Table 5.
Previous Idiopathic Thrombocytopenic Purpura Treatment and Eradication Effect on Platelet Response 12 Months after Eradication

Treatment	Eradication Effect	No. of Cases	Platelet Response, n (%)		
			Complete Remission	Partial Remission	No Response
No treatment	Yes	40	11 (28)	16 (40)	13 (33)
	No	7	3 (43)	1 (14)	3 (43)
Steroid treatment	Yes	52	12 (23)	24 (46)	16 (31)
	No	18	2 (11)	3 (17)	13 (72)
Other treatment	Yes	18	3 (17)	6 (33)	9 (50)
	No	5	0	0	5 (100)
Splenectomy	Yes	12	2 (17)	5 (42)	5 (42)
	No	3	0	0	3 (100)
Total	Yes	122	28 (23)	51 (42)	43 (35)
	No	33	5 (15)	4 (12)	24 (73)

respectively, of cases showing adverse effects. These problems were the main adverse reactions, and except in 1 case in which the tendency to bleed became more serious, the patients were able to overcome these events and complete the eradication schedule.

4. Discussion

The incidence of *H pylori* infection in the general population of Japan is approximately 10% to 40% until 40 years of age, after which it increases to approximately 80% [18]. Because the incidence of *H pylori* infection in patients with ITP does not seem to differ from that in the general population of Japan, the incidence of *H pylori* infection in ITP is not considered a specific feature of the disease.

H pylori infection did not influence the severity of the clinical features of ITP. This retrospective study revealed that the *H pylori*–positive group had a higher mean age and incidence of hyperplastic bone marrow megakaryocytes, but there was no evidence to explain the bone marrow findings.

The success rate of *H pylori* eradication in *H pylori*–positive ITP cases was slightly lower than that found in gastric diseases without ITP in Japan. The former was 78%, and the latter was 82% to 91% [19]. ITP treatment just before *H pylori* eradication did not influence this rate. Eradication in *H pylori*–positive ITP cases induced a significant increase in platelet count in the successful eradication group compared with that of the unsuccessful eradication group.

These findings suggest that *H pylori* infection is involved in the mechanisms of thrombocytopenia in most ITP patients in Japan who are older than 41 years. However, in the younger generation, other, unknown factors must be involved in the development of ITP.

Approximately 33% of the eradication failure group had an increase in platelet count. Although it is difficult to explain this response, a possibility is the occurrence of false-positive results of the ^{13}C UBT, because an insufficient period of time had passed to allow for an accurate result, or from inadequacy in the ^{13}C UBT itself. The other possibility is that reduction of *H pylori* organisms shuts down the circuit of platelet immunity or that another, unknown mechanism is at work [20,21].

The management of ITP initially or immediately prior to eradication treatment did not influence the increase in platelet count. That is, the platelet response rate in nontreatment groups was higher than in any treatment group, but there was no significant difference between them. This evidence suggests that refractory ITP cases with *H pylori* infection should be eradicated prior to other treatment procedures.

Examination of the clinical course after *H pylori* eradication clearly showed that a significant increase in platelets began 1 month after eradication and gradually continued to increase to a plateau. This finding suggests that the effect of eradication therapy on platelet counts can be predicted at an early stage. Several cases relapsed within 6 months, but in the remaining cases, platelet counts continued to increase for 12 months, even in the eradication failure group. The low incidence of relapse is one of the benefits of eradication therapy in *H pylori*–positive ITP cases.

In some cases, although eradication was successful, NR cases occurred. Several possibilities may account for this result. One possibility is that the UBT gave false-negative results in some cases or that small numbers of organisms remained after treatment and continued to stimulate an immune reaction against the platelets, even though the UBT gave negative results. An alternative is that *H pylori*–false-positive cases were present. Eleven cases of *H pylori* infection had been detected initially by serum antibody test. The other possibility is that this organism is not involved in the thrombocytopenia in some *H pylori*–positive ITP cases and another mechanism is taking place in these thrombocytopenia cases, because many other persons with *H pylori* infection do not develop thrombocytopenia.

This study confirmed previous reports with large-scale retrospective analyses. That is, eradication of *H pylori* is effective in significantly increasing platelet count, and this organism is involved in the pathogenesis of some ITP cases [4,12]. However, the mechanisms of thrombocytopenia that affect some persons with *H pylori* infection are unknown.

Several specific proteins derived from *H pylori*, such as blood group Ag–binding adhesin, *H pylori* neutrophil activation factor, lipopolysaccharide (LPS), and CagA (cytotoxin-associated gene A) protein, which is produced by the Cag pathogenicity island gene, induce colonization of *H pylori*, neutrophil infiltration, and cellular immunity. These agents may be involved in the development of gastritis, gastric ulcer, and MALT lymphoma [5,22]. However, it is not understood why localized *H pylori* infection at the gastric mucosa develops into immune thrombocytopenia in some cases.

The products derived from microorganisms such as LPS, bacterial DNA, and viruses are able to act as adjuvants and induce immune reactions to unrelated antigens [23]. Whether this phenomenon is due to the ability of the organisms to activate innate immunity and induce lymphocyte response in a nonspecific way, by molecular mimicking, or both has not been resolved. It recently was reported that platelet eluates from *H pylori*–positive ITP cases reacted with CagA protein [24]. This evidence seems to support the molecular mimicking mechanism in ITP development with *H pylori* infection. However, further investigations are required to confirm this hypothesis.

Although many persons in the world have *H pylori* infection, only a very small proportion develop thrombocytopenia. This discrepancy can be explained, as a working hypothesis, by the multiplicity of infective *H pylori* strains, diversity of host immune responses, differences in the age of infected persons, and environmental factors. In addition, genetic effects are likely to play a significant role in the development of thrombocytopenia [25,26].

Taken together, *H pylori* infections were involved in most thrombocytopenic patients older than 40 years in Japan. In addition, eradication therapy proved to be significantly effective in increasing platelet count in cases of short duration of ITP, and this effect was found even in cases in which splenectomy was not effective. The increase occurred soon after eradication and continued for a long period without ITP treatment in many cases, preventing bleeding events and improving the quality of life in more than 60% of *H pylori*–positive ITP patients. From these results, it is highly recommended that eradication therapy be used as a first line of treatment in *H pylori*–positive ITP cases because the treatment schedule is economical and patients are not subject to adverse effects such as those of steroid and other immunosuppressive therapies.

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Helicobacter pylori Infection and Idiopathic Thrombocytopenic Purpura

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Abstract

A treatment strategy for idiopathic thrombocytopenic purpura (ITP) is considered with the aim of cure or management of the bleeding tendency. In 1998, Gasbarrini et al reported a high prevalence of *Helicobacter pylori* infection in patients with ITP and showed that platelet recovery occurred after eradication therapy in most cases. Since then, many studies were performed to evaluate eradication therapy. This article discusses the incidence of *H pylori* infection in ITP, characteristic clinical features in *H pylori*–positive ITP, the effectiveness of eradication on platelet count increase, and the mechanisms of development of ITP by *H pylori* infection. Overall, there was a positive association between *H pylori* infection and ITP, and eradication of bacterium was accompanied by a significant increase in platelet counts in more than 50% of *H pylori*–positive ITP cases. These findings suggest that *H pylori* infection is involved in the mechanisms of thrombocytopenia in most cases of ITP in middle-aged and older patients. This approach could be beneficial to some ITP patients, but there were some uncertainties raised. To confirm the effectiveness of eradication therapy in *H pylori*–positive ITP, prospective studies conducted in several countries with a new treatment protocol are required, with a large number of ITP cases and longer follow-up.

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Key words: ITP; *Helicobacter pylori* infection; Eradication; Molecular mimicry

1. Introduction

Idiopathic thrombocytopenic purpura is a disorder that is characterized by platelet destruction caused by an antiplatelet antibody and results in platelet phagocytosis via the reticuloendothelial system [1-3]. Immunosuppressive drugs such as glucocorticoids or splenectomy are recommended to suppress the platelet destruction mechanisms.

In 1998, Gasbarrini et al reported that eradication therapy in *Helicobacter pylori*–infected immune thrombocytopenic purpura (ITP) cases had reduced thrombocytopenia after 2 and 4 months [4]. Since then, eradication therapy in *H pylori*–positive ITP cases is drawing attention because it increases platelet counts easily. But the relationship between *H pylori* infection and ITP is not clear.

The incidence of *H pylori* infection in ITP, whether the characteristic clinical features are present or not in *H pylori*–positive ITP, the effectiveness of eradication on platelet count response, and the mechanisms of ITP in *H pylori* infection are discussed in this article.

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2. *H pylori* Infection and Generalized Diseases

H pylori is a Gram-negative bacterium that is the main cause of gastritis and peptic ulcers. It has also been implicated in the development of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. There is strong evidence epidemiologically that *H pylori* increases the risk of gastric cancer, and *H pylori* infection has been classified as a type I (definite) carcinogen since 1994 [5].

On the other hand, several diseases other than digestive diseases have been found to be associated with *H pylori* infection. In cardiovascular disease, an *H pylori*–specific DNA sequence was found in atherosclerotic plaque, and this organism was implicated in the development and/or progression of the disease. Iron deficiency anemia that was refractory to iron administration improved after *H pylori* eradication. Additionally, monoclonal gammmopathy disappeared after eradication therapy. These findings suggest a relation between *H pylori* infection and these generalized diseases [6]. Furthermore, many studies have reported the presence of

H pylori infection with autoimmune diseases such as chronic thyroiditis, Sjogren disease, rheumatoid arthritis, and ITP [7-9]. These relations are intriguing, and it is important to analyze the pathogenesis of these diseases and to improve their management.

3. The Incidence of *H pylori* Infection in ITP and Clinical Characteristics of *H pylori*–Positive ITP

H pylori infections are found worldwide, but the infection rate varies between countries and races within the same country. Epidemiological studies suggest that the overall prevalence of *H pylori* infection might correlate with economic conditions, age, and the spread of water filtration plants [5].

It is thought that *H pylori* is transferred to gastric mucosal surfaces by oral infection by the bacterium, and infection is established easily in infants and young children with immature immune systems. It seems likely that in industrialized countries direct transmission from person to person by vomitus, saliva, or feces predominates; additional transmission routes, such as water, may be important in developing countries. This evidence suggests that sanitary conditions and socioeconomic conditions of newborn children will affect the *H pylori* infection rate. That is, the general infection rate in the middle-aged population of developing countries is more than 80%, whereas this figure is approximately 40% in industrialized countries, and the rate is less than 10% for children younger than 10 years [5].

The *H pylori* infection rate in ITP is high in Japan and Italy, with 70% to 90% of middle-aged adult ITP patients infected. There is no evidence that the infection rate is higher in ITP patients than in the general population. The *H pylori* infection rate in ITP was the same in both sexes for all age groups [10-14].

The high incidence of *H pylori* infection in middle-aged and older ITP patients in Japan can be explained as follows. The people in these age groups passed through the unsanitary and poor economic conditions occurring after World War II, and consequently the people born in Japan in this period have an increased chance of *H pylori* infection. If true, this theory may indicate that a long incubation period is required to develop thrombocytopenia involving *H pylori* infection. Further epidemiological studies will be required to clarify this explanation and to determine whether or not *H pylori*–positive ITP will decrease in the near future.

There were no *H pylori*–positive ITP cases in children in north Europe, but in Taiwan *H pylori* was positive in 9 of 22 ITP cases in children (40%), and in 5 cases platelet counts recovered after *H pylori* eradication [15,16]. This evidence supports the idea that infection rates in children vary between countries, similarly to adult cases.

Because the incidence of *H pylori* infection in patients with ITP did not differ from that seen in the general population in Japan or Italy, *H pylori* infection in ITP is not a specific feature of the disease.

Are there any differences between *H pylori*–positive and –negative ITP patients? The reported studies showed no difference between them in sex ratio, duration of ITP, or clinical symptoms such as a bleeding tendency and initial platelet counts [10-13,17]. However, most reports pointed out that *H pylori*–positive ITP patients were significantly older than *H pylori*–negative patients, which reflects the age distribution of *H pylori* infection in the general population. These clinical findings suggest that *H pylori* infection does not influence the severity or clinical features of ITP.

The gastric mucosal lesions of *H pylori*–positive ITP cases showed pangastritis or remarkable atrophic gastritis at the corps area, findings similar to non-ITP cases. Also, the *H pylori* strains were not different from those in peptic ulcer cases in Japan [18]. These findings showed that there were no specific *H pylori* strains that produce thrombocytopenia.

4. Eradication Effects on Platelet Counts in *H pylori*–Positive ITP Cases

The eradication of *H pylori* was accompanied by a significant increase in platelet counts in most ITP cases, as reported by Gasbarrini et al in 1998 [4]. Since then, many reports about the effects of eradication therapy on platelet response in *H pylori*–positive ITP cases have been published. These are listed in Table 1 [10-13,17-25]. As seen in Table 1, the effectiveness of eradication on platelet counts varied between countries. Reports from Spain, France, and the United States showed little or no platelet response after eradication [17,20,21]. The majority of *H pylori*–positive ITP patients in Italy and Japan showed increased platelet counts after *H pylori* eradication of up to 50% to 80% [10-13,18,22,23,25]. The cause of this discrepancy can be explained by the differences in immunological backgrounds between populations or the difference of antigenicity or degree of antigen expression of infecting *H pylori*, but there was no clear evidence to support these explanations.

Most eradications were carried out with a standard 1- or 2-week regimen, entailing administration of clarithromycin (400-800 mg/d twice daily), amoxicillin (1500 mg/d twice daily), and lansoprazole (60 mg/d twice daily). The overall platelet response rate was 51%; however, it is difficult to compare the responses between studies because the evaluations of platelet response, the follow-up periods, the time of evaluation, and the previous treatments before eradication differed from study to study.

A nation-wide study in Japan had the following results [25]. Eradication therapy was performed in 207 cases, and 161 cases (78%) became *H pylori* negative. A total of 122 cases in which eradication had succeeded were further evaluated 12 months after eradication.

Complete response (CR) occurred in 28 cases (23%), with platelet counts greater than $15 \times 10^4/\mu L$ without any therapy for ITP; partial response (PR) in 51 cases (42%), with platelet counts greater than $5 \times 10^4/\mu L$ or more than 3 times the pretreatment platelet counts; and no response (NR) in the remaining 43 cases (35%). Most PR cases showed platelet counts from $10 \times 10^4/\mu L$ to $15 \times 10^4/\mu L$ and were followed up without any ITP treatment. In NR cases, the platelet counts did not change significantly from the pre- to the posteradication period. In 6 cases of the NR group, however, increased platelet counts occurred over a 6-month period and then returned to previous levels (relapsed cases), even though eradication had succeeded.

Table 1.
Idiopathic Thrombocytopenic Purpura and *Helicobacter pylori* Infection (Reported Studies)

Author (Country)	Year	No. of Cases	*H pylori* Infection Rate	Eradication-Effective Rate	Platelet-Response Cases/ Eradication-Effective Cases, n (%)*	Median Follow-up, mo
Adult cases						
Gasbarrini et al (Italy)	1998	18	11 (61%)	8/11 (73%)	8 (100%)	4
Emilia et al (Italy)	2001	30	13 (43%)	12/13 (92%)	6 (50%)	8.3
Jarque et al (Spain)	2001	56	40 (71%)	23/32 (72%)	3 (13%)	24
Veneri et al (Italy)	2002	35	25 (71%)	15/16 (94%)	11 (73%)	11.7
Kohda et al (Japan)	2002	48	27 (56%)	19/19 (100%)	12 (63%)	14.8
Hino et al (Japan)	2003	30	21 (70%)	18/21 (86%)	10 (56%)	15
Hashino et al (Japan)	2003	22	14 (64%)	13/14 (93%)	5 (39%)	15
Ando et al (Japan)	2003	61	50 (82%)	27/29 (93%)	16 (59%)	11
Michel et al (USA)	2004	76	16 (21%)	14/16 (93%)	0/14 (0%)	11.5
Veneri et al (Italy)	2004	21	19 (91%)	19/21 (91%)	14 (74%)	18.1
Takahashi et al (Japan)	2004	20	15 (75%)	13/15 (87%)	7 (54%)	4
Fujimura et al (Japan)	2004	435	300 (69%)	161/207 (78%)	79/122 (65%)	>12
Ando et al (Japan)	2004	20	17 (85%)	15/17 (88%)	10 (67%)	24
Sato et al (Japan)	2004	53	39 (74%)	27/32 (84%)	15 (56%)	6M
Kurtoglu et al (Turkey)	2004	38	26 (68%)	—	—	—
Total		963	633 (66%)	384/463 (83%)	196 (51%)	>12.8
Child cases						
Rajantie et al (Finland)	2003	17	0/17 (0%)	—	—	—
Yang et al (Taiwan)	2003	22	9 (41%)	—	5/9 (56%)	24
Fujimura et al (Japan)	2004				9/33 (27%)*	>12

*For Fujimura et al, n (%) given for platelet response in eradication-ineffective adult cases.

On the other hand, in several cases platelet counts increased even though eradication did not succeed (5 cases [15%] in CR and 4 cases [12%] in PR of 33 unsuccessful eradication cases) (Table 1). There was no clear evidence to explain these data, but we speculated that this result may have been due to false-positive ^{13}C urea breath test (UBT) results, an inadequate amount of time allowed to judge the effect of the eradication, the eradication drug itself acting to increase platelet counts, or spontaneous remission. These cases must be collected and followed up for a long time to observe whether their platelet counts maintain a high level or not, and/or the ^{13}C UBT test results must be reexamined to reconfirm eradication failure.

The results of this nationwide retrospective study confirmed the previous reports on large-scale groups and also indicated that the eradication effect on platelet count continues for long time without medication and that relapse cases are few.

As in *H pylori* infection–negative ITP cases, eradication effects on platelet count were not found in secondary immune thrombocytopenias such as systemic lupus erythematosus or Evans syndrome, despite *H pylori* infection being positive [11,17]. This observation indicates that the eradication effect on platelet count is a limited phenomenon in *H pylori*–positive ITP cases.

The eradication effects on *H pylori*–positive ITP are summarized as follows: (1) platelet count response rates were significantly high in the successful eradication group [12,14,22,23,25]; (2) platelet counts increased by more than 51%, but the response grade is variable between studies; (3) the increment of platelet counts began 1 month after eradication therapy in responsive cases [10-12, 25]; (4) cases of relapse were few [10,25]; (5) disease duration of ITP was shorter in the platelet count response group than in the nonresponse group [25]; (6) in some cases in which previous platelet counts were below $1 \times 10^4/\mu L$, platelet counts tended to increase to below $5 \times 10^4/\mu L$ after eradication, but clinical improvements were observed [25]; (7) most platelet response cases did not require additional medication for ITP [4,10-13,25]; (8) platelet count response was also found in cases refractory to steroids or splenectomy [10-13,23,25]; (9) previous treatments before eradication did not influence the response rate for platelet counts [10,13,23,25]; (10) platelet count response rates were not different in any age group [15,25]; (11) the eradication effects on platelet counts were found in primary ITP, not in secondary ITP [11]; and (12) bacterial eradication and platelet recovery were accompanied by the disappearance of platelet autoantibodies (platelet autoantibody immunoglobulin G [PAIgG]) in most cases [11-13,22].

5. The Mechanisms of Immune Thrombocytopenia with *H pylori* Infection

Although clinical observations suggest the involvement of *H pylori* infection in the pathogenesis of ITP, there is no clear evidence to explain the mechanism.

In *H pylori,* it is known that the vacuolating cytotoxin Vac A is secreted as an exotoxin, causes the release of cytochrome c, and induces apoptosis, which then induces tissue damage [5]. Cag A, which is encoded by the *cag* pathogenicity island (*cag*-PAI), translocates into the host cells and is phosphorylated and binds to SHP-2 tyrosine phosphatase, thus inducing cellular response and cytokine production by

the host cell and also producing a high antigenicity [5]. The gastric lesions such as gastritis or ulcer formation can be explained by this process.

Why do these generalized immune reactions occur? Generally speaking, the products of microorganisms act as adjuvants and subsequently stimulate the innate immune response, producing inflammatory cytokines and perhaps helping to establish pathological regions [25]. Microbial products can induce T-cell responses to self as well as foreign antigens [26]. Several reports cite the association between the onset of various autoimmune diseases and infections such as rheumatic fever (Streptococci infection) or multiple sclerosis (Epstein-Barr virus/capsid) [27].

It was reported, however, that there was no difference between the *H pylori*–infected and –noninfected groups in the lymphocyte subset of peripheral blood or the appearance in organs of nonspecific (antinuclear antibody) or specific autoantibodies (such as microsome antibody or smooth muscle antibody) [28,29]. Also, in *H pylori*–infected ITP cases, the cytokine profiles in blood such as interferon γ, interleukin (IL)-2, IL-4, and IL-6 were not different from those in noninfected ITP cases [12].

Several hypotheses have been suggested concerning the mechanisms by which *H pylori* infection may cause ITP.

5.1. Involvement of Lewis antibody

High Lewis antibody titer was detected in some *H pylori*–infected cases. This antibody binds to several tissues that have cross antigen and may cause immune reactions. For example, H^+-K^+–adenosine triphosphatase of parietal cells having an epitope of the Lewis antibody causes parietal cell damage and produces atrophic gastritis [30,31]. One hypothesis is that this Lewis antibody binds to platelets nonspecifically and induces thrombocytopenia [32].

5.2. Molecular Mimicry and Epitope Progression

Infectious agents affect the ability of T-cells to detect self antigens by cross reaction (molecular mimicry) [27]. If the agent peptide codes are closely related to a peptide of the host, active responses may induce T-cells, which can then react against cells bearing the cross-reacting self antigen. In *H pylori* infection, Cag A antigen might be one of the candidates for pathological immune reactions. There might be cross mimicry between *H pylori* and platelet antigens. Takahashi et al reported that the platelet eluates of 12 of 18 *H pylori*–positive ITP cases could recognize Cag A protein with high specificity, and that cross-reactive antibodies disappeared following eradication in patients showing a platelet response according to the immunoblotting method. Additionally, platelet eluates from eradication-failure ITP cases did not decrease the reaction. On the other hand, platelet eluates from *H pylori*–negative ITP cases reacted to Cag A protein, indicating that the platelet surface antigen peptide that reacts to the platelet antibody (PAIgG) is very similar to the Cag A protein [22].

Takahashi et al also observed that *H pylori* infection might induce an imbalanced immune reaction, because the T-helper 1/T-helper 2 (Th1/Th2) ratio in T-cells was elevated in eradication-effective, platelet count–response cases [33]. However, these observations were not consistent with other reports. The French group reported that, according to immunoblotting results, platelet eluates from 3 *H pylori*–positive ITP patients did not cross react with Cag A protein [21]. Another report showed no change in the Th1/Th2 ratio in eradication-effective cases [12]. These discrepancies might have been caused by the difference of bacterial strains. Antigen mimicry between Cag A and the platelet membrane must be confirmed by further studies from various countries in the future.

Although negative evidence is present, the antigen-mimic theory offers an attractive explanation of the clinical evidence. For example, Cag A protein, which has antigen mimicry with a peptide from a platelet membrane protein, may activate helper T-cells or autoreactive T-cells and initially produce B-cell proliferation for antibody production. Some of these antibodies will cross react with the platelet membrane, causing thrombocytopenia. If this phenomenon continues for a long time, epitope progression will occur and another epitope may stimulate B-cell clones to produce another platelet membrane autoantibody [27].

This epitope progression theory is a convenient explanation of the clinical observations of a variety of platelet responses after eradication, such as some cases becoming CR and other cases PR or NR, and the platelet response being found in the shorter disease-duration group [25]. Because antibodies to the platelet membrane, which are different from the crossing epitope of *H pylori*, will be produced over time, these antibodies may not disappear after eradication, and therefore immunosuppressive treatment may be required for platelet counts to recover completely. This hypothesis must be confirmed by clinical and laboratory studies.

5.3. Platelet Activation by Anti–H pylori Antibody and von Willebrand Factor Causes Thrombocytopenia

It was reported [34] that some *H pylori* strains had induced platelet aggregation in the presence of von Willebrand Factor (vWF) and anti–*H pylori* antibodies. That is, *H pylori* may interact with platelets through vWF and IgG (anti-*H pylori* antibody), and their corresponding receptors, GPIb/IX/V complex and FcγRIIA, which colocalize with GPIb on platelets. The interaction between platelets and *H pylori* may contribute to the pathogenesis of *H pylori* associated with peptic ulcers by inducing platelet activation locally, or thrombocytopenia by causing chronic platelet consumption.

6. Genetic Influences in Development of Thrombocytopenia in *H pylori* Infection

Although a large number of people in the world are infected with *H pylori*, a very small number develop thrombocytopenia. This important point was investigated according to genetic background. Veneri et al compared the HLA class II allele patterns of ITP patients with and without *H pylori* infection [35]. They found a lower frequency of HLA-DRB1*11 and HLA-DQB1*03 in typical ITP with-

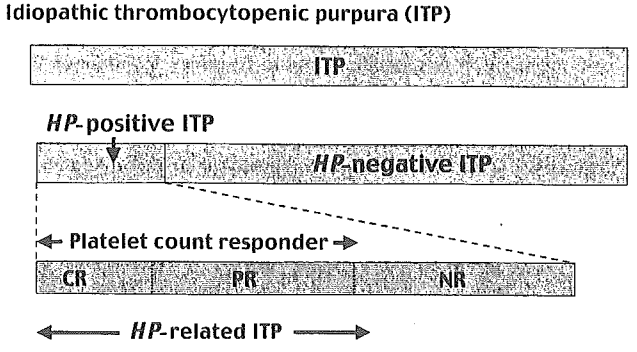

Figure 1. Concept of *Helicobacter pylori* (HP)-related idiopathic thrombocytopenic purpura (ITP). HP-related ITP cases make up part of the total number of ITP cases. HP-related ITP should be differentiated from HP-positive ITP. HP-related ITP cases are delimited here to show the HP eradication effect on platelet count recovery. CR indicates complete response; PR, partial response; NR, no response.

out *H pylori* infection than in healthy controls. But in *H pylori*–positive ITP cases, these HLA allele levels were similar to healthy controls, and HLA-DRB1*03 levels were lower and HLA-DRB1*14 levels higher than those of *H pylori* infection–negative ITP cases.

These observations suggest that persons who have one of the HLA allelic patterns described above may develop ITP with *H pylori* infection. However, because the HLA system is different among various human races, these results are not general. For example, ITP cases in Japanese people showed a higher frequency of HLA-DRB1*04 than healthy controls.

Genetic background is an important factor to investigate in the mechanisms by which *H pylori* may cause ITP, and disease susceptibility of HLA must be examined in each race [36].

7. Future Directions in the Treatment of *H pylori*–Positive ITP Cases

Many clinical reports have suggested that *H pylori*–positive ITP has some characteristic features. Eradication therapy in this group is often accompanied by an increase in platelet counts of more than 50%, suggesting an important advance in ITP treatment [37]. This eradication-effective group may contribute to stratifying different subgroups of ITP, termed *H pylori*–related ITP (Figure 1). It is recommended that eradication therapy be selected as a first line of treatment in *H pylori*–positive ITP cases because the side effects are tolerable, the treatment schedule is short, there is an economic benefit, and there are fewer adverse effects than occur in steroid or other immunosuppressive therapies. A prospective study should confirm the effectiveness of eradication therapy and establish the disease entity of *H pylori*–related ITP.

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To the editor:

Protein S–K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients

Deep vein thrombosis (DVT) is a multifactorial disease caused by interactions between acquired risk factors and coagulation abnormalities.[1] In whites, the factor V–Leiden and the prothrombin-20210G>A are widely recognized as genetic risk factors for DVT. However, these 2 mutations are not present in Japanese populations, and little is known about the genetic risk factors for DVT in these populations. In this study, we evaluated the genetic contributions of 5 polymorphisms in Japanese DVT patients. The plasminogen-A620T mutation, formerly referred to as plasminogen-Tochigi, and the protein S–K196E mutation, formerly referred to as protein S–Tokushima, exhibited decreased activities of plasminogen and protein S despite normal antigen levels.[2-4] The ADAMTS13-P475S mutation exhibited low von Willebrand factor–cleaving activity in vitro.[5] The factor XII–4C>T substitution in the 5′-untranslated region, formerly referred to as 46C>T, showed decreased plasma levels of both antigen and activity.[6] The plasminogen activator inhibitor-1 (PAI-1) 4G/5G polymorphism is related to in vitro differences in transcription activity.[7] We genotyped subjects for these 5 polymorphisms and compared their genotypic frequencies between 161 DVT patients and 3655 population-based controls. The protocol for this study was approved by the ethical review committee, and only those subjects who provided written informed consent for genetic analyses were included in this study. All participants of this study were Japanese. The controls were from a general population randomly selected from the residents of Suita City located in the second largest urban area in Japan (the Suita Study).[8] One hundred sixty-one DVT patients, 78 men and 83 women, were registered by the Study Group of Research on Measures for Intractable Diseases, working under the auspices of the Ministry of Health, Labor, and Welfare of Japan. Six centers (Tochigi, Tokyo, Nagoya, Kyoto, and 2 in Osaka) participated in this study. The patients' mean age was 49.5 years (range, 12-87 years) and their mean body mass index was 23.6 ± 3.3. Thirteen percent of patients had a family history of thrombosis, and 16% of the patients had recurrent thrombosis.

Of all the polymorphisms tested, only the frequency of protein S–K196E was statistically different between the 2 groups ($\chi^2 = 38.3$, $P < .001$) (Table 1). No other frequency differences were statistically significant. Two DVT patients were homozygous for the protein S–196E allele; however, no homozygotes were identified in the control group. One patient with the 196EE genotype first developed DVT following surgery at age 47, while the other patient developed DVT during pregnancy at age 32.

The mutant protein S with the E allele has already been intensively studied as protein S–Tokushima.[11] The protein S mutant showed the reduced activated protein C cofactor activity compared with wild-type protein S, suggesting a direct link between the protein S–K196E mutation and the development of DVT. By the genotyping of the general population, the protein S–196E allele frequency was estimated as 0.009. Thus, a substantial portion of the Japanese population harbors this mutant allele and is at higher risk for DVT.

Table 1. Numbers and genotypic frequencies of protein S–K196E mutation in the DVT and control groups

Genotypes	General population, no. (%)	DVT group, no. (%)
Additive model*		
KK	3585 (98.2)	146 (90.7)
KE	66 (1.8)	13 (8.1)
EE	0 (0.0)	2 (1.2)
Total	3651 (100.0)	161 (100.0)
Dominant model†		
KK	3585 (98.2)	146 (90.7)
KE + EE	66 (1.8)	15 (9.3)
Total	3651 (100.0)	161 (100.0)

DNA genotyping was performed by the TaqMan allele discrimination method.[9] We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide + 1, and the initial Met residue is denoted as amino acid + 1, resulting in the renaming of several mutant alleles.[10] Comparisons between the DVT cases and the controls were analyzed using a χ^2 test with the genotypes as independent variables (indicated by P and OR) or using multiple logistic analyses with the genotypes as independent variables and age and sex as covariates (indicated by P' and OR′).

*For comparison of general population to DVT group, P was not determined.

†For comparison of general population to DVT group, $P < .001$; OR = 5.58 (3.11-10.01); $P' < .001$; OR′ = 4.72 (2.39-9.31).

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Adenovirus-Mediated Transfer of Human Placental Ectonucleoside Triphosphate Diphosphohydrolase to Vascular Smooth Muscle Cells Suppresses Platelet Aggregation In Vitro and Arterial Thrombus Formation In Vivo

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Background—Platelet-rich thrombus formation is a critical event in the onset of cardiovascular disease. Because ADP plays a significant role in platelet aggregation, its metabolism is important in the regulation of platelet activation and recruitment. Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) is a key enzyme involved in vascular ADP metabolism. We recently isolated 2 isoforms of E-NTPDase from the human placenta. The present study examined whether these isoforms suppress platelet aggregation and thrombus formation after adenovirus-mediated gene transfer to vascular smooth muscle cells (SMCs).

Methods and Results—We constructed adenovirus vectors expressing human placental E-NTPDase isoforms I (AdPlac I) and II (AdPlac II) or bacterial β-galactosidase (AdLacZ). Vascular SMCs infected with AdPlac I expressed significant NTPDase activity and inhibited the platelet aggregation induced by ADP and collagen in vitro. In contrast, SMCs infected with AdPlac II and AdLacZ did not exert antiplatelet effects. To investigate the antithrombotic and antiproliferative effects of placental E-NTPDase isoform I in vivo, we generated thrombosis in rat carotid arteries by systemically administered rose Bengal and transluminal green light 5 days after gene transfer and examined neointimal growth 3 weeks after thrombus formation. Blood flow in AdLacZ-infected arteries rapidly deteriorated and vanished within 96±18 seconds of occlusive thrombus formation. In contrast, blood flow in AdPlac I–infected arteries was preserved for at least 10 minutes during irradiation. In addition, thrombus formation and subsequent neointimal growth were obviously suppressed.

Conclusions—The local expression of placental E-NTPDase in injured arteries might prevent arterial thrombosis and subsequent neointimal growth. (***Circulation.* 2005;111:808-815.**)

Key Words: platelets ■ thrombosis ■ genetics ■ muscle, smooth ■ arteries

Thrombus formation is a critical event in the onset of cardiovascular disease. Thrombotic occlusion still occasionally arises after vascular interventions, such as angioplasty and stent implantation. Platelets play a pivotal role in the development of arterial thrombosis. Therefore, antiplatelet agents, including aspirin, ADP receptor blockers, and platelet glycoprotein (GP) IIb/IIIa antagonists, are systemically administered to prevent cardiovascular events.[1] Although some of these agents effectively reduce cardiovascular events,[1] they can also produce systemic hemorrhagic side effects.[2]

Platelet adhesion to injured vascular walls leads to platelet activation and the release of additional agonists such as ADP, serotonin, and thromboxane A_2, which cause further platelet recruitment to injured sites. Because ADP plays a key role in platelet aggregation,[3] its metabolism in the blood is important in the regulation of platelet activation and recruitment. Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase; EC 3.6.1.5, or CD39) is a major metabolic enzyme of ADP in the vasculature.[4] Originally identified in B lymphocytes,[5] E-NTPDase is a membrane-bound enzyme that rapidly hydrolyzes both ATP and ADP to AMP and thereby inhibits platelet aggregation.[6–8] Makita et al[9] purified human placental E-NTPDase (Plac I) as an isoform of CD39 that alternatively differed at the *N*-terminus from that of CD39. Matsumoto et al[10] recently isolated another truncated variant

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of placental E-NTPDase (Plac II). Although these structural differences might affect their enzymatic activities,[11,12] the properties of the 2 isoforms remain unknown.

The present study used adenovirus-mediated gene transfer into vascular smooth muscle cells (SMCs) in vitro and in vivo to investigate whether the 2 isoforms of placental E-NTPDase inhibit platelet aggregation, thrombus formation, and neointimal growth.

Methods

Preparation of Recombinant Adenovirus With Placental E-NTPDase

Replication-defective E1$^-$ and E3$^-$ adenoviral vectors encoding Plac I (AdPlac I) and Plac II (AdPlac II) were generated with the Adeno-X expression system (Clontech) according to the manufacturer's instructions.[13] In brief, cDNA clones of Plac I (1554 bp) and Plac II (921 bp) were isolated at the Department of Blood Transfusion Medicine of Nara Medical University[10] and subcloned into the mammalian expression cassette pShuttle. Recombinant pShuttle was digested and inserted into the Adeno-X viral DNA. The recombinant viruses were propagated in HEK293 cells. Viral titers were determined by limiting dilution as plaque-forming units (PFU).[14] We also constructed control recombinant adenovirus encoding bacterial β-galactosidase (AdLacZ).

Cell Culture

Arterial SMCs were isolated from explanted thoracic aortas of Sprague-Dawley rats. Cells were cultured in SmGM2 growth medium (Sanko Junyaku) containing 5% fetal bovine serum and antibiotics. Confluent cells were immunopositive to anti-smooth muscle actin antibody (Dako Japan).

In Vitro Gene Transfer

Cells from passages 3 to 6 were incubated with the adenovirus vectors in the serum-free SmGM2 medium at the indicated multiplicity of infection (MOI). After incubation for 24 hours at 37°C, cells were washed twice with sterile phosphate-buffered saline and incubated in complete medium until assay.

Western Blots

Four days after infection, cells were lysed in Tris-buffered saline (pH 8.0) containing 150 mmol/L NaCl, 1% Nonidet P-40, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 12 000*g* for 5 minutes, the supernatant was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 4% to 12% gradient gels, and then separated proteins were electrophoretically transferred to an Immobilon membrane (Millipore). Nonspecific binding was blocked with 5% skimmed milk, and then the membrane was incubated overnight at 4°C with a monoclonal anti-human placental E-NTPDase antibody (YH34). This novel antibody was produced in mice by a standard procedure at the Department of Blood Transfusion Medicine of Nara Medical University. Purified placental E-NTPDase used as an immunogen was prepared on an immunoadsorbent column as described.[9] Western blotting showed that YH34 bound with high affinity to the purified enzyme (≈80 kDa) under both nonreducing and reducing conditions (data not shown). We determined the immunoglobulin subclass and idiotype of YH34 (IgG_1-κ) by using an isotyping kit (Serotec). We purified YH34 IgG from mice ascites on a protein A–Sepharose CL-4B column (Amersham Bioscience Corp). The antibody recognizes the external domain of both Plac I and Plac II. Specific binding was visualized with a horseradish peroxidase–conjugated goat anti-mouse IgG (Nacalai Tesque) and an enhanced ChemiLuminescence reagent (DuPont NEN).

Analysis of NTPDase Activity

The activity of NTPDase in transfected SMCs was determined by measuring extracellular ATP and ADP concentrations with luciferin-luciferase.[15] In brief, cells (5×10^3) were seeded in 96-well plates and cultured in serum-free SmGM2 medium with 0.1% bovine serum albumin for 2 days. After 2 washes with phosphate-buffered saline, 100 μL of firefly luciferase ATP assay mixture (ATP determination kit, Molecular Probes) was added to each well. Luminescence was measured with a microplate luminometer (Fluoroskan Ascent FL, Labsystems). After 10 minutes of equilibration, 100 nmol/L exogenous ATP was added, and luminescence was measured at 1-minute intervals for up to 15 minutes. ATPase activity, calculated by measuring the degradation of exogenous ATP, was expressed as picomoles of inorganic phosphate per minute per milligram.

We estimated the ADP concentration by converting ADP to ATP.[16] After the cells were washed and the firefly luciferase ATP assay mixture was added, 100 nmol/L exogenous ADP was added to each well. After a 5-minute incubation, 1 U pyruvate kinase and 1 mmol/L phosphoenolpyruvate were added and luminescence was measured. ADPase activity, calculated by measuring the degradation of exogenous ADP, was expressed as picomoles of inorganic phosphate per minute per milligram.

Platelet Aggregation

We evaluated platelet aggregation in the presence of SMCs by using a modification of a described method.[17] Uninfected or infected SMCs detached by EDTA-collagenase were washed 3 times. Blood samples collected in 3.8% sodium citrate (9:1, vol/vol) were centrifuged at 900 or 3000 rpm for 10 minutes to prepare platelet-rich plasma (PRP) or platelet-poor plasma (PPP), respectively. PRP (5×10^8 platelets) and SMCs (5×10^2 cells) were incubated in siliconized cuvettes at 37°C in a PA-20 aggregometer (Kowa) that had been calibrated with PRP and PPP for 0% and 100% transmission, respectively. Thereafter, either ADP (10 μmol/L, final concentration) or collagen (10 μg/mL, final concentration) was added to the cuvettes, and platelet aggregation was measured.

Animal Care

The Animal Care Committee of Miyazaki Medical College (No. 1998-025-6) approved the study protocols. We used 59 male Sprague-Dawley rats weighing 400 to 500 g. The animals received humane care according to the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National (Bethesda, Md) Institutes of Health (NIH publication No. 86-23, revised 1985). Aseptic surgery proceeded under general anesthesia induced by an intraperitoneal injection of pentobarbital (50 mg/kg body weight).

In Vivo Gene Transfer Into Injured Arteries

The common carotid arteries of the rat were exposed and isolated by temporary ligation at a distance of 1.5 cm. A 31-gauge needle was inserted into the proximal side of the segment. Thereafter saline was flushed into the segment, and then air was infused at a rate of 50 mL/min for 3.5 minutes to denude endothelial cells.[18] After the segment was filled with 0.1 mL of saline containing AdPlac I or AdLacZ (final titer, 5×10^8 PFU) or saline alone (n=6 each) for 30 minutes, the mixture was aspirated and blood flow was restored.[19] The vessels harvested 2 to 5 days after gene transfer were perfusion-fixed in 4% paraformaldehyde and embedded in paraffin by standard procedures. We detected the expression of human placental E-NTPDase protein by immunohistochemistry (EnVision+ kits) by using the primary monoclonal antibody YH34. The negative control included nonimmune mouse IgG_1 instead of YH34.

NTPDase Activity in Vessels

Carotid arteries without perfusion-fixation were excised and homogenized with a Polytron PT3000 (Kinematica) in Tris-buffered saline (pH 7.4) containing aprotinin and phenylmethylsulfonyl fluoride.[20,21] The homogenates were then incubated with the firefly luciferase ATP assay mixture. Extracellular ATP and ADP concentrations were

determined by measuring luminescence as described earlier. Protein concentrations of homogenates were determined by the bicinchoninic acid assay with bovine serum albumin as the standard.

Arterial Thrombus Formation and Neointimal Growth

Thrombus formation was produced in rat common carotid arteries 5 days after gene transfer by photochemical exposure under anesthesia.[22] Blood flow in the distal side of the arteries was recorded with a transit-time blood flowmeter (T106, Transonic Systems Inc) with a PowerLab system (AD Instruments Pty Ltd). After baseline blood flow was established, rose Bengal (20 mg/kg; Wako) was slowly injected into the jugular vein, and the carotid arteries were irradiated with green light (wavelength, 540 nm) with a xenon lamp (Hamamatsu Photonics) equipped with heat-absorbing and green filters. Blood flow was measured during irradiation for 10 minutes, and then the arteries were perfusion-fixed and stained with hematoxylin and eosin/Victoria blue for histological examination. To evaluate the neointimal growth 3 weeks after thrombus formation, the areas (μm^2) of neointima and media were measured with an image analyzing system (Axio Vision 2.05, Carl Zeiss) by 2 investigators (K.M. and K.H.) who were blinded to the treatment assignment.

Ex Vivo Platelet Aggregation, Prothrombin Time, and Activated Partial Thromboplastin Time

To confirm a systemic antithrombotic effect by local gene transfer, we evaluated platelet aggregation in response to either ADP or collagen ex vivo as described earlier. Prothrombin time and activated partial thromboplastin time were measured with a coagulation timer (Behring Fibrintimer, Behring Diagnostics) before and 5 days after gene transfer.

Statistical Analysis

All data are presented as mean±SE. An unpaired Student *t* test and ANOVA with Bonferroni multiple comparisons were used for comparisons between groups. A value of $P<0.05$ was considered significant.

Results

Expression of Human Placental E-NTPDases and NTPDase Activity of Infected SMCs In Vitro

We examined whether SMCs infected with AdPlac I, AdPlac II, or AdLacZ can produce biologically active E-NTPDases. Figure 1 shows 82- and 50-kDa immunoreactive bands that were detected from 2 days after gene transfer in lysates of cells infected with AdPlac I and AdPlac II, respectively.[9] These proteins persisted for at least 8 days thereafter (data not shown) but were undetectable in lysates of parental SMCs or of those infected with AdLacZ.

The ATPase activities of uninfected SMCs and of those infected with AdLacZ were 77.5±5.5 and 79.3±8.2 pmol $Pi \cdot min^{-1} \cdot mg^{-1}$, respectively (Figure 2A). In contrast, the ATPase activity of SMCs infected with AdPlac I was significantly high at an MOI of 100. We found that the ADPase activity of SMCs infected with AdPlac I was also high in an MOI-dependent manner (Figure 2B); however, the activities of ATPase and ADPase in AdPlac II–infected SMCs were similar to those of parental and AdLacZ-infected SMCs.

Platelet Aggregation With Infected SMCs In Vitro

We investigated whether the expression of Plac I and Plac II in SMCs inhibits platelet aggregation in vitro. Platelet aggregation induced by ADP was significantly and MOI-dependently suppressed in the presence of SMCs infected with AdPlac I, but not when the cells were infected with AdPlac II or AdLacZ (Figure 3A). Collagen-induced platelet aggregation was significantly suppressed in the presence of SMCs infected with AdPlac I at an MOI of 100 but not at that of 10 (Figure 3B). AdPlac I–infected SMCs dose-dependently suppressed platelet aggregation (data not shown), whereas SMCs infected with either AdPlac II or AdLacZ did not affect ADP/collagen-induced aggregation.

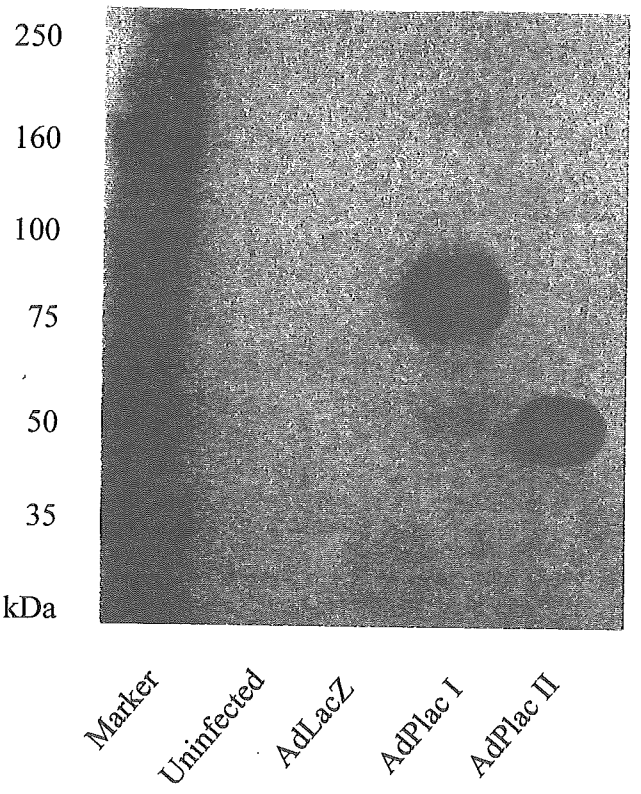

Figure 1. Expression of human placental E-NTPDases in infected SMCs 4 days after gene transfer. Proteins of whole-cell extracts from SMCs infected or not with AdLacZ, AdPlac I, or AdPlac II were separated by SDS-PAGE and immunoblotted against monoclonal anti-human placental E-NTPDase antibody YH34.

Inhibition of Arterial Thrombus Formation and Neointimal Growth in Arteries Infected With AdPlacI In Vivo

Five days after gene transfer, Plac I protein was broadly expressed in the medial SMCs of arteries infected with AdPlac I (Figure 4A) but was undetectable in control arteries infected with AdLacZ (Figure 4C). Arteries infected with AdPlac I were immunonegative for Plac I protein when the antibody was replaced with nonimmune mouse IgG_1 (Figure 4B). Figure 5 shows NTPDase activities in infected arteries (Figure 5). Two days after endothelial denudation with a saline infusion and AdPlac I infection, ATPase and ADPase activities were significantly reduced as compared with those of normal carotid arteries (control) by endothelial denudation. Five days after infection with AdPlac I, however, ATPase and ADPase activities were 2.0- and 1.7-fold higher, respectively,

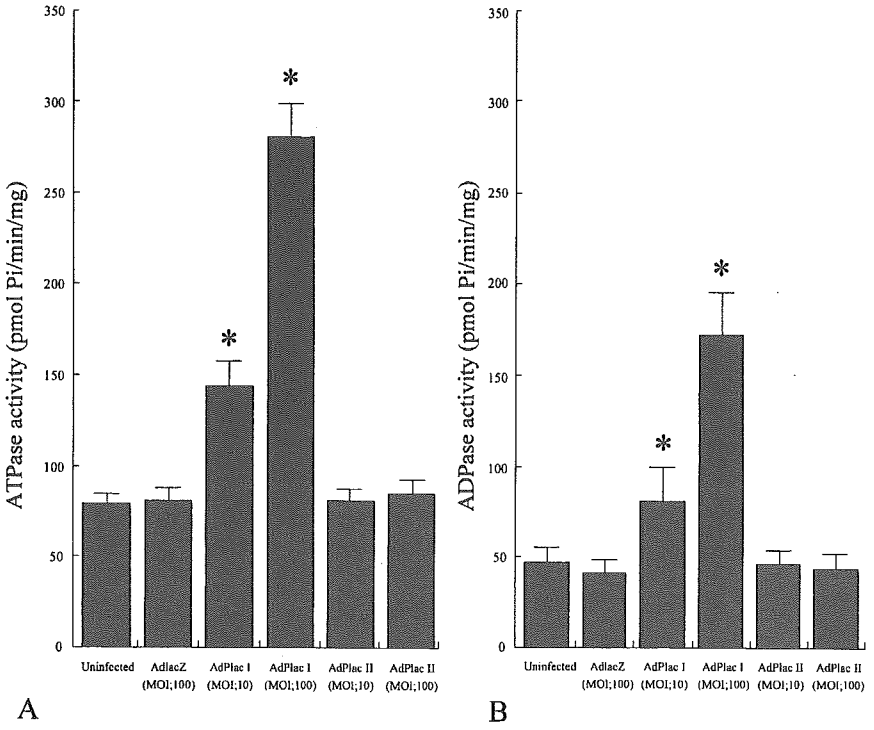

Figure 2. NTPDase activity of infected SMCs. Activities of NTPDase in transfected SMCs were determined by measuring extracellular ATP and ADP concentrations. Activities of ATPase (A) and ADPase (B) were determined in SMCs infected or not with AdLacZ at MOI 100, AdPlac I at MOI of 10 or 100, or AdPlac II at MOI of 10 or 100 (n=5 each; *$P<0.05$ vs AdLacZ).

than those after AdLacZ infection. The numbers of mRNA copies of Plac I per 10^6 copies of the housekeeping gene, glyceraldehyde 2-phosphate dehydrogenase, 2 and 5 days after gene transfer were 15.2±3.5 and 1456±65, respectively. These results indicated that Plac I protein expressed in carotid arteries had biological NTPDase activity in the vascular wall.

Blood flow in arteries infected with AdLacZ rapidly deteriorated after irradiation and vanished within 96±18 seconds (n=6, Figure 6A). In contrast, blood flow in arteries infected with AdPlac I was preserved for at least 10 minutes during green light irradiation (n=6, Figure 6B). Histological analyses revealed that occlusive thrombi, mainly consisting of aggregated platelets, had blocked arteries infected with AdLacZ (Figure 6C), whereas only small mural thrombi had developed in arteries infected with AdPlac I (Figure 6D). The neointimal growth 3 weeks after thrombus formation was significantly reduced by AdPlac I infection. Area of neointima and neointima/media ratio in arteries infected with AdLacZ versus AdPlac I were 97 500±14 000 μm^2 versus 45 300±5900 μm^2 (n=8 each, $P<0.01$) and 1.03±0.13 versus 0.41±0.05 (n=8 each, $P<0.001$), respectively.

Platelet Function and Blood Coagulation in Rats Infected With AdPlac I

Platelet aggregation induced by ADP and collagen, prothrombin time, and activated partial thromboplastin time did not significantly differ between rats infected with AdPlac I and AdLacZ (see Table).

Discussion

We have demonstrated that human placental E-NTPDase isoform I expressed in vascular SMCs hydrolyzed ATP/ADP, prevented platelet aggregation in vitro, and significantly suppressed photochemically induced arterial thrombus formation, as well as subsequent neointimal growth, in vivo. Placental E-NTPDase isoform II expressed in SMCs did not exert antiplatelet effects.

Fresh platelet-rich thrombi frequently develop in association with cardiovascular events, including unstable angina and acute myocardial infarction, as well as immediately after interventions such as angioplasty, stent implantation, and atherectomy.[23,24] Platelet activation induced by ADP plays a pivotal role in arterial thrombus formation.[3] In normal vessels, ADP is rapidly metabolized to AMP by E-NTPDase on the endothelial cell surface, which is subsequently converted to adenosine by the 5′-nucleotidase, also localized on the endothelial cell membrane. Recent in vivo and in vitro gene transfer studies of NTPDase have shown that increased NTPDase activity in the vasculature confers vascular protective effects and also survival benefits on cardiac grafts by blocking thrombotic sequelae.[25,26] Therefore, retaining high NTPDase activity in vascular SMCs should reduce the incidence of thrombus formation after vascular injury.

Vascular E-NTPDase was identified along with CD39, which has 2 putative transmembrane domains and an extracellular domain containing an enzymatically active region.[11,12] The extracellular domain contains 5 apyrase conserved regions (ACRs), of which ACR-1, -4, and -5 are important for maintaining enzymatic activity.[12] Plac I also has 2 putative transmembrane domains and an extracellular domain like CD39, whereas Plac II lacks ACR-5 and a putative transmembrane sequence in the *C*-terminal region.[10] Our results indicate that ACR-5 is essential for NTPDase activity.

Thrombus formation was almost completely suppressed in arteries infected with AdPlac I. Makita et al[9] showed that placental E-NTPDase blocks platelet aggregation under low shear stress (12 dyne/cm^2) but did not significantly inhibit

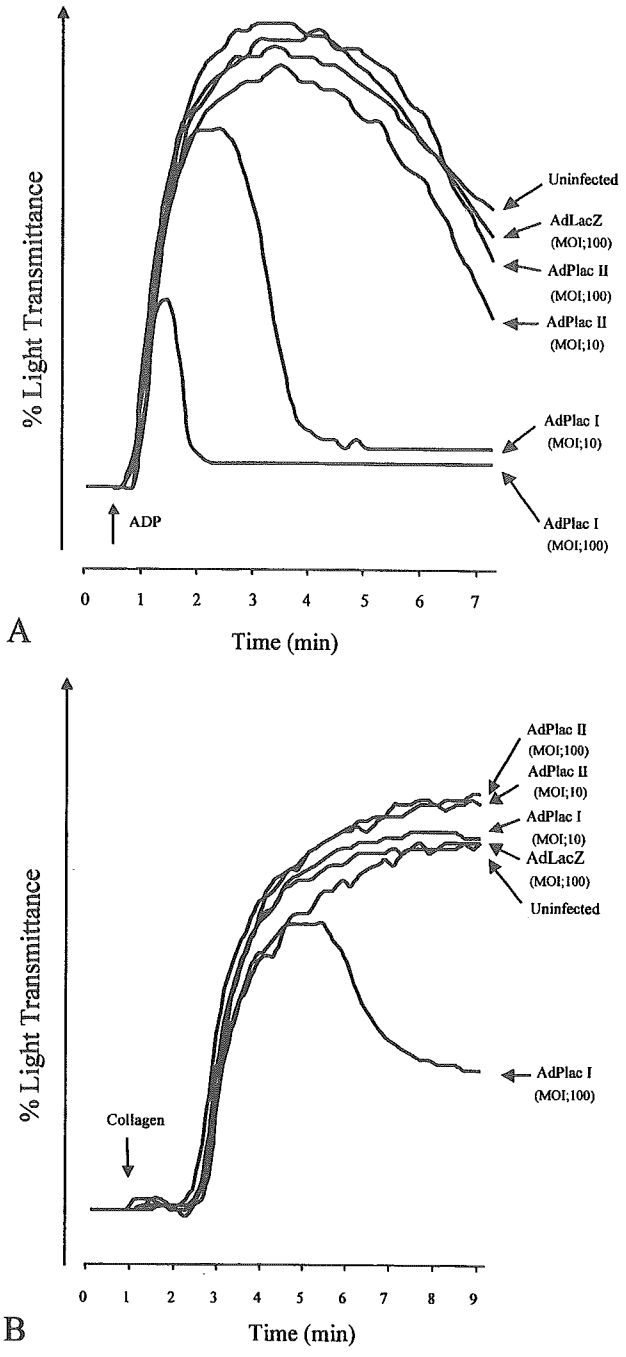

Figure 3. Platelet aggregation in presence of SMCs induced by ADP and collagen. ADP (A) or collagen (B) was added to PRP in presence of uninfected SMCs or SMCs infected with AdLacZ (MOI=100), AdPlac I (MOI=10 and 100), and AdPlac II (MOI=10 and 100), and platelet aggregation was measured.

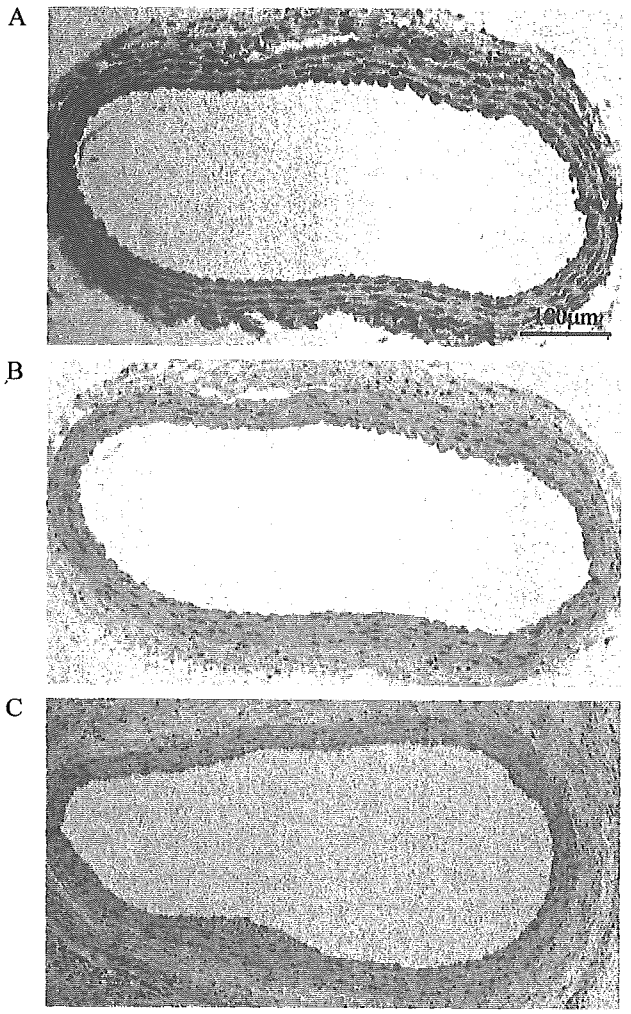

Figure 4. Immunohistochemical staining for human placental E-NTPDase in infected carotid arteries. Representative immunohistochemical microphotographs of rat carotid arteries infected with AdPlac I (A and B) or AdLacZ (C). SMCs in whole wall are immunopositive for human placental E-NTPDase in artery infected with AdPlac I (A) but not with AdLacZ (C). Replacing antibody with nonimmune mouse IgG_1 generated immunonegative reactions (B).

initial aggregation under high shear stress (108 dyne/cm^2), although platelets disaggregated during the later phase in vitro. These results imply that E-NTPDase plays an antithrombotic role under relatively low flow conditions. The finding that this enzyme localizes on syncytiotrophoblasts and in endothelial cells of the umbilical vein, rather than the artery,[9] might be consistent with the results in vitro; however, the present study showed that arterial thrombus formation under high flow conditions was obviously suppressed in vessels infected with AdPlac I. Interactions between von Willebrand factor and the platelet membrane receptors GP Ib and IIb/IIIa are crucial for platelet aggregation when blood flow is high,[27] and ADP and its receptors are key mediators of such interactions.[28,29] Thrombus formation was photochemically induced in the present study.[22] Photochemical exposure produces highly reactive oxygen species that react with cell membrane lipids to cause endothelial cell damage and platelet activation. In this animal model therefore, ADP released from damaged tissue and activated platelets played a critical role in thrombus formation. These lines of evidence indicate that placental E-NTPDase isoform I overexpression in injured vessels is highly antithrombotic, even under high blood flow, which is characteristic of stenosed atherosclerotic arteries.

A key advantage of the local expression of E-NTPDase in the rat model was the absence of systemic side effects.

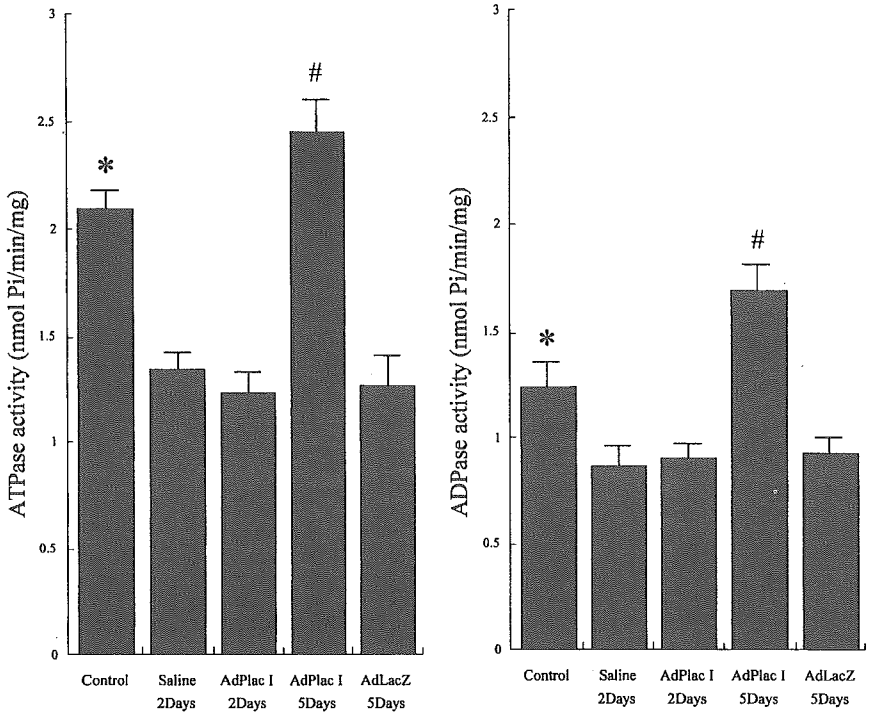

Figure 5. Activities of NTPDase in infected carotid arteries. Homogenized arteries were incubated for 5 minutes with exogenous ATP or ADP, and then ATPase and ADPase activities were determined. Control indicates rat normal carotid artery; saline 2 days, 2 days after endothelial denudation with saline infusion (n=5 each; *$P<0.05$, control vs saline 2 days and AdPlac I 2 days; #$P<0.05$, AdPlac I 5 days vs AdLacZ 5 days).

Animal studies have shown that a new soluble form of the extracellular region of CD39 (solCD39) has systemic antithrombotic effects[30,31]; however, antiplatelet therapy also tends to induce systemic bleeding.[2] This is an important limitation to clinical applicability and indicates a potential advantage of local gene transfer into injured vessels. Our study demonstrated that E-NTPDase expressed on SMCs inhibited platelet aggregation induced not only by ADP but also by collagen, although a high viral titer was required. Thus, local expression of E-NTPDase in diseased arteries should reduce the incidence of cardiovascular events without side effects.

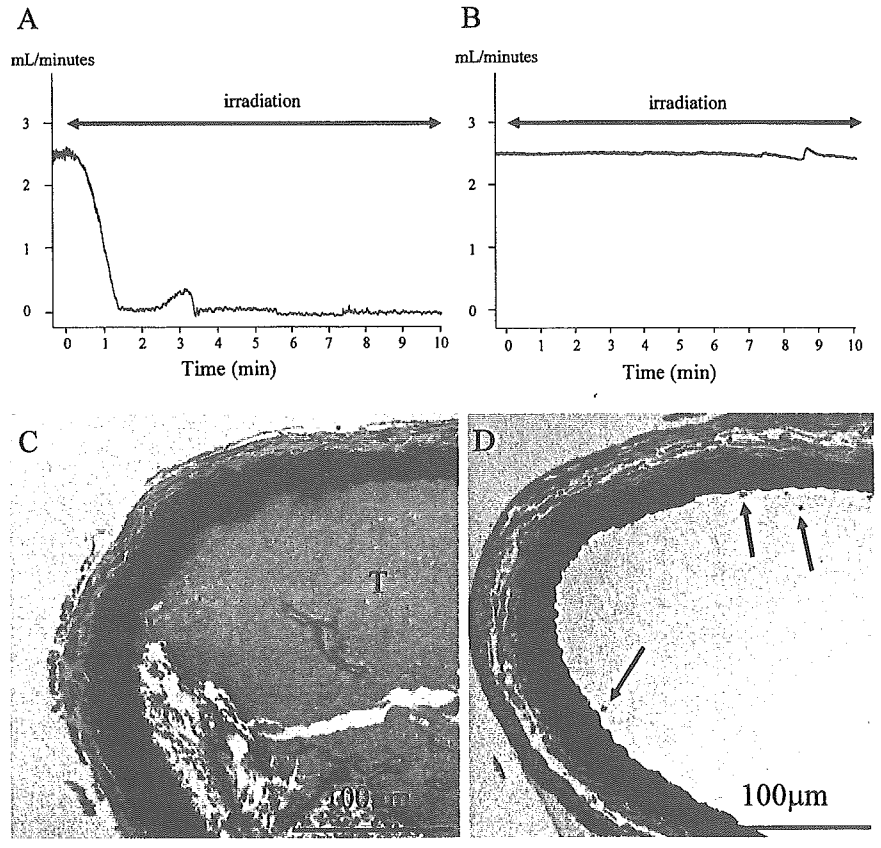

Figure 6. Arterial thrombus formation in arteries injured by air. Thrombus formation induced by photochemical exposure in rat common carotid arteries 5 days after gene transfer. Blood flow of carotid arteries infected with AdLacZ (A) or AdPlac I (B) was recorded during irradiation for 10 minutes. Histologically occlusive thrombus (T) formed in arteries infected with AdLacZ (C), whereas small mural thrombi (arrows) developed in those infected with AdPlac I (D). Results were similar in 6 rats.

Platelet Aggregation and Coagulation Parameters

	Before Virus Infection	AdLacZ	AdPlac I
% Maximum platelet aggregation			
ADP (10 μmol/L)	83.3±3.8	82.0±3.5	84.0±3.5
Collagen (10 μg/mL)	91.3±5.3	90.8±3.3	89.8±4.3
Coagulation parameters			
Prothrombin time, s	8.6±0.5	8.9±0.2	8.6±0.5
Activated partial thromboplastin time, s	14.6±0.5	14.5±0.5	14.8±0.6

Abbreviations are as defined in text.
n=4 each, $P>0.1$.

The endothelium predominantly expresses E-NTPDase and, to a lesser extent, so do other vascular cells.[32] The enzyme in vascular SMCs would modulate vascular tone via P2 purinoceptors.[33] The present study detected placental E-NTPDase isoform I expression by immunohistochemistry in whole walls of arteries infected with AdPlac I. Antiplatelet ability is thought to depend mainly on the NTPDase activity of luminal SMCs. Gangadharan et al[21] reported that adenovirus-mediated CD39 gene transfer augments NTPDase activity in rabbit iliac arteries injured with balloons but does not significantly reduce platelet deposition on the injured luminal surface. They detected CD39 expression only in luminal SMCs. Although the methods of gene transfer and/or species differences might be relevant factors in explaining this discrepancy, the powerful inhibitory effect against occlusive thrombus formation in AdPlac I–infected arteries might depend not only on antiplatelet function but also on reduced vasoconstriction in injured vessels. We evaluated total NTPDase activities of the vessel wall, which includes potential sources of the enzyme. We could not evaluate native E-NTPDase of rat vascular SMCs. Occlusive thrombi were photochemically induced in all arteries infected with AdLacZ, and the level of E-NTPDase/CD39 mRNA expression in rat carotid arteries was very low (data not shown). These results suggest that native E-NTPDase in SMCs would not have putative antithrombotic roles in vivo.

In addition to thrombus formation, subsequent neointimal growth was suppressed in arteries infected with AdPlac I. Other studies in vitro have revealed that purinergic signaling modulates the proliferation and death of SMCs and endothelial cells.[34] Extracellular ATP and ADP released from platelets, as well as injured or activated SMCs and endothelial cells, is mitogenic for intimal SMCs via P2Y receptors[35] and synergistically acts with growth factors such as platelet-derived growth factor and basic fibroblast growth factor.[35,36] The neointima in the present study was exclusively composed of SMCs and extracellular matrix, suggesting that increased NTPDase activity on SMCs suppresses neointimal growth via the inhibition of SMC proliferation. In addition, thrombus itself contributes to neointimal formation and plaque progression.[37] Taken together, increased NTPDase activity on SMCs directly and/or indirectly might contribute to neointimal growth after thrombosis.

In summary, the present study showed that transfer of the adenovirus-mediated human placental E-NTPDase gene into vascular SMCs eliminates platelet aggregation induced by ADP and collagen as well as occlusive thrombus formation in injured arteries. In the clinical setting of cardiovascular events, not only the development of platelet-rich thrombi but also the rapid activation of the blood coagulation system plays a significant role in thrombus formation. The antithrombotic efficacy of AdPlac I infection should be evaluated in arteries with atherosclerosis.

Acknowledgments

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ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

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Decreased Activity of Plasma ADAMTS13 May Contribute to the Development of Liver Disturbance and Multiorgan Failure in Patients with Alcoholic Hepatitis

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Background: The pathogenesis of alcoholic hepatitis (AH) remains unclear and the prognosis of severe alcoholic hepatitis (SAH) is very poor. Deficiency of von Willebrand factor (VWF)-cleaving protease (VWF-CP/ADAMTS13) results in an increase of the plasma unusually large VWF multimer and leads to platelet clumping, which causes microcirculatory disturbance and finally multiorgan failure. The aim of this study was to explore the potential role of ADAMTS13 on the development of liver disturbance and multiorgan failure in AH.

Methods: The activity of plasma ADAMTS13 and its clinical correlation were determined in 14 patients with AH, 4 with SAH (Maddrey score, mean 62), and 10 with alcoholic liver cirrhosis (LC).

Results: The activity of the plasma ADAMTS13 significantly decreased in patients with AH (mean 59%, $p < 0.001$), SAH (17%, $p < 0.001$) and LC (76%, $p < 0.02$) as compared with the healthy subjects (102%, n = 60). The activity was markedly lower in SAH than in AH ($p < 0.02$) and LC ($p < 0.02$). In three nonsurvivors with SAH who had multiorgan failure, it was extremely low (4.5%, 5.0%, and 16.0%, respectively), but in a survivor with SAH it remained mild decrease (44%). In AH, the protease activity increased at the recovery stage (42% → 75%, $p < 0.05$). In the univariate analysis, the activity correlated with 10 clinical variables including functional liver capacity, inflammation signs, renal function, and platelet count in patients with AH and SAH. Among these, multivariate analysis showed that serum total bilirubin and C-reactive protein independently correlated with the protease activity.

Conclusion: The activity of plasma ADAMTS13 markedly decreased in SAH in addition to AH. The activity was closely related to hyperbilirubinemia and inflammation signs, and was extremely low in nonsurvivors with SAH and multiorgan failure. The marked decrease of plasma ADAMTS13 may, in part, contribute to not only the progression of liver disturbance in AH, but also the development of multiorgan failure in SAH through microcirculatory disturbance.

Key Words: ADAMTS13, Alcoholic Hepatitis, Severe Alcoholic Hepatitis, Microcirculatory Disturbance, Multiorgan Failure

ALCOHOLIC HEPATITIS (AH) is a potentially life-threatening complication of alcoholic abuse, and its severe form, severe alcoholic hepatitis (SAH), frequently develops multiorgan failure with the manifestation of acute hepatic failure, which is associated with high morbidity and mortality (Maddrey et al., 1978; Ishii et al., 1993; Mookerjee et al., 2003). The pathogenesis of AH remains unclear, although various factors including metabolism of ethanol to toxic products, endotoxin-induced Kupffer cell activation and subsequent stimulation of cytokine network, and oxidant stress have been implicated (Haber et al., 2003).

Recently, much attention has been paid on a special plasma protease termed von Willebrand factor (VWF)-cleaving protease (VWF-CP) related to the occurrence of the thrombotic thrombocytopenic purpura (TTP) (Furlan et al., 1997; Tsai and Lian, 1998; Fujimura et al., 2002), a multiorgan disorder characterized by Moschcowitz's pentad (Moschcowitz, 1924); thrombocytopenia, renal dysfunction, fluctuating neurological symptoms, microangiopathic

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Table 1. Clinical Data of Patients with Alcoholic Hepatitis and Liver Cirrhosis

Variable	Alcoholic hepatitis	Severe alcoholic hepatitis	Alcoholic liver cirrhosis	Normal range
Age (yr)	50.1[a] (23 ~ 67)	44.7[a] (35 ~ 61)	61.0* (53 ~ 71)**	
Sex (male/female)	13/1	3/1	7/3	
Serum total bilirubin (mg/dl)	2.8 (0.4 ~ 11.9)	14.4[a,f] (8.0 ~ 24.3)	1.6 (0.5 ~ 4.9)	0.3 ~ 1.1
Aspartate aminotransferase (IU/L)	212[c] (46 ~ 673)	427[c, d] (292 ~ 709)	42 (19 ~ 92)	12 ~ 32
Alanine aminotransferase (IU/L)	119[a] (25 ~ 407)	116[c] (78 ~ 165)	40 (10 ~ 69)	5 ~ 36
Lactate dehydogenase (IU/L)	306 (162 ~ 420)	620[a, d] (298 ~ 836)	247 (120 ~ 426)	116 ~ 230
γ-Glutamyl transpeptidase (IU/L)	548[c] (156 ~ 1041)	590[a] (181 ~ 1000)	119 (17 ~ 251)	11 ~ 69
White blood cell count (/mm^3)	6557[a] (3000 ~ 10800)	12075[a] (3500 ~ 26600)	3850 (1900 ~ 5900)	3900 ~ 9800
Polymorphonuclear neutrophil (/mm^3)	4502[a] (1462 ~ 9167)	10877[a, d] (3220 ~ 25004)	2469 (893 ~ 4720)	2000 ~ 7500
Hemoglobin (g/dl)	12.4 (8.4 ~ 16.5)	8.5[a, d] (7.3 ~ 10.3)	11.1 (8.8 ~ 14.3)	13.5 ~ 17.6
Platelet count (x10^4/mm^3)	16.2[c] (7.8 ~ 38.3)	9.8 (2.8 ~ 16.4)	7.6 (3.9 ~ 18.3)	13.1 ~ 36.2
C-reactive protein (mg/dl)	1.1 (0.1 ~ 5.0)	4.8[a,d] (1.7 ~ 12.2)	0.5 (0.1 ~ 2.7)	0 ~ 0.6
Serum albumin (g/dl)	3.9[a] (2.7 ~ 4.5)	2.5[a,e] (1.8 ~ 3.0)	3.3 (2.6 ~ 4.0)	3.8 ~ 5.0
Prothrombin time (%)	77 (63 ~ 98)	35[a, e] (27 ~ 38)	68 (37 ~ 94)	70 ~ 100
Blood urea nitrogen (mg/dl)	11 (4 ~ 20)	33 (11 ~ 89)	13 (8 ~ 21)	8 ~ 20
Serum creatinine (mg/dl)	0.8 (0.4 ~ 1.2)	2.5 (0.4 ~ 4.7)	0.8 (0.6 ~ 1.09)	0.3 ~ 0.9
Liver cirrhosis (+/−)	6/8	4/0	10/0	
Outcome (alive/dead)	14/0	1/3	10/0	

*The data are expressed as mean. **Parentheses indicate range.
[a]$p<0.05$,[b]$p<0.01$, and [c]$p<0.005$ vs. liver cirrhosis, respectively.
[d]$p<0.05$,[e]$p<0.01$, and [f]$p<0.005$ vs. alcoholic hepatitis, respectively.

hemolytic anemia, and fever. Congenital or acquired deficiency of VWF-CP activity can cause TTP (Furlan et al., 1997; Tsai and Lian, 1998; Fujimura et al., 2002). VWF not only plays an essential role in primary hemostasis through its anchoring action of platelets, but also is known as a marker of endothelial activation and an acute phase reactant (Ruggeri 1997). In patients with fulminant hepatic failure and liver cirrhosis, a remarkably high level of the plasma VWF has been noted (Langley et al., 1985; Albornoz et al., 1999), but its pathogenesis related to clinical significance has not been fully elucidated. The immunostaining with anti-VWF polyclonal antibody is positive in the liver from an early stage of alcoholic liver diseases, presumably in association with transformation to vascular endothelial cells (Urashima et al., 1993).

Unusually large VWF multimer (UL-VWFM), produced in and released soon from the vascular endothelial cells, is assumed to interact with the circulating platelets and leads to platelet clumping under elevated high shear stress, because in general a larger VWF multimer has more potent biological activities (Moake et al., 1986; Ruggeri 1997). The UL-VWFM has been often found in the plasma of patients with TTP (Moake et al., 1986). In the normal circulation, however, UL-VWFM is rapidly degraded into smaller VWFM by VWF-CP, which splits the Tyr842-Met843 bond of the subunit (Furlan et al.,1996; Tsai 1996). VWF-CP is now identified as a metalloproteinase belonging to the ADAMTS (*A D*isintegrin-like *A*nd *M*etalloproteinase domain, with *T*hrombo*S*pondin type-1 motif) family, termed ADAMTS13 (Zheng X et al., 2001; Soejima et al., 2001; Plaimauer et al., 2002), which is produced in the liver, exclusively in the perisinusoidal cells (Lee et al., 2002).

On the basis of these findings, it is particularly interesting to evaluate the plasma activity of ADAMTS13 in the patients with AH. We demonstrated that the protease activity significantly reduced in the patients with hepatic veno-occlusive disease after stem cell transplantation throughout their clinical course (Park et al., 2002). Subsequently, it has been shown that plasma ADAMTS13 activity is significantly low in the patients with acute hepatitis (Kavakli 2002) and liver cirrhosis (Mannucci et al., 2001), but without addressing its clinical significance.

In this study, we determined the plasma activity of ADAMTS13 in patients with AH and alcoholic liver cirrhosis (LC), and thereby, tried to explore a potential role of the protease activity related to the severity of liver disturbance especially in association with the development of multiorgan failure in patients with SAH.

MATERIALS AND METHODS

Patients

The study was carried out on 14 patients with AH (13 men and 1 woman; mean age: 50.1 year, range 23 to 67 years), 4 patients with SAH (3 men and 1 woman; mean age: 44.7 year, range 35 to 61 years) and 10 patients with alcoholic LC (7 men and 3 women; mean age: 61.0 year, range 53 to 71) (Table 1). The severity of alcoholic LC was Child A in 5, Child B in 3 and Child C in 2 according to Child-Pugh's criteria (Pugh et al., 1973). All patients were originally admitted into our hospital between June 2001 and January 2004. Patients with a known history of coagulopathies, sepsis, or platelet disorders were excluded from this study. The diagnosis of AH and SAH was based on the physical findings, laboratory tests, and often confirmed by the liver histology according to the Diagnosis Criteria for Alcoholic Liver Injury, established by Takada Group in Ministry of Education Comprehensive Research A (1993). The severity of SAH was estimated according to Maddrey score (Carithers et al., 1989). Hepatic encephalopathy was graded according to the classification of Trey et al. (1966). All subjects gave informed consent to participate in the study. The study protocol was approved by the Nara Medical University Hospital Ethics Committee.

Assays of ADAMTS13 Activity

Blood was taken from the patients on and/or during admission in plastic tubes with 1/10th volume of 3.8% sodium citrate as an anticoagulant. In 4 patients with AH, a second plasma sample was taken between 7