, because adiponectin binds to collagen types I, III, and V.[23] However, mouse recombinant adiponectin (40 μg/mL) did not inhibit the adhesion of platelets onto collagen, indicating that the inhibitory effect of adiponectin is not mediated by the inhibition of platelet binding to collagen (data not shown). At a high shear rate (1000 s^{-1}), the thrombus grew rapidly and fully covered the surface within 3 to 4 minutes. Under such strong stimuli, we did not detect any difference in thrombus formation between WT and APN-KO mice, probably because of the full activation of platelets.

Adiponectin Inhibits the Enhanced Platelet Aggregation in APN-KO Mice

In platelet aggregation studies, PRP obtained from APN-KO mice showed significantly enhanced platelet aggregation in response to low doses of agonists (ADP 2.5 μmol/L, collagen 2.5 μg/mL, and protease-activated receptor 4–activating peptide [PAR4-TRAP] 75 μmol/L), as compared with WT mice (Figure 3). The maximal platelet aggregation was achieved at higher concentrations of agonists, and the enhanced platelet aggregation in APN-KO mice was not apparent at these high doses of agonists, probably because of the full activation of platelets.

To confirm the inhibitory effect of adiponectin on platelet aggregation in vitro, we mixed 1 volume of PRP obtained from APN-KO mice with 4 volumes of platelet-poor plasma (PPP) obtained from APN-KO mice injected with either Ad-βgal or Ad-APN to adjust platelet counts to $300\times10^3/\mu L$. As shown in Figure 4A, the in vitro supplementation of PPP containing adiponectin attenuated the enhanced platelet aggregation. Similarly, in vitro administration of mouse recombinant adiponectin (40 μg/mL) to PRP from APN-KO mice attenuated the enhanced platelet aggregation (Figure 4B).

Expression of Adiponectin Receptors in Platelets and Effects of Adiponectin Deficiency on $\alpha_{IIb}\beta_3$ Activation and P-Selectin Expression

To reveal the effect of adiponectin on platelets, we examined whether platelets possess transcripts for adiponectin receptors AdipoR1 and AdipoR2 by using RT-PCR. As shown in Figure 5A, platelets from APN-KO, as well as WT mice, contained mRNAs for AdipoR1 and AdipoR2. We also confirmed that the human megakaryocytic cell line CMK, as well as carefully isolated human platelets, possessed mRNAs for AdipoR1 and AdipoR2. We next examined the effects of adiponectin deficiency on $\alpha_{IIb}\beta_3$ activation and α-granule secretion at various concentrations of agonists by flow cytometry. However, neither the platelet $\alpha_{IIb}\beta_3$ activation induced by ADP nor P-selectin expression induced by PAR4-TRAP showed significant difference between WT and APN-KO mice (n=4; Figure 5B and 5C).

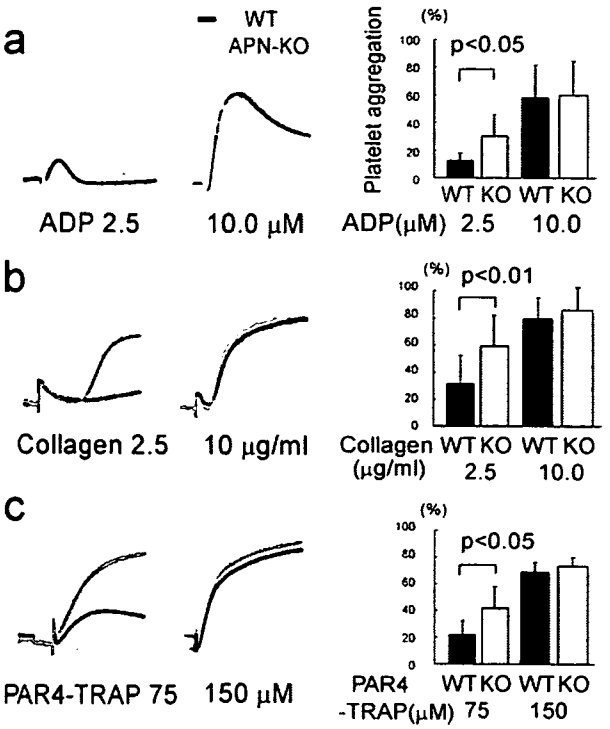

Figure 3. Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ($300\times10^3/\mu L$) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a; n=4), collagen (b; n=4), or PAR4-TRAP (c; n=3). As compared with WT mice, platelet aggregation was enhanced in APN-KO mice at low concentrations of agonists.

Adiponectin Adenovirus Attenuates Thrombus Formation in WT Mice

Because WT mice have large amounts of adiponectin in their plasma, we, therefore, examined whether adiponectin overexpression could additionally inhibit thrombus formation, as well as platelet function, in WT mice. After the administration of Ad-APN or Ad-βgal into WT mice, the plasma adiponectin levels in Ad-APN-infected mice reached ≈4 times higher than those in Ad-βgal-infected WT mice (8.5±0.6 μg/mL for Ad-βgal and 37.0±14.8 μg/mL for Ad-APN; n=5). As shown in Figure 6A, platelet aggregation in PRP induced by collagen or PAR4-TRAP was significantly attenuated by the overexpression of adiponectin. Similarly, in vitro administration of human recombinant adiponectin (40 μg/mL) to human PRP attenuated the platelet aggregation response to 2.5 μg/mL collagen (Figure 6B). Moreover, in the He-Ne laser–induced carotid artery thrombus model, the overexpression of adiponectin significantly inhibited thrombus formation in WT mice ($4.38\pm0.75\times10^7$ arbitrary units for Ad-βgal and $2.75\pm0.61\times10^7$ arbitrary units for Ad-APN; n=7; $P<0.05$; Figure 6C).

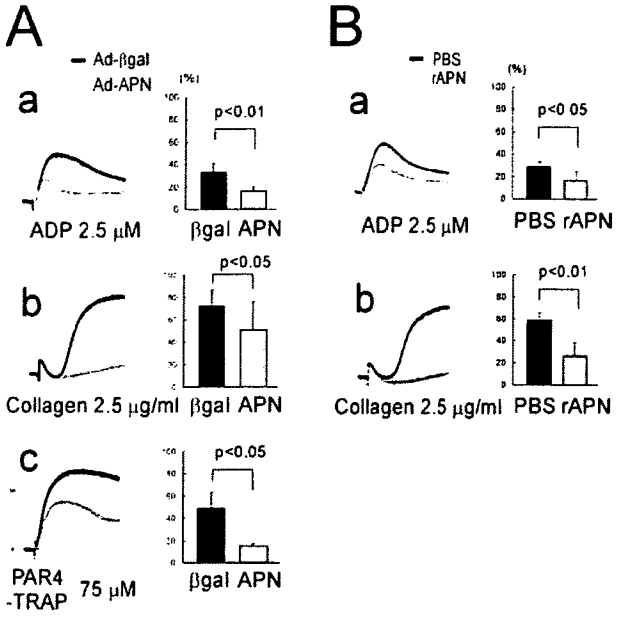

Figure 4. Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with ≈4 volumes of PPP from APN-KO mice injected with Ad-βgal (black line) or Ad-APN (gray line) to obtain a platelet concentration of $300\times10^3/\mu L$. Platelets were stimulated with indicated agonists (n=4). (B) Mouse recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP from APN-KO mice. Platelets were adjusted to 300×10^3 platelets/μL and stimulated with indicated agonists (n=4).

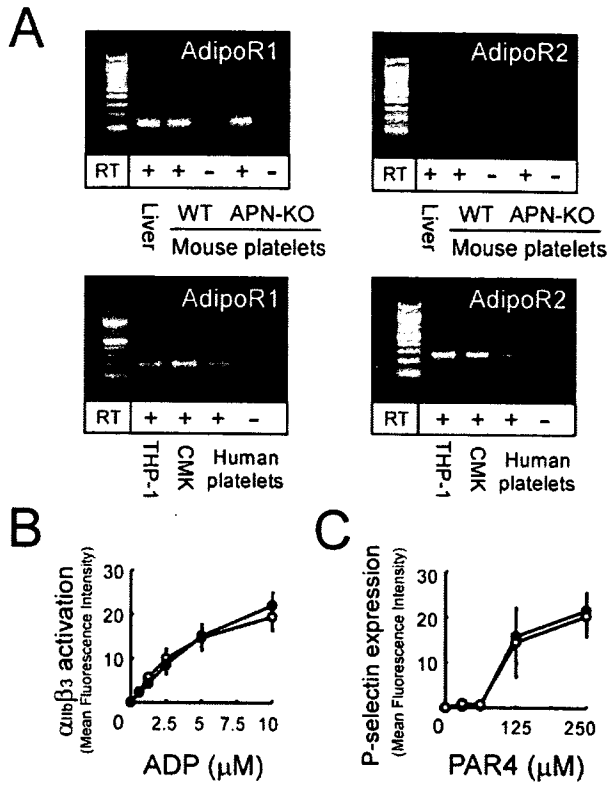

Figure 5. Expression of adiponectin receptors and effects of adiponectin deficiency on platelet function. (A, top) Expressions of transcripts for adiponectin receptors, AdipoR1 (133-bp fragments) and AdipoR2 (156-bp fragments), in platelets from WT or APN-KO mice were examined by RT-PCR. The liver was used as a positive control. (Bottom) Expressions of transcripts for adiponectin receptors, AdipoR1 (196-bp fragments) and AdipoR2 (243-bp fragments), in CMK cells, as well as human platelets, were examined by RT-PCR; 100-bp DNA Ladder (New England Biolabs) was used as a marker. Effects of adiponectin deficiency on (B) $\alpha_{IIb}\beta_3$ activation and (C) α-granule secretion. PRP obtained from WT (●) or APN-KO (○) mice in the presence of phycoerythrin-JON/A mAb or FITC-anti-P-selectin mAb was stimulated with the indicated agonist and then analyzed by flow cytometry without any washing. There were no significant differences in platelet $\alpha_{IIb}\beta_3$ activation or P-selectin expression between WT and APN-KO mice (n=4).

Discussion

In the present study, we have newly revealed an antithrombotic effect of adiponectin. APN-KO male mice (8 to 12 weeks old) fed on a normal diet showed no significant differences in platelet counts and coagulation parameters compared with WT mice. In the He-Ne laser–induced carotid artery thrombus model, APN-KO mice showed an accelerated thrombus formation, and adenovirus-mediated supplementation of adiponectin attenuated this enhanced thrombus formation. Platelet aggregometry and the real-time observation of in vitro thrombus formation on a type I collagen-coated surface under flow conditions showed the enhanced platelet function in APN-KO mice. Moreover, adenovirus-mediated overexpression of adiponectin attenuated in vivo thrombus formation, as well as the in vitro platelet aggregation response, even in WT mice. Thus, the present data strongly suggest that adiponectin possesses an antithrombotic potency.

We have demonstrated that low concentrations of adiponectin are associated with the prevalence of CAD in men, which is independent of well-known CAD risk factors.[8] Pischon et al[9] have recently shown that high concentrations of adiponectin are associated with a lower risk of myocardial infarction in men, which is also independent of inflammation and glycemic status and can be only partly explained by differences in blood lipids. These clinical studies suggest that the protective effect of adiponectin on the development of CAD may be primary rather than secondary through the protection of metabolic abnormalities, such as insulin resistance. Indeed, APN-KO mice fed on a normal diet did not show any abnormalities in plasma glucose, insulin, or lipid profiles.[10,12] Although the atherosclerotic and thrombotic processes are distinct from each other, these processes appear to be interdependent, as shown by the term *atherothrombosis*. The interaction between the vulnerable atherosclerotic plaque, which is prone to disruption, and thrombus formation is the cornerstone of acute coronary syndrome (ACS).[24] In this context, our present data strongly suggest that adiponectin deficiency (or hypoadiponectinemia) may directly contribute to the development of ACS by enhanced platelet thrombus formation.

Although APN-KO fed on a normal diet showed no significant differences in major metabolic parameters, they showed delayed clearance of FFA in plasma, elevated plasma TNF-α concentrations (≈40 pg/mL in APN-KO; ≈20 pg/mL in WT), and elevated CRP mRNA levels in white adipose tissue.[12,25] In addition, recombinant adiponectin increased NO production in vascular endothelial cells.[26] To rule out any

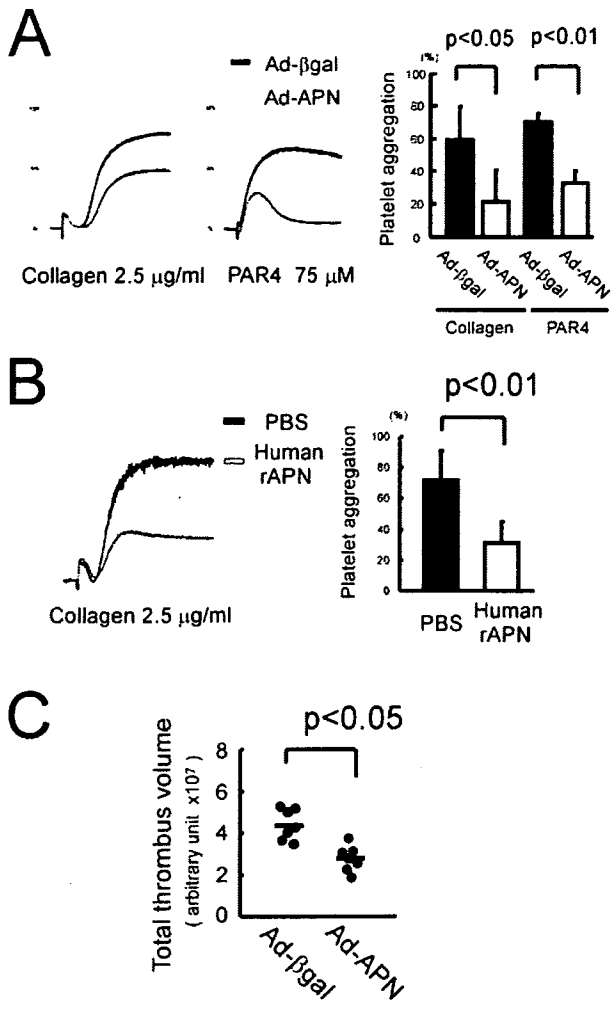

Figure 6. Overexpression of adiponectin additionally attenuates thrombus formation in WT mice. (A) Platelet aggregation in PRP obtained from WT mice injected with either Ad-βgal or Ad-APN. PRP ($300\times10^3/\mu L$) obtained from WT mice injected with either Ad-βgal (black line) or Ad-APN (gray line) was stimulated with collagen or PAR4-TRAP (n=4). Administration of Ad-APN significantly attenuated platelet aggregation in WT mice. (B) Human recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP ($300\times10^3/\mu L$) from control subjects. Platelets were stimulated with collagen (n=7). (C) He-Ne laser–induced thrombus formation in WT mice injected with either Ad-βgal or Ad-APN. Administration of Ad-APN in WT mice additionally reduced the total thrombus volume in the carotid artery thrombus model (n=7, $P<0.05$).

effect of adiponectin on vascular cells, we examined in vitro thrombus formation on a type I collagen-coated surface under flow conditions, as well as platelet aggregation in APN-KO mice. Thus, the enhanced platelet function in APN-KO mice was still evident even in the absence of vascular cells. Moreover, human and mouse recombinant adiponectin attenuated the aggregation response obtained from control human subjects and from APN-KO mice, respectively. Thus, adiponectin inhibits platelet function. However, the mechanism by which adiponectin attenuates platelet aggregation and arterial thrombus formation in vivo remains unclear. During thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial matrices, such as collagen, and then become activated and aggregate to each other.[16] The thrombus formed in APN-KO mice appeared to be stable and more resistant to the increased shear stress, without affecting the initiation time for thrombus formation in carotid artery injury experiments, as well as in flow chamber perfusion experiments. In addition, preincubation of collagen with recombinant adiponectin did not inhibit platelet adhesion on collagen under static conditions. Thus, it is unlikely that the inhibitory effect of adiponectin is mediated by the inhibition of platelet binding to collagen. These characteristics are quite distinct from C1q-TNF–related protein-1, which belongs to the same family as adiponectin and inhibits thrombus formation by interfering with platelet–collagen interaction.[27] We confirmed that transcripts for AdipoR1 and AdipoR2 were present in mouse and human platelets and CMK cells. Although the platelet–platelet interaction appeared to be enhanced in APN-KO mice, we did not detect any difference in agonist-induced $\alpha_{IIb}\beta_3$ activation or P-selectin expression between APN-KO and WT mice by flow cytometry. Based on these results, it is possible that adiponectin may inhibit $\alpha_{IIb}\beta_3$-mediated intracellular postligand binding events. Alternatively, previous studies have shown that adiponectin is physically associated with many proteins, including α_2-macroglobulin, thrombospondin-1 (TSP-1), and several growth factors.[5,23,28] Interestingly, TSP-1, after secretion from platelet α granules, may participate in platelet aggregation by reinforcing interplatelet interactions through direct fibrinogen-TSP-fibrinogen and TSP-TSP crossbridges.[29,30] In this context, it is also possible that it may interfere with interplatelet interactions in platelet aggregation. Additional studies to clarify the mechanism of adiponectin are currently under way.

In conclusion, our present study revealed that adiponectin acts as an endogenous antithrombotic factor. Although it is possible that the in vivo antithrombotic effect of adiponectin may be partly mediated by its action on vascular cells, our present data clearly indicate that adiponectin affects platelet function in the absence of vascular cells. In addition, the overexpression of adiponectin in WT mice attenuates in vivo thrombus formation, as well as the in vitro platelet aggregation response. Our data provide a new insight into the pathophysiology of ACS in nonobese, as well as obese, subjects, and adiponectin (and its derivatives) may be a new candidate for an antithrombotic drug.

Acknowledgments

This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan; from the Ministry of Health, Labor, and Walfare in Japan, Astellas Foundation for Research on Metabolic Disorder, Tokyo, Japan; and Mitsubishi Pharma Research Foundation, Osaka, Japan.

References

1. Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. *Cell.* 2001;104:531–543.
2. Friedman JM. Obesity in the new millennium. *Nature.* 2000;404:632–634.
3. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science.* 1993;259:87–91.

4. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature*. 2001;409:307–312.
5. Matsuzawa Y, Funahashi T, Kihara S, Shimomura I. Adiponectin and metabolic syndrome. *Arterioscler Thromb Vasc Biol*. 2004;24:29–33.
6. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol*. 2000;20:1595–1599.
7. Zoccali C, Mallamaci F, Tripepi G. Novel cardiovascular risk factors in end-stage renal disease. *J Am Soc Nephrol*. 2002;13:134–141.
8. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol*. 2003;23:85–89.
9. Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rhimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA*. 2004;291:1730–1737.
10. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med*. 2002;8:731–737.
11. Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y, Matsuzawa Y. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*. 2000;96:1723–1732.
12. Matsuda M, Shimomura I, Sata M, Arita Y, Nishida M, Maeda N, Kumada M, Okamoto Y, Nagaretani H, Nishizawa H, Kishida K, Komuro R, Ouchi N, Kihara S, Nagai R, Funahashi T. Role of adiponectin in preventing vascular stenosis. *J Biol Chem*. 2002;277:37487–37491.
13. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2002;106:2767–2770.
14. Kato H, Honda S, Yoshida H, Kashiwagi H, Shiraga M, Honma N, Kurata Y, Tomiyama Y. SHPS-1 negatively regulates integrin $\alpha_{IIb}\beta_3$ function through CD47 without disturbing FAK phosphorylation. *J Thromb Haemost*. 2005;3:763–774.
15. Sano T, Oda E, Yamashita T, Shiramasa H, Ijiri Y, Yamashita T, Yamamoto J. Antithrombotic and anti-atherogenic effects of partially defatted flaxseed meal using a laser-induced thrombosis test in apolipoprotein E and low-density lipoprotein receptor deficient mice. *Blood Coagul Fibrinolysis*. 2003;14:707–712.
16. Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A. Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. *Blood*. 1999;94:968–975.
17. Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K. Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem*. 2004;279:1304–1309.
18. Tomiyama Y, Kashiwagi H, Kosugi S, Shiraga M, Kanayama Y, Kurata Y, Matsuzawa Y. Abnormal processing of the glycoprotein IIb transcript due to a nonsense mutation in exon 17 associated with Glanzmann's thrombasthenia. *Thromb Haemost*. 1995;73:756–762.
19. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomiza T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature*. 2003;423:762–769.
20. Bluher M, Fasshauer M, Kralisch S, Schon MR, Krohn K, Paschke R. Regulation of adiponectin receptor R1 and R2 gene expression in adipocytes of C57BL/6 mice. *Biochem Biophys Res Commun*. 2005;329:1127–1132.
21. Kashiwagi H, Honda S, Tomiyama Y, Mizutani H, Take H, Honda Y, Kosugi S, Kanayama Y, Kurata Y, Matsuzawa Y. A novel polymorphism in glycoprotein IV (replacement of proline-90 by serine) predominates in subjects with platelet GPIV deficiency. *Thromb Haemost*. 1993;69:481–484.
22. Chinetti G, Zawadski C, Fruchart JC, Staels B. Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARα, PPARγ, and LXR. *Biochem Biophys Res Commun*. 2004;314:151–158.
23. Okamoto Y, Arita Y, Nishida M, Muraguchi M, Ouchi N, Takahashi M, Igura T, Inui Y, Kihara S, Nakamura T, Yamashita S, Miyagawa J, Funahashi T, Matsuzawa Y. An adipocyte-derived plasma protein, adiponectin, adheres to injured vascular walls. *Horm Metab Res*. 2000;32:47–50.
24. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes. *N Engl J Med*. 1992;326:242–250.
25. Ouchi N, Kihara S, Funahashi T, Nakamura T, Nishida M, Kumada M, Okamoto Y, Ohashi K, Nagaretani H, Kishida K, Nishizawa H, Maeda N, Kobayashi H, Hiraoka H, Matsuzawa Y. Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation*. 2003;107:671–674.
26. Chen H, Montagnant M, Funahashi T, Shimomura I, Quon MJ. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem*. 2003;278:45021–45026.
27. Meehan WP, Knitter GH, Lasser GW, Lewis K, Ulla MM, Bishop PD, Hanson SR, Fruebis J. C1q-TNF related protein-1 (CTRP1) prevents thrombus formation in non-human primates and atherosclerotic rabbits without causing bleeding. *Blood* 2002;100:23a.
28. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem*. 2005;280:18341–18347.
29. Leung LL. Role of thrombospondin in platelet aggregation. *J Clin Invest*. 1984;74:1764–1772.
30. Bonnefoy A, Hantgan R, Legrand C, Frojmovic MM. A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIbIIIa receptor. *J Biol Chem*. 2001;276:5605–5612.

Journal of Thrombosis and Haemostasis, 4: 2010–2013

ORIGINAL ARTICLE

Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers

R. KIMURA,* T. SAKATA,† Y. KOKUBO,‡ A. OKAMOTO,† A. OKAYAMA,‡ H. TOMOIKE‡ and T. MIYATA*

**Research Institute; †Laboratory of Clinical Chemistry; and ‡Department of Preventive Cardiology, National Cardiovascular Center, Suita, Osaka, Japan*

To cite this article: Kimura R, Sakata T, Kokubo Y, Okamoto A, Okayama A, Tomoike H, Miyata T. Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers. *J Thromb Haemost* 2006; 4: 2010–13.

See also Okada H, Yamazaki T, Takagi A, Murate T, Yamamoto K, Takamatsu J, Matsushita T, Naoe T, Kunishima S, Hamaguchi M, Saito H, Kojima T. *In vitro* characterization of missense mutations associated with quantitative protein S deficiency. This issue, pp 2003–9.

Summary. *Background:* Protein S (PS) is an anticoagulant protein that functions as a cofactor for activated protein C (APC), and congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT). Recently, we and others identified the K196E missense mutation in the second epidermal growth factor-like domain of PS as a genetic risk factor for DVT in the Japanese population. The incidence of this mutation is high in the Japanese population. *Objectives:* In the present study, we investigated the relationship between plasma PS activity and the presence of the K196E mutation. *Patients and methods:* We measured PS activity as a cofactor activity for APC in 1862 Japanese individuals and determined the PS K196E genotype in this population. *Results:* Individuals heterozygous for the mutant E-allele had lower plasma PS activity than wildtype subjects (mean ± SD, 71.9 ± 17.6%, $n = 34$ vs. 87.9 ± 19.8%, $n = 1828$, $P < 0.0001$). However, the PS activity of several heterozygous individuals ($n = 8$) was greater than the population average. In contrast, multiple wildtype subjects ($n = 26$) had PS activity less than 2 SD below the population mean, indicating that other genetic or environmental factors affect PS activity. *Conclusions:* Plasma PS activity itself is not suitable for identifying PS 196E carriers and other methods are required for carrier detection.

Keywords: deep vein thrombosis, missense mutation, protein S.

Introduction

Protein S (PS) is an important regulator of coagulation that serves as a cofactor for activated protein C (APC), the anticoagulant protease that proteolytically degrades activated factor (F) V and FVIII [1]. Individuals with homozygous or compound heterozygous deficiency for PS develop disseminated thrombosis after birth, and heterozygosity for PS deficiency increases the risk of deep vein thrombosis (DVT) [2,3].

Recently, we and others identified that a PS missense mutation prevalent in the Japanese population, which causes Lys196 to be replaced by Glu (K196E mutation, formerly known as PS Tokushima, and referred to as K155E mutation), is a genetic risk factor for the development of DVT [4,5]. This mutation lies within the second epidermal growth factor-like domain of PS, and, *in vitro*, K196E mutant PS has decreased APC cofactor activity and poorly accelerates prothrombinase inactivation [6–8]. This missense mutation was originally identified in Japanese patients with PS deficiency suffering from DVT [9,10]. However, the plasma PS activity in individuals with this mutation remained controversial. In one report, PS activity was decreased in carriers of the K196E mutation with normal PS levels [9]. In contrast, another study found PS activity within the normal range in affected individuals [10].

We identified 66 heterozygotes and no homozygotes for the mutant PS 196E-allele from a population of 3651 individuals [5]. Therefore, the frequency of the mutant E-allele in the Japanese population was about 0.009. Extrapolating from these values, we estimated that approximately one out of every 55 Japanese individuals is heterozygous for the E-allele [11]. Thus, a substantial number of Japanese carry the E-allele for PS and are at increased risk for the development of DVT. Given the relatively high frequency of this mutation and its strong correlation with DVT, it may be advisable to screen individuals for the presence of this mutation so that carriers can avoid additional environmental risk factors associated with DVT. An appropriate screening test is lacking, however, and we hypothesized that plasma PS activity levels may directly correlate with PS genotype. If this were the case, genetic testing

Correspondence: Toshiyuki Miyata, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012 ext. 2512; fax: +81 6 6835 1176; e-mail: miyata@ri.ncvc.go.jp

Received 27 March 2006, accepted 1 June 2006

would not need to be undertaken to determine the PS genotype of a large population.

In this study, we examined the relationship between PS activity and the presence of the K196E mutation. The mean PS activity of individuals heterozygous for the K196E mutation was significantly less than that of wildtype individuals. However, there was substantial overlap in PS activity between these populations, and, thus, PS activity is not an appropriate method to differentiate K196E carriers from the general population.

Methods

We previously measured the PS activity in a population of Japanese individuals as part of the Suita Study, and we determined their genotype with respect to the PS K196E mutation [5,12]. The ability of PS to act as a cofactor for PC activation was measured on the basis of the activated partial thromboplastin time assay using Staclot PS (Diagnostica Stago, Asnières, France) [12]. The plasma levels of PS activity were expressed as percentages of the levels obtained from commercially available standard human plasma (Behringwerke, Marburg, Germany). The intra-assay coefficient of variation for PS activity was 6.9% (n = 10). The PS K196E genotype was determined by the TaqMan genotype discrimination method [5], using the primers 5′-ACCACTGTTCCTGTAAAAATGGTTT/5′-TGTGTTTTAATTCTACCATCCTGCT and the probes 5′-VIC-CAAATGAGAAAGATTGTAAAG-MGB (the mutant E-allele)/5′-FAM-CAAATAAGAAAGATTGTAAAG-MGB (the wild-type allele). The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. PS activity was measured in 2690 population individuals [12] and the genotype was determined in 3651 individuals [5]. The 1862 individuals with both known PS activity and genotype were used for analysis in this study. Plasminogen activity was previously measured using the chromogenic assay method with streptokinase as the activator and the specific substrate S-2251 (Chromogenix AB, Stockholm, Sweden) [13]. Plasminogen activity was determined in 4517 individuals [13], and the plasminogen A620T mutation genotype was determined in 3295 out of 4517 individuals by the TaqMan method using the primers 5′-TGTGGAGGCACCTTGATATCC/5′-TGTCAATTGTCCCCTAAACATACTTC and the probes 5′-VIC-TGTTGACTACTGCCCACT-MGB (the mutant T-allele)/5′-FAM- TGTTGACTGCTGCCCACT-MGB (the wild-type allele). Analysis of variance was used to compare mean values between groups by Student's t-test using JMP v 5.1 software (SAS Institute Inc., Cary, NC, USA).

Results

We measured the PS activity in 1862 individuals of known PS genotype, and we compared the activity of wildtype and heterozygous individuals. Within this population, 1828 subjects harbored the wildtype allele while 34 were heterozygous for the K196E mutation. No individuals were homozygous for the mutant E-allele. Within the total population, the mean ± SD PS activity was 87.6% ± 19.9%.

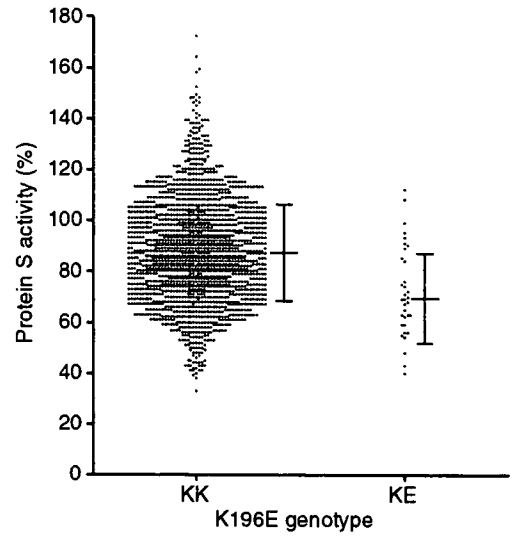

Fig. 1. Protein S (PS) activity in wild-type and K196E heterozygous individuals. Mean ± SD PS activity in heterozygous and wild-type individuals was 71.9% ± 17.6% (n = 34) and 87.9% ± 19.8% (n = 1828) (P < 0.0001), respectively.

Individuals heterozygous for the K196E mutation had reduced plasma PS activity compared to individuals with the KK genotype (mean ± SD, 71.9% ± 17.6%, n = 34 vs. 87.9% ± 19.8%, n = 1828, P < 0.0001) (Fig. 1). However, several heterozygous individuals with the mutant E-allele (n = 8) had measured PS activity greater than the total population average, while 26 wildtype subjects had PS activity at least 2SD less than the population mean (47.8%). Thus, PS activity does not appear to be a useful surrogate marker for PS genotype.

To determine whether an individual's genotype for any coagulation related protein could be determined by measuring the activity of the respective factor, we further examined the genotype and plasma activity of plasminogen in 3295 subjects. We identified 92 individuals heterozygous for the plasminogen A620T mutation, and the plasma plasminogen activity of these individuals was significantly less than wildtype individuals. Furthermore, there was little to no overlap between the measured plasminogen activities of wildtype and heterozygous individuals. Thus, the concept we originally wished to test was validated (Fig. 2).

There are well-documented gender- and age-related differences in PS activity [14], and this was true for our study population as reported [11] (Fig. 3A). When we examined the relationship between PS activity, genotype, and age, we observed decreased PS activity across all ages for individuals with the KE-genotype (Fig. 3B).

Discussion

DVT is a multi-factorial disease caused by the interaction of environmental and genetic factors. In Caucasian populations,

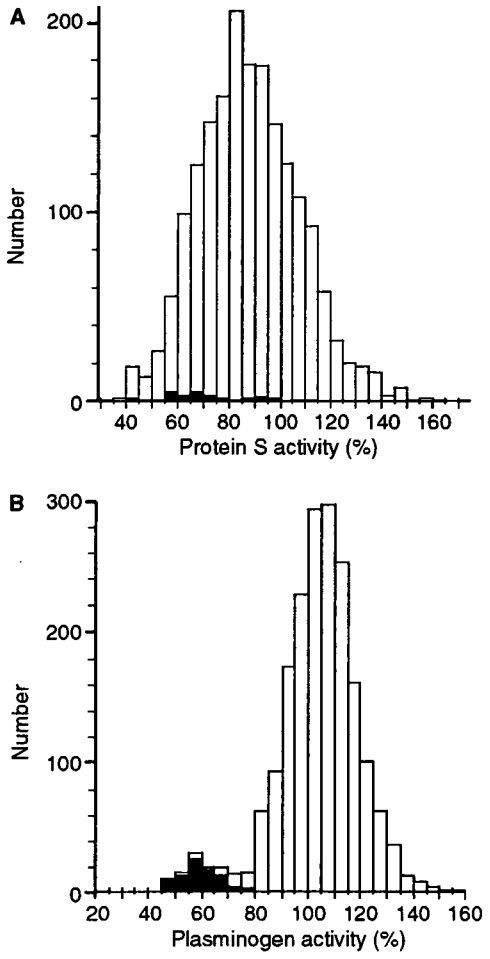

Fig. 2. Histogram representation of protein S (PS) (A) and plasminogen (B) activity in wildtype and heterozygous individuals. PS activity was measured in 1862 individuals, and plasminogen activity was measured in 3295 individuals. Activity was divided into groups by 5% increments, and mutation carriers are shown in closed bars.

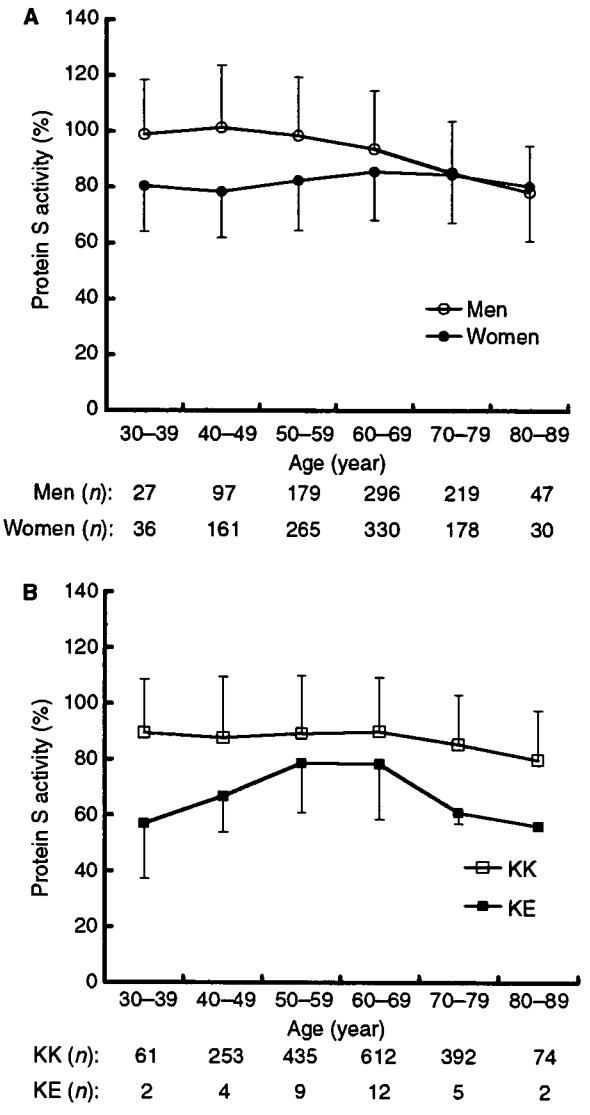

Fig. 3. Protein S (PS) activity divided in sex, age, and genotype. Open circles and closed circles in (A) show the mean PS activity in men and women, respectively. Open squares and closed squares in (B) show the mean PS activity in wild-type (KK-genotype) and heterozygote (KE-genotype). Error bars represent SD.

the FV Leiden (FVL) mutation, R506Q mutation in FV, is an important risk factor for the development of DVT. FVL carriers can be readily identified using the APC resistance test [15]. A FVL carrier will exhibit a prolonged clotting time in an activated thromboplastin time assay following the addition of APC. The incidence of this particular mutation varies in different ethnic populations [16,17] and is not observed in the Japanese [18]. In contrast, the PS K196E mutation present in the Japanese population is a genetic risk factor for DVT [4,5]. Therefore, a plasma assay for detecting PS 196E carriers should be developed. To understand the relation of the PS activity with the K196E mutation, we examined the PS activity and the K196E genotype in the Japanese population enrolled in the Suita Study.

The plasma PS activity in individuals with the PS K196E mutation remained controversial [6,9,10]. In one report, four members in a family who carried this mutation showed the PS activity with 37%, 72%, 101%, and 77%, respectively [10]. In a second family in this report, two members carried this mutation with the PS activity with 87% and 92%. On the basis of these results, the authors suggested this mutation as a phenotypically neutral polymorphism. In contrast, another study identified the same mutation correlated with low PS activity [6,9]. In this study, the authors identified this mutation in three patients with DVT. In addition, four individuals who did not show history of thrombosis were carriers of this mutation. All of these carriers showed low PS activity (mean ± SD, 43.1% ± 9.1%). Thus, so far, the relationship between the plasma PS activity and K196E mutation has not been settled. To address this issue, we have measured the PS activity and determined the genotype in the general Japanese population. As the results, we found that individuals heterozygous for the PS K196E mutation had reduced plasma PS activity compared to wildtype subjects, but this difference was relatively small and did not sufficiently differentiate between the two genotypes. In contrast, plasma

plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

Acknowledgements

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We would like to express our highest gratitude to Mr Y. Sakaguchi, the mayor of Suita city, and Dr S. Kitamura, President of the National Cardiovascular Center, for their support of the genome project. We would like to express our gratitude to Dr O. Hishikawa, Dr K. Kawanishi, Dr Y. Kotani, Mr T. Fujii, and Dr T. Mannami for their continuous support of our population survey in Suita city. We thank the members of the Satsuki-Junyukai.

Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

References

1 Dahlback B. Blood coagulation. *Lancet* 2000; **355**: 1627–32.
2 Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlback B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. *Thromb Haemost* 1996; **76**: 651–62.
3 Bucciarelli P, Rosendaal FR, Tripodi A, Mannucci PM, De Stefano V, Palareti G, Finazzi G, Baudo F, Quintavalla R. Risk of venous thromboembolism and clinical manifestations in carriers of antithrombin, protein C, protein S deficiency, or activated protein C resistance: a multicenter collaborative family study. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1026–33.
4 Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005; **38**: 908–15.
5 Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Nishigami K, Chiku M, Hayashi T, Kokubo Y, Okayama A, Tomoike H, Ikeda Y, Miyata T. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006; **107**: 1737–8.
6 Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. *Blood* 1994; **83**: 683–90.
7 Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S (Tokushima) (Lys155–>Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta* 1995; **1272**: 159–67.
8 Hayashi T, Nishioka J, Suzuki K. Characterization of dysfunctional protein S-Tokushima (K155–>E) in relation to the molecular interactions required for the regulation of blood coagulation. *Pol J Pharmacol* 1996; **48**: 221–3.
9 Shigekiyo T, Uno Y, Kawauchi S, Saito S, Hondo H, Nishioka J, Hayashi T, Suzuki K. Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. *Thromb Haemost* 1993; **70**: 244–6.
10 Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, Saito H. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. *Thromb Res* 1993; **70**: 395–403.
11 Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis in Japanese, importance of protein S K196E mutation. *Int J Hematol* 2006; **83**: 217–23.
12 Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost* 2004; **2**: 1012–3.
13 Okamoto A, Sakata T, Mannami T, Baba S, Katayama Y, Matsuo H, Yasaka M, Minematsu K, Tomoike H, Miyata T. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic diseases of plasminogen deficiency in the Japanese: the Suita Study. *J Thromb Haemost* 2003; **1**: 2397–403.
14 Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. *Thromb Haemost* 1995; **74**: 1271–5.
15 Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–8.
16 Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995; **346**: 1133–4.
17 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.
18 Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995; **74**: 1381–2.
19 Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 2004; **103**: 1192–201.
20 Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Munoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. *Arterioscler Thromb Vasc Biol* 2003; **23**: 508–11.
21 Garcia de Frutos P, Alim RI, Hardig Y, Zoller B, Dahlback B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 1994; **84**: 815–22.

International Journal of
HEMATOLOGY

Review Article

Genetic Risk Factors for Deep Vein Thrombosis among Japanese: Importance of Protein S K196E Mutation

Toshiyuki Miyata,[a] Rina Kimura,[a] Yoshihiro Kokubo,[b] Toshiyuki Sakata[c]

[a]Research Institute, [b]Department of Preventive Cardiology, and [c]Laboratory of Clinical Chemistry, National Cardiovascular Center, Suita, Osaka, Japan

Received December 16, 2005; received in revised form January 12, 2006; accepted January 17, 2006

Abstract

There is mounting evidence that mutations associated with a given disease arise with different frequencies among ethnic groups, thus ethnicity-specific studies are needed to identify causative mutations and properly assess risk. In particular, ethnic differences in the genetic background of thrombophilia have been reported. We recently conducted a large-scale analysis of the plasma activities of proteins C, S, antithrombin, and plasminogen within the Japanese general population. We found age- and sex-related differences and estimated the prevalence of deficiencies of protein C (0.13%), antithrombin (0.15%), protein S (1.12%), and plasminogen (4.29%). We also evaluated the genetic contribution to deep vein thrombosis and found that protein S mutation K196E is a genetic risk factor in the Japanese population. We estimated allele frequency to be 0.009, suggesting that 1 of 12,000 Japanese may be homozygous for the E allele, thus possibly as many as 10,000 individuals. Accordingly, a substantial proportion of the Japanese population carries the protein S E allele and is at risk of developing deep vein thrombosis. Given the frequency of this mutation and its strong correlation with deep vein thrombosis, it may be valuable to conduct a large-scale screening for this allele and advise concerned persons to avoid environmental risk factors known to be associated with deep vein thrombosis.

Int J Hematol. 2006;83:217-223. doi: 10.1532/IJH97.05187

Key words: Protein S; Deep vein thrombosis; Genetic risk; General population

1. Two Natural Anticoagulant Pathways: Protein C System and Protease Inhibitor System

Regulation of coagulation is achieved by a finely tuned balance between procoagulant and anticoagulant potencies. Generation of the multifunctional protease thrombin is a key event resulting from activation of the blood coagulation system. To regulate thrombin formation in plasma, 2 anticoagulant systems act in synergy. The first is known as the protein C anticoagulant pathway, the second as the heparan sulfate–dependent protease inhibitor system [1,2]. The protein C system controls 2 critical reactions: activation of factor X and activation of prothrombin. In this system, the thrombin-thrombomodulin complex activates protein C bound to its endothelial cell receptor, which is constitutively expressed. Resulting activated protein C (APC) has a relatively long half-life in circulation (approximately 20 minutes) and proteolytically inactivates activated factors V (FVa) and VIII (FVIIIa). Protein S accelerates inactivation of FVa and FVIIIa by APC. In the protease inhibitor system, antithrombin and tissue factor pathway inhibitor neutralize key coagulation proteases, in particular activated factors VII, IX, and X, in addition to thrombin. Inactivation of these proteases is heparan sulfate–dependent and occurs on the endothelium, lowering the potency of coagulant activity. Thus 2 systems involving a total of 6 proteins mainly control coagulation. Genetic or acquired deficiencies of any of these proteins may lead to vein thrombosis. Deficiency in protein C, protein S, or antithrombin is a major risk factor for vein thrombosis among white people [2,3]. Lack of data concerning the prevalence of these deficiencies in the general population of other ethnic groups renders it hazardous to extrapolate risk factors for vein thrombosis.

Correspondence and reprint requests: Toshiyuki Miyata, PhD, Director, Department of Etiology and Pathogenesis, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan; 81-66833-5012; fax: 81-66835-1176 (e-mail: miyata@ri.ncvc.go.jp).