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ORIGINAL ARTICLE

AMP-activated protein kinase as a promoting factor, but complement and thrombin as limiting factors for acquisition of cytoprotection: implications for induction of accommodation

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accommodation, AMP-activated protein kinase, complement regulatory protein, complement-mediated cytotoxicity, cytoprotective gene, signal transduction pathway.

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Summary

Accommodation has been termed as a condition without graft rejection even in the presence of antidonor antibody. We previously reported an *in vitro* accommodation model, which demonstrated that preincubation of A/B antigen-expressing endothelial cells with anti-A/B antibody resulted in ERK inactivation followed by resistance to complement-mediated cytotoxicity through the induction of complement regulatory genes. However, under the *in vivo* condition, the effects of complement and coagulation system cannot be ignored. The purpose of this study is to find effective ways to navigate accommodation by exploring the relevant signal transduction. Preincubation with a low level of complement or thrombin failed to induce resistance to complement-mediated cytotoxicity. AMP-activated protein kinase (AMPK) activators such as resveratrol, AICAR and metformin protected endothelial cells against complement-mediated cytotoxicity through the increase in CD55, CD59, haem oxygenase-1 (HO-1) and ferritin heavy chain (ferritin H) genes, all of which were attenuated by AMPKα knock-down. Resveratrol counteracted the inhibitory effect of pretreated complement and thrombin on acquisition of resistance to complement-mediated cytotoxicity through AMPKα. AMPK regulation in endothelial cells could become the potential strategy to induce accommodation in clinical pro-inflammation and pro-coagulation.

Introduction

'Accommodation' has been widely demonstrated to be one of the key issues for the success of organ transplantation [1]. However, the mechanisms of accommodation have yet to be elucidated. Accommodation has been termed a protective condition even in the presence of antidonor antibody [2]. Recent effective immunosuppressive agents have reduced the incidence of acute cellular rejection and improved graft survival [3]. To alleviate the organ shortage, the energy of clinicians has been invested in ABO-incompatible (ABO-I) and HLA-incompatible (HLA-I) transplantation. Such transplantation with antidonor antibodies was previously considered to be contraindicated, but is now safely conducted, if patients are properly selected, by a desensitization regimen including plasmapheresis,

anti-CD20 mAb, splenectomy and intravenous immunoglobulin therapy [4,5]. Attention has been directed towards not short-term, but long-term graft outcome, because subclinical rejection has reportedly progressed. Identification of accommodation mechanisms and development of effective induction methods are presently the most vital challenges for successful ABO-I/HLA-I kidney transplantation.

Many researchers have indicated that the difference in signalling pathways triggered by antidonor antibody binding could determine endothelial response, influencing graft outcome, whether rejection or accommodation. Low levels of anti-HLA antibody ligation activate the PI3K/AKT survival signalling pathway, resulting in anti-apoptotic (Bcl-2) and cytoprotective gene [ferritin heavy chain (ferritin H) and haem oxygenase-1 (HO-1)] induction, whereas high levels activate the ERK pathway, leading to endothelial activation and graft rejection [6–8]. Furthermore, we have reported that anti-A/B ligation on endothelial cells expressing A/B antigens could reduce the ERK pathway accompanied by endothelial activation and increase the expression of complement regulatory proteins such as CD55 and CD59, which might be responsible for favourable results even in C4d deposition in ABO-I kidney transplantation, unlike HLA-I transplantation [9]. Thus, we found that endothelial cells could acquire resistance to complement-mediated cytotoxicity, so-called graft accommodation by pretreatment with anti-A/B antibody ligation for 24 h. However, in line with clinical situations, we need to consider complement and coagulation system in such an *in vitro* accommodation model. The possibility has been suggested that endothelial cell activation caused by coagulation and complement might be a strong inhibitor of successful transplantation [10]. ERK activation might explain one mechanism underlying the disturbance of accommodation by complement and thrombin, because its activation triggers endothelial dysfunction, resulting in organ rejection [11]. However, the paradigm of the signalling pathway still remains unable to provide a theoretical model of accommodation in clinical settings.

AMP-activated protein kinase (AMPK) is an important enzyme as a sensor of cellular energy balance and metabolic status [12]. The AMPK pathway could control the intracellular signalling pathway including PKC, mTOR and NF-kappaB [13]. AMPK activation has a protective role in ischaemia–reperfusion injury [14] and the oxidative stress damage by inducing HO-1 expression through Nrf2/ARE axis [15], resulting in anti-inflammatory, anti-apoptotic and cytoprotective function on endothelial cells [16]. AMPK-activating agents are now anticipated to exhibit various protective effects against graft injury. In this study, we investigated whether thrombin and complement would hamper anti-A and anti-HLA antibody-induced accommodation on endothelial cells, and in addition, an AMPK activator such as resveratrol was found to enhance accommodation.

Materials and methods

Cell culture and materials

The human endothelial-like immortalized cell line, EA.hy926, derived from the fusion of human umbilical vein endothelial cells with the lung carcinoma cell line A549, was obtained from ATCC and maintained in DMEM supplemented with 10% FBS (Hyclone, Logan, UT, USA). Blood type A antigen-expressing EA.hy926 cells (EA.hy926/A) were established by the overexpression of A transferase previously [9]. Mouse monoclonal anti-A IgG and IgM were purchased from BD Biosciences (San Jose, CA, USA) and Ortho Clinical Diagnostics (Raritan, NJ, USA), respectively. Resveratrol, AICAR, metformin and everolimus were purchased from Sigma-Aldrich (St Louis, MO, USA).

Western blotting

Western blots were performed using whole-cell lysates. Antibodies against AKT, phospho-AKT (Ser473), ERK, phospho-ERK (Thr202/Tyr204), AMPKα, phospho-AMPK α (Thr172), AMPKβ1/2, S6K, phospho-S6K (Thr389) and GAPDH (all from Cell Signaling, Danvers, MA, USA) were used with a working dilution in Can Get signal solution I (Toyobo, Tokyo, Japan). Secondary anti-rabbit IgG-HRP antibodies (Cell Signaling) were used at 1:2000 dilutions in Can Get signal solution II (Toyobo). Signals were observed via ECL® Western Blotting Detection Reagents (Amersham–GE Healthcare, Pittsburgh, PA, USA).

Flow cytometry

For the detection of CD55 or CD59 expression, harvested cells were incubated with FITC-labelled anti-CD55 (eBioscience, San Diego, CA, USA) or FITC-labelled anti-CD59 (Beckman, Marseille, France) for 30 min at 4 °C. As a negative control, FITC-labelled-mouse IgG1 (R&D Systems, Minneapolis, MN, USA) was used. Stained cells were then washed twice with PBS and analysed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Complement-mediated cytotoxicity assay

Complement-mediated cytotoxicity was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cells were plated at 0.5–1.0 × 10^4 cells per well in 96-well culture plates 1 day prior to the assay in 2% FBS DMEM medium. Cells were incubated

with 10% rabbit serum (One Lambda, Canoga Park, CA, USA) as a complement source or heat-inactivated rabbit serum for 30 min, then washed with PBS and incubated with 0.5 mM MTT containing FBS-free DMEM medium for 1 h. The 540 nm absorbance was measured by microplate reader Vient XS (DS Pharma Biomedical, Osaka, Japan). Values were expressed as a percentage of cytotoxicity calculated as follows: %cytotoxicity = [1−test OD/control OD] × 100. All assays were performed in duplicate.

Quantitative Real-Time PCR

Total RNA was extracted from cells using the Trizol (Invitrogen, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was carried out using an iCycler Bio-Rad (Hercules, CA, USA). Total RNA was reverse-transcribed using 1 μM oligo (dT) and high-capacity reverse transcriptase (Takara, Tokyo, Japan), according to the manufacturer's instructions. cDNA of ferritin H, HO-1 and Kruppel-like factor 2 (KLF2) were amplified and normalized to the level of β-actin. Quantitative real-time PCR was performed in the Mx3000P quantitative PCR system (Stratagene, La Jolla, CA, USA) using SYBR premix Ex taq (TAKARA BIO, Shiga, Japan).

In vitro assay for modelling accommodation

The flowchart of the *in vitro* accommodation model was shown in Fig. 1. Cytoprotection (resistance to complement-mediated cytotoxicity) was previously observed after preincubation with a saturation level of anti-A/B or a low level of anti-HLA antibodies [9]. After cells were preincubated with a saturation level (1 μg/ml) of anti-A/B IgG, a low level (1 μg/ml) of anti-HLA IgG or none for 24 h, CD55/59 expression and HO-1/Ferritin H/KLF2 mRNA were measured by flow cytometry and quantitative real-time PCR, respectively.

For cytotoxicity assay, the total amount of cell-bound antibodies was fixed at 1 μg/ml for anti-A/B IgG or 10 μg/ml for anti-HLA IgG. Therefore, after preincubation with 1 μg/ml of anti-A/B IgG or 1 μg/ml of anti-HLA IgG for 24 h, cells were treated with additional antibodies (0 μg/ml of anti-A/B IgG or 9 μg/ml of anti-HLA IgG) for 1 h, followed by incubation with 10% rabbit serum (complement). Cells treated with 10% rabbit serum immediately after the total amount of anti-A/B IgG (1 μg/ml) or anti-HLA IgG (10 μg/ml) without 24-h preincubation were defined as positive controls (no pretreatment). Cells treated with only 10% rabbit serum without antibody binding were defined as negative controls. During the preincubation for 24 h, complement (1%, 2%), thrombin (0.1, 0.5 U/ml) and/or AMPK activators such as resveratrol (25, 50 μM), AICAR (0.5, 1 mM) and metformin (0.5, 1, 2 mM) or everolimus (0.5, 1, 5, 10 ng/ml) were added to examine their effects.

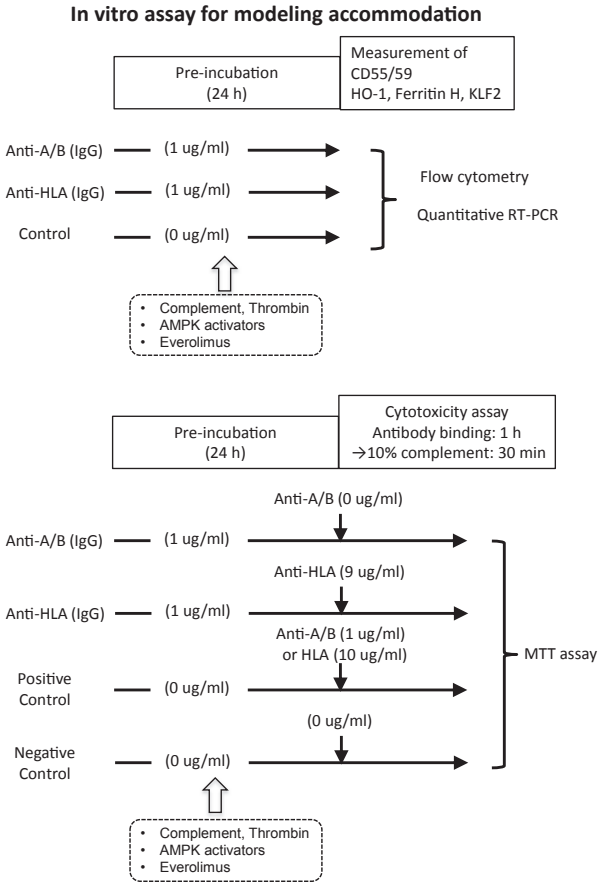

Figure 1 Flowchart of *in vitro* assay for modelling accommodation. (see text for details).

Small interfering RNA (siRNA) transfection

AMPKα siRNA (Dharmacon, Lafayette, CO, USA) were transfected into cells by Accell siRNA transfection method. Transfected cells were suspended in DMEM medium containing 2% FBS and incubated for 48 h. To detect the expression of AMPKα, whole-cell lysates were subjected to Western blotting with anti-AMPKα, anti-AMPKβ1/2 and anti-GAPDH (all from Cell Signaling).

Statistical analysis

All experiments were repeated at least three times. The results were represented as a mean ± SD. The statistical significance was calculated by Student's *t*-test or Welch's *t*-test from three independent experiments. P values < 0.05 were considered statistically significant.

Results

A low level of complement or thrombin prevents anti-A antibody-induced cytoprotection and ERK inactivation in endothelial cells

We first investigated the effect of complement and thrombin at low levels on acquisition of anti-A antibody-induced resistance to complement-mediated cytotoxicity using an *in vitro* accommodation model that was reported previously [9]. EA.hy926/A cells were treated with a saturation level of anti-A antibody (IgG; Fig. S1a and b, and IgM) for 24 h with or without complement or thrombin. Anti-A antibody (IgG and IgM) ligation on EA.hy926/A showed resistance to complement-mediated cytotoxicity (Fig. 2a and b), as observed in a previous experiment using anti-A/B IgM antibody. However, in contrast, the presence of

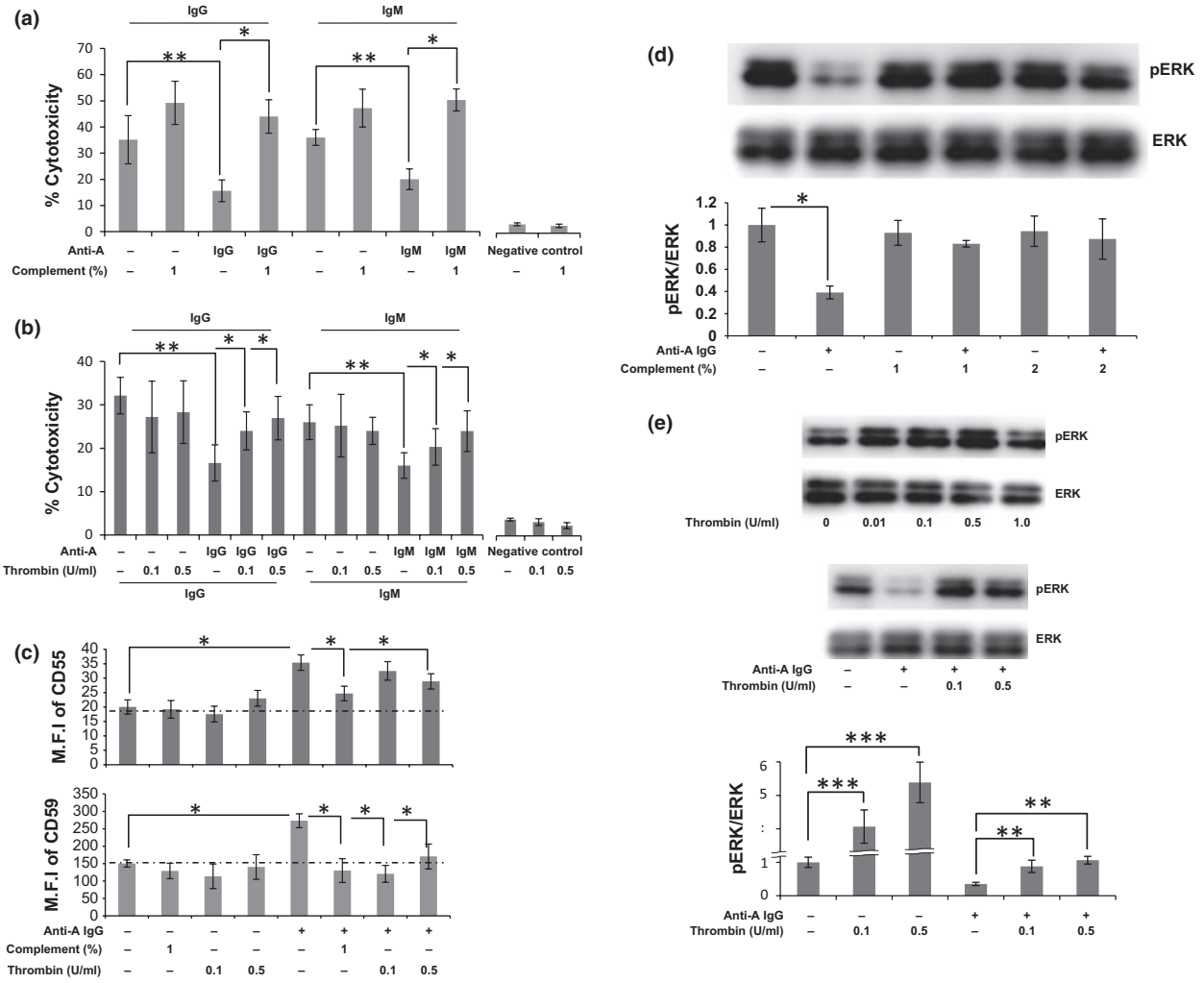

Figure 2 Inhibition of anti-A antibody-induced cytoprotection by complement and thrombin. (a, b) EA.hy926/A cells were pretreated with anti-A IgG (1 μg/ml), IgM antibody (2 mg/ml) or none (no pretreatment) with or without 1% complement (a) and 0.1 or 0.5 U/ml of thrombin (b) for 24 h. Then, cells were incubated with 10% complement for 30 min and subjected to cytotoxicity assay. To fix cell-bound antibody levels, cells pretreated without anti-A antibody (untreated cells) were incubated with the same amount of anti-A antibody for 1 h, immediately before 10% complement incubation. Cell viability was measured by MTT assay. Negative control means only 10% rabbit complement was added without antibody. (c) EA.hy926/A cells were pretreated with 1 μg/ml of anti-A IgG antibody in the presence or absence of 1% complement and 0.1 or 0.5 U/ml of thrombin for 24 h. Cells were harvested and subjected to flow cytometry to measure CD55 and CD59 protein expression shown by M.F.I. (d) EA.hy926/A cells were preincubated with 1 μg/ml of anti-A IgG antibody in the presence or absence of 1 or 2% complement. (e) EA.hy926/A cells were treated with several amounts of thrombin for 15 min (upper) or 0.1 or 0.5 U/ml of thrombin in the presence of anti-A IgG antibody for 1 h (middle). Cells were harvested and subjected to Western blotting with anti-phospho-ERK, anti-ERK. A representative Western blotting figure was shown. The ratio of phospho-protein against intact protein was quantified and shown (fold increases in *y*-axis) from at least three independent experiments (lower). All of the data represent means ± SD ($n \geq 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

1% complement (Fig. 2a) and 0.1 or 0.5 U/ml of thrombin (Fig. 2b) inhibited the acquisition of anti-A antibody-induced cytoprotection against complement-mediated cytotoxicity, although such a low level of complement or thrombin alone did not exhibit any cytotoxicity against EA.hy926/A for 24 h (Fig. S2a–c). In the following experiments, as anti-A IgG and IgM antibodies showed similar cell response results, we expressed only data on anti-A IgG antibody-induced cytoprotection. We also measured CD55/59 expression in this experiment. Figure 2c shows that anti-A ligation by itself induced CD55/59, but the existence of 1 or 2% complement and 0.1 or 0.5 U/ml of thrombin attenuated the expression of those proteins. Because ERK inactivation following anti-A antibody ligation was shown to be involved in cytoprotection of *in vitro* accommodation models, we next monitored the effect of complement and thrombin on ERK signalling pathway. EA.hy926/A cells were incubated with anti-A IgG antibody in the presence or absence of 1% or 2% complement and 0.1 or 0.5 U/ml of thrombin. Anti-A antibody ligation of EA.hy926/A cells inactivated ERK within 1 h, which was hampered in the presence of 1% or 2% complement (Fig. 2d). Complement treatment without antibody did not affect ERK activation. On the other hand, thrombin more rapidly and strongly activated ERK within 15 min (Fig. 2e). 0.1 or 0.5 U/ml of thrombin reversed anti-A IgG antibody-induced ERK inactivation.

AMPK activation protects endothelial cells against complement-mediated cytotoxicity

To demonstrate the effect of AMPK on endothelial cells, EA.hy926/A cells were treated with resveratrol, AICAR and metformin, all of which were known as AMPK activators. All of the reagents induced AMPKα phosphorylation at Thr172 within 2 h (Fig. 3a). Because AMPK activation has been reported to be associated with ERK and mTOR signalling pathway, Western blotting was carried out to investigate signalling pathway-related molecules. Statistical analysis showed that resveratrol, AICAR and metformin reduced AKT and S6K phosphorylation, but none had any effect on ERK phosphorylation. AMPK activator inhibited S6K phosphorylation to a lesser extent than the mTOR-specific inhibitor, everolimus (Fig. 3b).

Next, we investigated whether AMPK activator had a protective effect against complement-mediated cytotoxicity. Western blotting result confirmed that siRNA against AMPKα down-regulated its expression (Fig. 3c). After knock-down of AMPKα, cells were incubated with AMPK activator for 24 h. Figure 3d showed that resveratrol (50 μM), AICAR (1 mM) and metformin (2 mM) protected the cells against complement-mediated cytotoxicity, but everolimus (10 ng/ml) had no effect. AMPKα knock-down by siRNA attenuated the cytoprotective effect induced by resveratrol, AICAR and metformin. Among these reagents, resveratrol has the strongest cytoprotective effect. Furthermore, resveratrol-induced cytoprotective effect was similarly observed in complement-mediated cytotoxicity caused by anti-HLA antibody (Fig. S3).

AMPK activation induces cytoprotective and complement regulatory genes

We investigated whether AMPK activation induced CD55/59 and HO-1/ferritin H expression. Because AMPK activator also dephosphorylated S6K (Fig. 3a), everolimus was included in this experiment. EA.hy926/A cells were treated with resveratrol, AICAR, metformin and everolimus for 24 h. CD55 expression was induced only by resveratrol (Fig. 4a). Although all reagents tested in this experiment induced CD59 expression, resveratrol was found to be the most potent inducer (Fig. 4a). HO-1/ferritin H mRNA expression was also induced by resveratrol, AICAR and metformin, but not everolimus (Fig. 4b). All of the reagents tested in this experiment induced mRNA expression of KLF2, which was reported to induce CD59 expression (Fig. 4b) [17].

AMPK activation by resveratrol recovers anti-A antibody-induced cytoprotection prevented by complement and thrombin

As resveratrol was found to induce HO-1/ferritin H and CD55/59 expression more strongly than other reagents, we focused on the potential benefit of resveratrol. After knock-down of AMPKα, cells were incubated with anti-A antibody in the presence of 1% complement or 0.5 U/ml of thrombin for 24 h. Although the cytoprotective effect induced by anti-A antibody ligation was inhibited in the presence of 1% complement or 0.5 U/ml of thrombin, resveratrol recovered it (Fig. 5a). AMPKα knock-down by siRNA counteracted the resveratrol-mediated beneficial effect. Resveratrol also induced CD59 (Fig. 5b) and HO-1/ferritin H/KLF2 mRNA even in the presence of complement or thrombin (Fig. 5c), which was considered to be AMPKα-dependent, because AMPKα knock-down blocked resveratrol-induced gene expression. CD55 expression was also induced by resveratrol. But AMPK involvement might be partial, because AMPKα knock-down could not completely inhibit CD55 expression (Fig. 5b).

Regarding the response after anti-B antibody ligation, IgM antibody similarly showed the induction of resistance to complement-mediated cytotoxicity and the beneficial effect of resveratrol on acquisition of cytoprotection even in the presence of complement or thrombin (data not shown). A low level of anti-HLA antibody ligation also

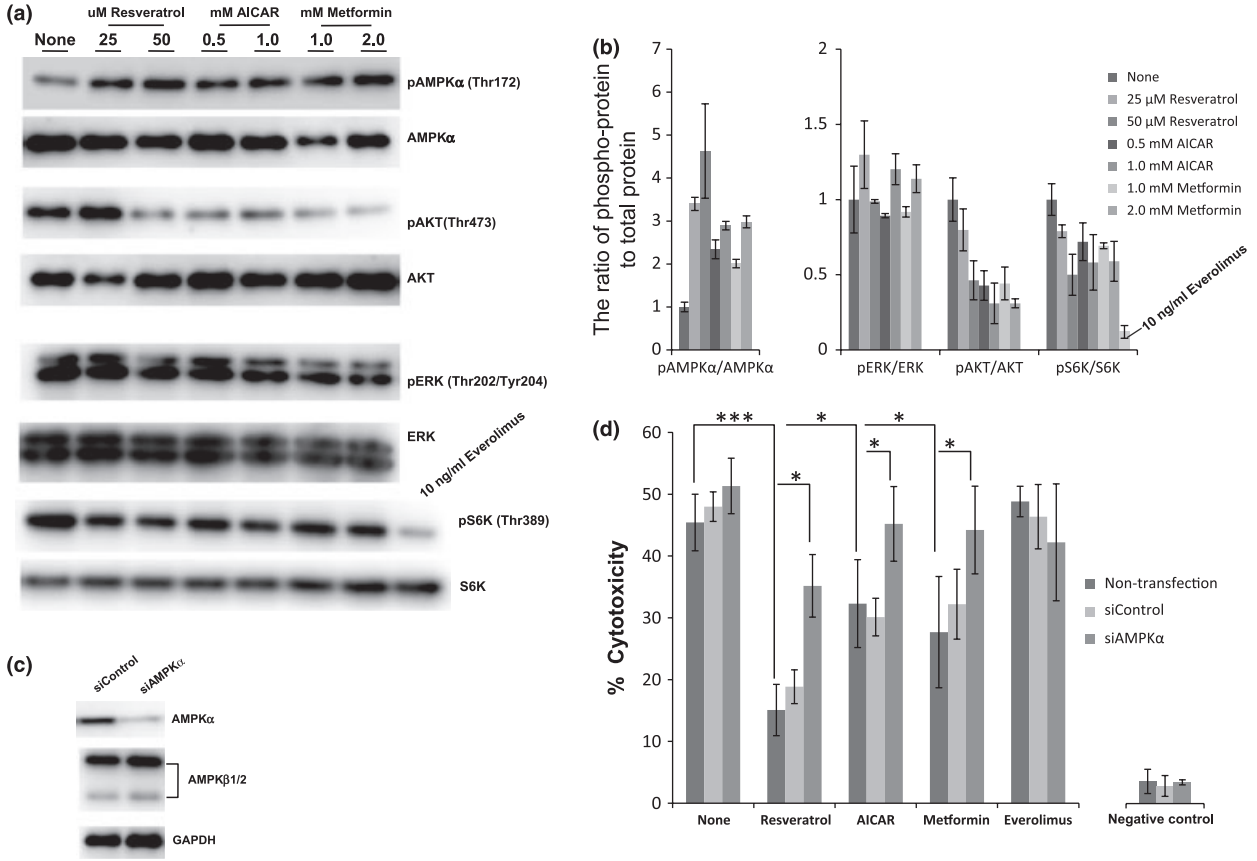

Figure 3 Protection of endothelial cells against complement-mediated cytotoxicity by AMP-activated protein kinase (AMPK) activator. (a) EA.hy926/A cells were incubated with the indicated amounts of resveratrol, AICAR, metformin and everolimus for 2 h, respectively. Cells were harvested and subjected to Western blotting with primary antibody against phospho-AMPKα, AMPKα, phospho-ERK, ERK, phospho-AKT, AKT, phospho-S6K and S6K. Representative Western blotting figure was shown. (b) The ratio of the strength of phospho-protein to total protein was quantified and shown. Each bar represents the mean ratio. (c) EA.hy926/A cells were transfected with siControl or siRNA against AMPKα and incubated for 48 h. Cells were harvested and subjected to Western blotting with anti-AMPKα, anti-AMPKβ1/2 and anti-GAPDH. Representative Western blotting figure was shown. (d) EA.hy926/A cells were transfected with siControl or siRNA against AMPKα and incubated for 48 h. Transfected and untreated cells were incubated with 50 μM of resveratrol, 1 mM of AICAR, 2 mM of metformin and 10 ng/ml of everolimus for the following 24 h. Then, cells were incubated with 1 μg/ml of anti-A IgG antibody for 1 h, followed by 10% complement incubation for 30 min. Cell cytotoxicity was measured by MTT assay. Harvested cells were subjected to Western blotting with anti-AMPKα, anti-AMPKβ1/2 and anti-GAPDH. Representative Western blotting figure was shown. Negative control means 10% rabbit complement were added without antibody. All of the data represent means ± SD ($n \geq 3$). *$P < 0.05$, ***$P < 0.001$.

showed the induction of resistance to complement-mediated cytotoxicity and the beneficial effect of resveratrol on acquisition of cytoprotection even in the presence of complement or thrombin via AMPK (Fig. 5d). Resveratrol induced HO-1/Ferritin H even in the presence of complement, which was partially AMPKα dependent, because AMPKα knock-down blocked the expression of those genes induced by resveratrol (Fig. 5e). Thrombin did not inhibit anti-HLA-mediated cytoprotective gene expression. AMPK knock-down blocked the expression of ferritin H, but not HO-1 induced by resveratrol (Fig. 5e). Collectively, these results revealed that AMPK could be a key molecule in induction of accommodation following HLA- and ABO-I transplantation.

Discussion

Several efforts have been made to define the mechanisms of accommodation, the condition of no graft injury even with the existence of antidonor antibody [2]. Both PI3K/AKT and ERK signalling pathways have been extensively investigated as potential candidates for relevant intracellular signal transduction following antigen–antibody ligation [18–20]. The concept of accommodation was summarized in Fig. 6. Low levels of anti-HLA antibodies were found to elicit cytoprotection through PI3K/AKT, whereas high levels of anti-HLA activated ERK, resulting in cell proliferation and activation in endothelial cells [21]. Our previous finding demonstrated that CD55/59 upregulation due to anti-A/B

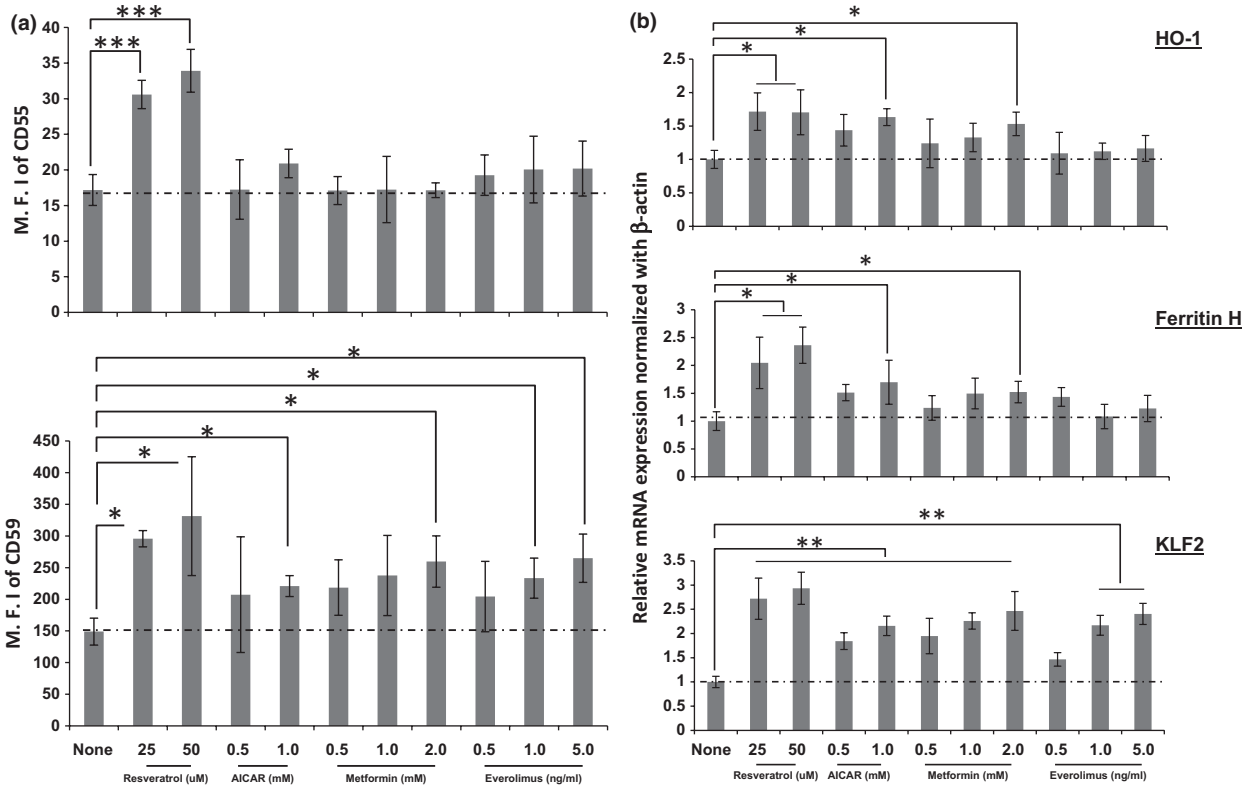

Figure 4 CD55 and CD59 proteins, and HO-1, ferritin H and Kruppel-like factor 2(KLF2) mRNA induction by AMP-activated protein kinase activators in endothelial cells. EA.hy926/A cells were preincubated with the indicated amounts of resveratrol, AICAR, metformin and everolimus for 24 h. (a) EA.hy926/A cells were harvested and subjected to flow cytometry to measure CD55 and CD59 protein expression shown by M.F.I. (b) Purified total RNA was subjected to quantitative RT-PCR to measure HO-1, ferritin H and KLF2 mRNA (normalized to β-actin mRNA), and the values were represented relative to nontreated cells. All of the data represent mean ± SD ($n \geq 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

antibody ligation-induced ERK inactivation is one of the critical mechanisms for the protection of endothelial cells against complement-mediated cytotoxicity [9]. Complement activation and thrombin generation have been considered as detrimental factors that would lead to rejection in transplantation [10,22]. In our *in vitro* experiment, the addition of a low level of complement or thrombin completely inhibited anti-A antibody-mediated ERK inactivation and CD55/59 induction resulting in failure to protect against complement-mediated cytotoxicity. From the viewpoint of accommodation, it was suggested that ERK might be the pivotal signalling pathway to be controlled and that due caution against complement and thrombin, known as a strong ERK activator, even at a low level [23,24], is needed during the induction period of accommodation in ABO-I or HLA-I transplantation. Pretreatment with double-filtration plasmapheresis immediately before transplantation, which can remove complement and coagulation factors with high molecular weight as well as immunoglobulins, might have served to enhance accommodation.

CD55/CD59 induction and membrane attack complex inhibition might be one of the most efficient agents to direct cell properties towards a protective condition in anti-donor antibody-positive transplantation [25,26]. CD55 was reportedly induced by TNF-α in a PKC-dependent manner [27]. HO-1 transgenic mouse showed higher ferritin H and CD55 expression in endothelial cells [28]. However, in both cases, CD59 expression was not changed, which implied that CD55 and CD59 expression could be regulated by independent mechanisms. Our current finding also showed that AICAR- or metformin-mediated AMPK activation upregulated only CD59, but that another AMPK activator, resveratrol, induced CD55 as well as CD59. In contrast, it was speculated that mTOR signalling pathway inhibition could be linked to the expression of KLF2 transcription factors, resulting in CD59 induction, but not cytoprotective gene expression. It was suggested that resveratrol might have an additional unknown intracellular signalling pathway related to CD55 expression.

AMP-activated protein kinase is one of the essential molecules found in all eukaryotes as a regulator of metabolism

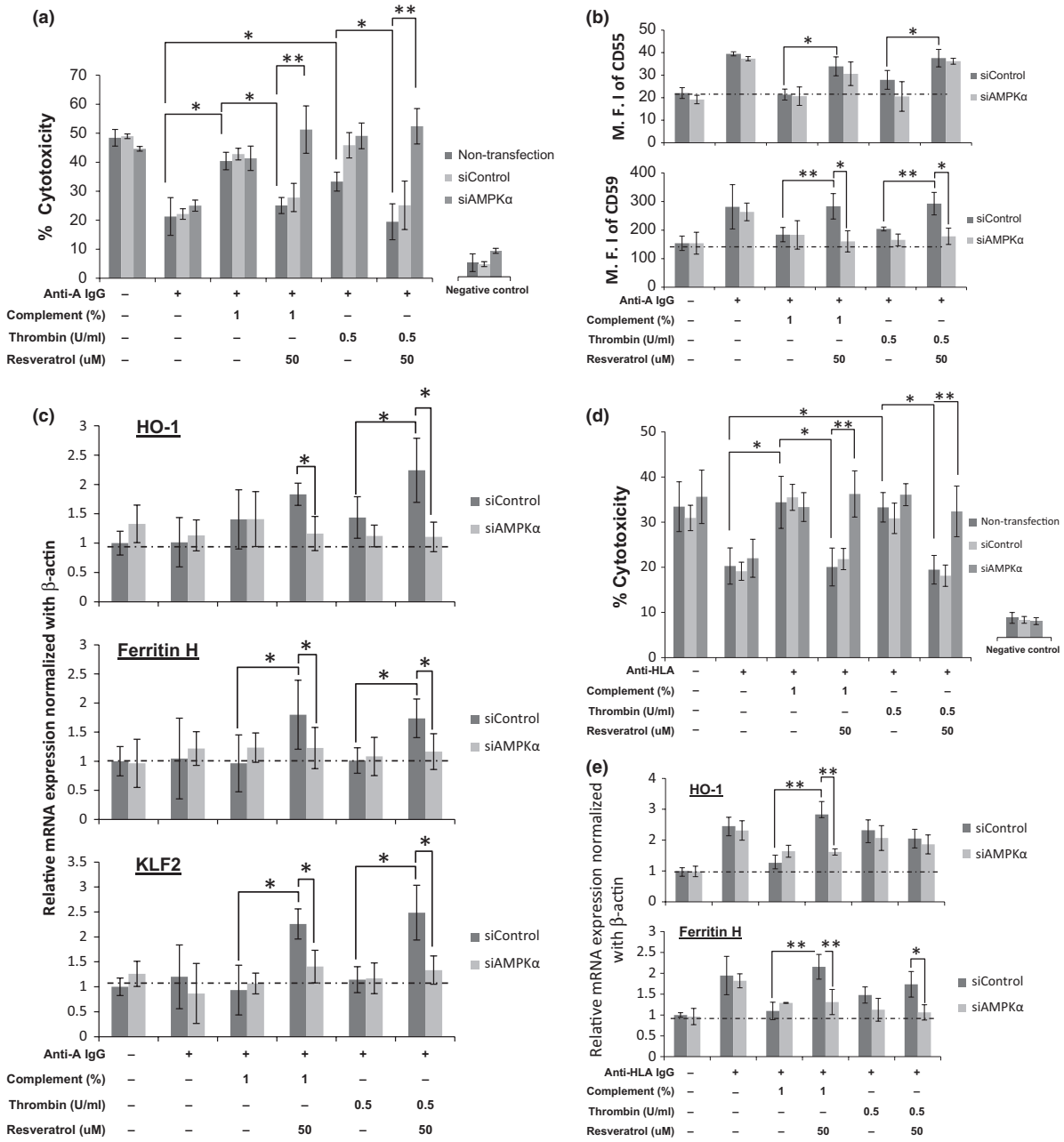

Figure 5 Beneficial effect of resveratrol on anti-A or anti-HLA antibody-induced cytoprotection in the presence of complement or thrombin. EA.hy926/A cells were transfected with nontargeting siRNA (siControl) or siRNA against AMPKα (siAMPKα) and incubated for 48 h. Cells were treated with 50 μM of resveratrol for 24 h, followed by incubation with 1 μg/ml of anti-A IgG (a, b, c) or 1 μg/ml of anti-HLA IgG (d, e) antibody in the presence or absence of 1% complement or 0.5 U/ml of thrombin for 24 h. (a, d) EA.hy926/A cells pretreated without anti-A/anti-HLA antibody (untreated cells) were incubated with the same amount of anti-A/anti-HLA antibody for 1 h, immediately before 10% complement incubation. Cell viability was measured by MTT assay. Negative control means 10% rabbit complement was added without antibody. (b) Harvested EA.hy926/A cells were subjected to flow cytometry to measure CD55 and CD59 protein expression shown by M.F.I. (c, e) Purified total RNA was subjected to quantitative RT-PCR to measure HO-1, ferritin H and Kruppel-like factor 2 mRNA (normalized to β-actin mRNA), and the values were represented relative to nontreated cells. All of the data represent mean ± SD ($n \geq 3$). *$P < 0.05$, **$P < 0.01$.

[13]. The preclinical experiment targeting AMPK activation has been shown to be an attractive therapeutic strategy for the treatment for diabetes, metabolic disorders and cancer [29]. AMPK has been reported to have a cytoprotective effect on endothelial cells [16] and inhibitory crosstalk with ERK signalling pathway in cardiac fibroblasts [30]. We

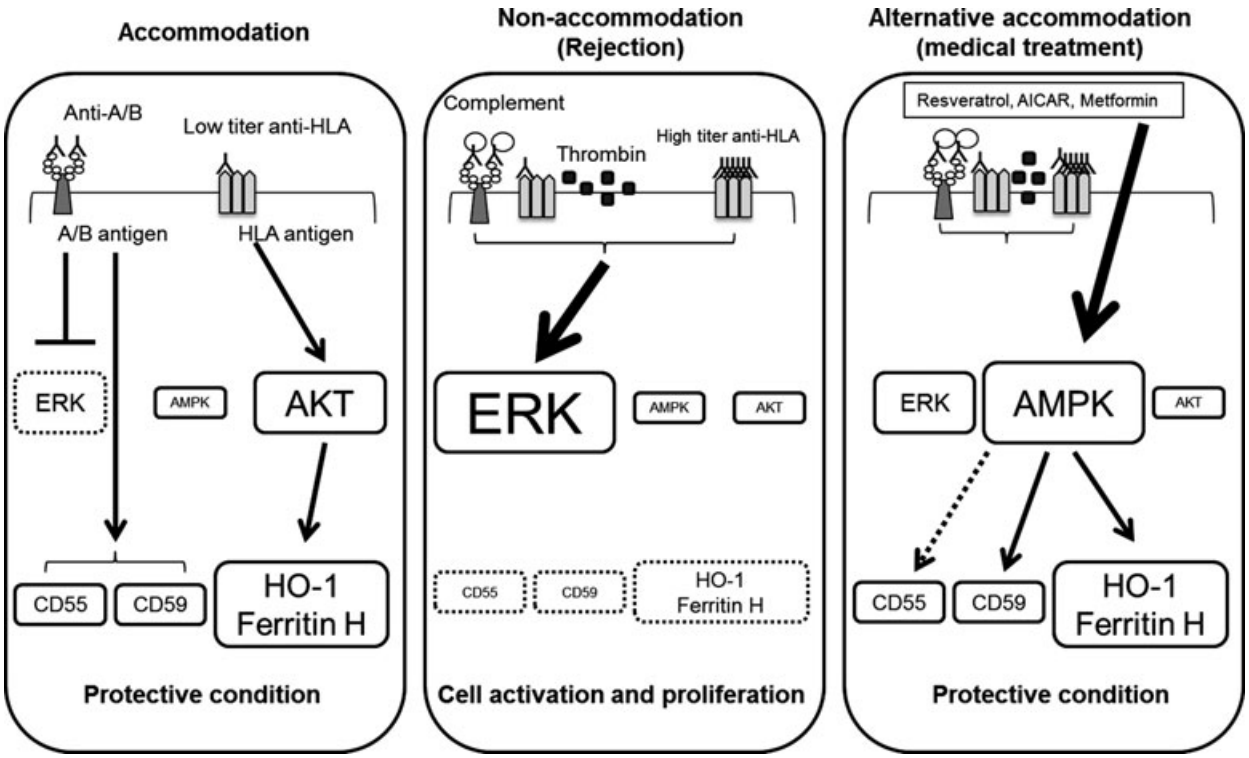

Figure 6 Model for the concept of accommodation in HLA-incompatible and ABO-incompatible transplantation and alternative accommodation via AMP-activated protein kinase activation (see text for details).

previously demonstrated Nrf2-mediated cytoprotective gene induction by a low level of anti-HLA antibodies could promote acquisition of the resistance against complement-mediated cytotoxicity [7]. Recent findings suggested that NF-kappaB was linked to kidney graft acceptance [31]. Both transcription factors have been shown to be modulated through AMPK activation by resveratrol [32,33], which led us to hypothesize that AMPK activation might contribute accommodation under the complement- and thrombin-mediated endothelial activated condition. In endothelial cells, AMPK activation has been linked to improvement of endothelial dysfunction caused by ischaemia–reperfusion [14], fluid shear stress [34] and oxidative stress [35]. Here, we showed that several AMPK activators used in our experiment, in particular resveratrol, play a significant role in establishing accommodation by inducing cytoprotective and complement regulatory genes, which resulted in protection against complement-mediated cytotoxicity (Fig. 6; right).

Resveratrol has also been reported to show multiple favourable effects on some diseases and health problems such as type II diabetes [36], Alzheimer's [37], cancer [38] and obesity in mouse models [39]. In transplantation, several studies provided strong evidence that resveratrol could extend graft survival [40,41]. Recent clinical study on cancer treatment indicated the feasibility of resveratrol oral administration at a high dose (5 g/body), which reached 4.24 μM of resveratrol and 18.3 μM of its metabolite resveratrol-3-*O*-sulphate in serum [42]. Therefore, it may be possible that resveratrol could demonstrate clinical benefit in organ transplantation. However, considering the concentration effect and tolerability, it is still controversial whether resveratrol could be used to promote accommodation. Furthermore, as other studies have shown that resveratrol could control cytokine expression [32] and regulate T-cell function [43], the effective and safe dose of resveratrol needs to be clarified by appropriate clinical study.

It is imperative to elucidate the mechanisms of accommodation, which may provide useful information on safe minimization of immunosuppressive agents after transplantation [1]. Cytoprotective genes, complement regulatory molecules and anti-apoptotic genes have been reported to be associated with accommodation and protection of endothelial dysfunction in transplantation procedure [9,44–46]. AMPK-mediated gene regulation described here can be one potential strategy to acquire resistance to the endothelial cell dysfunction caused by antibody and complement activation. Taken together, AMPK and ERK pathway-targeting therapy may be a

promising method for further drug development in the transplantation field. However, because all experiments have been conducted in the immortalized cell line, which might exhibit different biological properties from graft endothelium, and under *in vitro* conditions without inflammatory cells, platelets or a majority of serum components, further experimental setting will be required to demonstrate the identity of accommodation and the significance of AMPK under an *in vivo* situation.

In conclusion, (i) complement and thrombin could be limiting factors for the induction of accommodation after ABO-I and HLA-I transplantation because of potent activators of ERK signalling pathway; (ii) AMPK activator, in particular resveratrol, induced cytoprotective and complement regulatory molecules, resulting in the attenuation of complement-mediated cytotoxicity, which could promote accommodation in clinical settings; and (iii) AMPK and the ERK and PI3K/AKT pathways must be considered the key factors for accommodation.

Authorship

KI and T Kobayashi: designed the research, wrote the article and participated in data analysis. KI, YM and MH: performed the research. T Kuzuya, HO and AO: contributed the new reagent or analysis tools.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Anti-A IgG antibody binding and cytotoxicity to endothelial cells.

Figure S2. Cytotoxic effect of thrombin and complement on endothelial cells.

Figure S3. Protection of endothelial cells against complement-mediated cytotoxicity with anti-HLA antibody by resveratrol.

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