Table 2. Correlation between Plasma Vitamin C Concentration and Selected Factors ($N = 655$)

Factor	Correlation*	
	r	p
Age	−0.004	.91
Height	0.04	.27
Weight	−0.05	.19
Body mass index	−0.08	.054
Triceps surae muscle	0.001	.98
Serum albumin	−0.04	.33
Percent body fat	−0.12	.002
Handgrip strength	0.16	<.001
One leg standing with eyes open	0.15	<.001
Usual walking speed	0.14	<.001
Maximal walking speed	0.09	.036

Notes: Number of subjects is slightly different for the selected factors because of missing values.

*Age-adjusted Pearson's correlation coefficient between logarithm of vitamin C concentration and each factor.

subjects was 75.7 ± 4.1 years. The geometric mean (geometric standard deviation) of plasma vitamin C concentration was 8.9 (1.5) μg/mL. The prevalence of women eating vegetables everyday was 84.2% and those eating fruits everyday was 81.8%.

The age-adjusted geometric mean of plasma vitamin C concentration was significantly lower in subjects who had a medical history of hypertension (8.53 vs 9.22, $p = .0015$) and diabetes mellitus (7.59 vs 9.00, $p = .002$) as compared with those who did not. A history of stroke, heart attack, or hyperlipidemia was not associated with plasma vitamin C concentration. Subjects who took fruits every day had a significantly higher concentration of vitamin C than those who did not (9.14 vs 7.78, $p < .0001$). Vegetable intake, alcohol drinking habit and smoking habit were not related to plasma vitamin C concentration (not shown in table).

Table 2 shows the age-adjusted correlations between the plasma vitamin C concentration and selected factors. As shown, the plasma vitamin C concentration was positively but modestly correlated with handgrip strength, length of time standing on one leg with eyes open, as well as usual walking speed and maximal walking speed, and modestly inversely correlated with body mass index and percent body fat of the subjects.

Table 3 shows the relationship between plasma vitamin C concentration and each physical performance after adjusting for confounding factors. Results obtained after the adjustment for potential confounders confirmed that the plasma vitamin C concentration was correlated with the handgrip strength independently from the other factors (eg, p for trend = .0004 after adjusting for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake; Table 3). There was also a significant relationship between the plasma vitamin C level and the subject's length of time standing on one leg with eyes open after adjustments for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake (Table 3; p for trend = .049). We did not observe any significant association between the plasma vitamin C level and the usual or the maximal walking speed of the subjects.

A subanalysis using data from the 238 vitamin C supplement users showed almost null relationship between handgrip strength and plasma vitamin C concentration (data not shown).

Discussion

A previous study has shown an association between higher daily dietary intake of vitamin C and skeletal muscle strength in elderly people (3). Results described in the present study indicated that plasma vitamin C concentration was positively related with muscle and physical performance in community-dwelling elderly women. To the best of our knowledge, this is the first study showing a significant

Table 3. Relationship between Plasma Vitamin C Concentration and Physical Performance Adjusted for Potential Confounder

Physical performance	Quartile of plasma vitamin C level				
	Q1	Q2	Q3	Q4	
	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	p for trend
Handgrip strength (kg), *N*	154	159	154	152	
Age adjusted	17.70 ± 0.34	18.75 ± 0.33	18.75 ± 0.34	19.60 ± 0.34	.0001
Multivariate adjusted*	17.83 ± 0.34	18.83 ± 0.32	18.89 ± 0.33	19.60 ± 0.33	.0004
One leg standing with eyes open† (s), *N*	162	163	164	161	
Age adjusted	31.44 ± 1.71	33.98 ± 1.70	37.70 ± 1.70	37.83 ± 1.71	.003
Multivariate adjusted*	33.39 ± 1.74	34.08 ± 1.67	37.63 ± 1.67	37.50 ± 1.70	.049
Usual walking speed (m/s), *N*	146	154	145	147	
Age adjusted	1.13 ± 0.02	1.19 ± 0.02	1.23 ± 0.02	1.21 ± 0.02	.008
Multivariate adjusted*	1.18 ± 0.02	1.19 ± 0.02	1.22 ± 0.02	1.21 ± 0.02	.23
Maximal walking speed (m/s), *N*	146	154	154	147	
Age adjusted	1.70 ± 0.03	1.76 ± 0.03	1.82 ± 0.03	1.76 ± 0.03	.15
Multivariate adjusted*	1.76 ± 0.03	1.77 ± 0.03	1.80 ± 0.03	1.75 ± 0.03	.94

Notes: Values are least squares mean and *SE* adjusted for the factors by analysis of covariance. Q1–Q4: first to fourth quartile groups of plasma vitamin C concentration, respectively.

*Adjusted for age, body mass index, percent body fat, hypertension, diabetes mellitus and **fruit intake**.

†Length of time standing on one leg with eyes open.

correlation between plasma vitamin C concentration and handgrip strength and ability to stand on one leg with eyes open. We, however, were unable to find any relationship between skeletal muscle mass and plasma vitamin C concentration. Handgrip strength has been found to correlate well with the strength of other muscle groups and is thus a good indicator of overall strength (18). Consistent with this idea, handgrip strength was found to be a strong and consistent predictor of all-cause mortality and morbidity of Activities of Daily Living in middle-aged people (19). The handgrip test is considered an easy and inexpensive screening tool to identify elderly people at risk of disability. Handgrip strength, an indicator of overall muscle strength, is thought to predict mortality through mechanisms other than underlying disease that could cause muscle impairment (18,19). The one leg standing test is one of the balance tests (20). The test is a clinical tool to assess postural steadiness in a static position by quantitative measurement. Many studies have shown that the decreased one leg standing time is associated with declines in Activities of Daily Living and increases in other morbidities including osteoporosis and fall (20).

Our findings suggest that vitamin C may play an important role in maintaining physical performance and thereby may help to improve healthy life expectancy in the elderly. However, the usual and maximal walking speeds did not relate to plasma vitamin C concentration. Walking speed test may be an efficient tool in screening older persons with higher risk of mortality and may easily identify high-risk groups in the community (21). Walking is a rhythmic, dynamic, and aerobic activity of the large skeletal muscles that confers multifarious benefits with minimal adverse effects. Muscles of the legs, limbs, and lower trunk are strengthened, and the flexibility of their joints are preserved (22). One of the reasons why walking speed was not related to vitamin C concentration may be because walking requires coordinated movements of arms, legs, and many parts of the body rather than a simple muscle and balance function. Previous reports showed that walking balance function did not correlate with standing balance function (23). Although we did not find any clear association between walking and plasma vitamin C concentration in this study, vitamin C may still have effects on relatively simple strength and balance functions.

One of the possible explanations for the observed relationship between vitamin C and physical performance, especially handgrip strength and the ability to stand on one leg with eyes open, may be the potential protective effects of the antioxidant vitamins against muscle damage (4,11). Vitamin C is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not in humans (12). Vitamin C is an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized (12). Thus, vitamin C readily scavenges reactive oxygen and nitrogen species, thereby effectively protects other substrates from oxidative damage (10,24). Although habitual exercise reduces systemic inflammation and oxidative stress as the production of endogenous antioxidants are enhanced, acute exercise increases the generation of oxygen-free radicals and lipid peroxidation (4,25). Strenuous physical performance can increase oxygen consumption by 10- to 15-folds over the resting state to meet the energy demands and results in muscle injury (26). Prolonged submaximal exercise was shown to increase the amount of both whole-body and skeletal muscle lipid peroxidation by-products; in the case of the former, the increase was indicated by greater exhalation of pentane but not of ethane (4,27,28). Supplementation with vitamin C was shown to decrease the exercise-induced increase in the rate of lipid peroxidation (27,28). Several studies suggested that oxidative damage may play a crucial role in the decline of functional activity in human skeletal muscle with normal aging (15). Consistent with this idea, several studies showed significantly lower plasma vitamin C level in the elderly population than in the younger adult population (29–31). Because the plasma vitamin C levels in these apparently healthy elderly persons rose markedly after an oral dose of vitamin C, their initially low plasma levels can be attributed to the low intake rather than to an age-related physiological defect.

In fact, the relationship between handgrip strength and plasma vitamin C concentration was significantly different between supplement users and nonusers, that is, an almost null relationship in the former and a positive relationship in the latter (data not shown). This finding suggested that vitamin C supplementation did not have any beneficial effect on the physical performance and muscle strength despite the increased plasma level of vitamin C. A number of studies reported that vitamin C supplement users had significantly higher blood vitamin C concentration than non-users (29, 32, 33). Several studies have examined the effects of exercise on changes in the serum vitamin C concentration (34–36). Some other experimental studies have shown that vitamin C supplementation can reduce symptoms or indicators of exercise-induced oxidative stress (37–40). However, the results regarding vitamin C supplementation are equivocal, and most well-controlled intervention studies report no beneficial effect of vitamin C supplementation on either endurance or strength performance (41,42). Likewise, vitamin C restriction studies showed that a marginal vitamin C deficiency did not affect the physical performance (43). Although evidence from a number of studies show that vitamin C is a powerful antioxidant in biological systems in vitro, its antioxidant role in humans has not been supported by currently available clinical studies.

Vitamin C is especially plentiful in fresh fruits and vegetables. Plasma vitamin C concentration may be merely a marker for intake of other nutrients that are abundant in fruits and vegetables. However, the statistical adjustment for fruit intake did not attenuate the relationship between plasma vitamin C and physical performance (Table 3), suggesting that vitamin C did have some beneficial effects

independently of other nutrients. A number of biochemical, clinical, and observational epidemiologic studies have indicated that diets rich in fruits, vegetables, and vitamin C may be of benefit for the prevention of chronic diseases such as cardiovascular disease and cancer (44,45). Several cohort studies have examined associations between plasma vitamin C concentration and mortality from stroke or coronary heart disease (30,46,47). The effects of vitamin C supplementation are, however, still unclear. A pooled study suggested reduced incidences of coronary heart disease events with higher intake of vitamin C supplement (48), while another study showed that a high intake of vitamin C supplement is associated with an increased risk of mortality due to cardiovascular diseases in postmenopausal women with diabetes (49). A randomized placebo controlled 5-year trial, however, did not show any significant reduction in the mortality from, or incidence of, any type of vascular disease or cancer (50). These studies, in fact, have failed to demonstrate any benefit from such supplementation.

There are a number of potential weaknesses in our study that should be mentioned here. The subjects used in this study were not selected randomly from the study population, and they may be relatively healthy elderly women who were able to come to the health examination hall from their homes. A previous study assessed the correlation of antioxidants with physical performance and muscular strength (3) and demonstrated that a higher daily intake of vitamin C and carotene associated with skeletal muscle strength. However, we have no data regarding the presence of other dietary antioxidants in blood such as vitamin E, retinol, and carotene. In our questionnaire, participants were asked to respond "Yes" or "No" to whether they took supplements, and not about the frequency and quantity of intake of the supplements. Thus, we were unable to examine the reason why plasma vitamin C was not related to the handgrip strength in the supplement users by considering the dose of vitamin C they took.

This study was a cross-sectional study and, therefore, does not provide cause/effect relationships, although we demonstrated a significant correlation between physical performance and concentration of plasma vitamin C. Therefore, longitudinal follow-up studies and controlled clinical trials are necessary to confirm the role of plasma vitamin C and physical performance of the elderly women. These limitations should be considered in future studies.

In conclusion, we found a strong correlation of a higher plasma vitamin C concentration with handgrip strength and one leg standing time in community-dwelling elderly women. Although the elderly are prone to vitamin C deficiency, and they appear to have a higher dietary requirement for vitamin C, the beneficial effects of vitamin C supplementation to maintain physical performance in elderly people are equivocal and thus, need further in-depth studies.

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Vitamin K_1 (Phylloquinone) or Vitamin K_2 (Menaquinone-4) Induces Intestinal Alkaline Phosphatase Gene Expression

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Summary Alkaline phosphatase (ALP) hydrolyzes a variety of monophosphate esters into inorganic acid and alcohol at a high optimum pH (pH 8–10). Previously, we identified a significant increase of intestinal ALP (IAP) activity in the rat intestine on long-term dietary vitamin K supplementation. However, it was unclear whether the induction of ALP gene expression was caused by vitamin K intake. In the present study, we examined the effects of vitamin K on IAP gene expression. A total of 21 male ICR strain mice (7 wk old) were divided into three groups: control, PK, and MK groups. Mice were orally administered a 0.1-mL solution of physiological saline in the control group, phylloquinone (3 mg/kg mouse) in the PK group, and menaquinone-4 (3 mg/kg mouse) in the MK group. Four hours after administration, we determined the ALP activity of the intestinal mucosa in three areas (duodenum, jejunum, and ileum). In the MK groups, the levels of ALP activity in the jejunum increased significantly compared with the control. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) analysis using specific primers revealed that IAP mRNA expression was significantly enhanced in the jejunum in both PK and MK groups. Interestingly, vitamin K administration also increased the expression of pregnane X receptor mRNA. This is the first report concerning IAP mRNA expression induced by oral administration of vitamin K. The results support the possible involvement of vitamin K in the regulation of IAP mRNA expression as a novel pharmacological effect of vitamin K.

Key Words alkaline phosphatase, phylloquinone, menaquinone, mice, intestine

Alkaline phosphatase (ALP, EC 3.1.3.1) is an enzyme containing zinc which hydrolyzes monophosphate esters into inorganic phosphoric acid and alcohol at a high optimal pH (pH 8–10). The enzyme is distributed widely throughout the living world from bacteria to animals, excluding plants, and it exists in various tissues such as the intestine, liver, kidney, bone, placenta, stomach, and leukocytes.

In humans, four kinds of ALP isozyme have been identified: tissue-nonspecific ALP (liver/bone/kidney: TNSALP), intestinal ALP (IAP), placental ALP, and germ cell ALP (*1–4*). The TNSALP gene is located on chromosome 1 and consists of 12 exons and 11 introns, with the coding sequence beginning in the second exon.

A single gene for human IAP has been isolated, and the multiple forms of mRNA encoding human IAP are due to differences in polyadenylation (*2*). Although most species express a single IAP, several kinds of IAP have been identified in three species: the mouse (*5*), rat (*6, 7*), and cow (*8*).

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In rats, ALP is classified into two types: TNSALP and IAP. IAP is present in the membrane surrounding neutral fat droplets in the microvilli of the intestinal mucosa during fat absorption, and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (SLPs) (*9*). Two different cDNA clones, IAP-I and IAP-II, for rat IAP were isolated by Lowe et al. (*6*) and Strom et al. (*7*), respectively. Strom et al. found that the expression of IAP-II mRNA was specifically enhanced by $1\alpha,25(OH)_2D_3$ administration. The two isozymes are products of two distinct genes and their cDNA sequences show 79% homology at the amino acid level. Functional differences between IAP-I and IAP-II were suggested by the differing regulation of the expression of the two mRNAs (*10*), as well as by structural and catalytic differences (*11*).

In mice, five different ALP loci have been identified: TNSALP, IAP, embryonic ALP (EAP), *Akp6*, and *Akp-ps1*. These ALP genes code for different proteins: *Akp2* encodes TNSALP, *Akp3* encodes IAP, *Akp5* encodes EAP, *Akp6* encodes a novel IAP-like isozyme expressed globally in the gut (thus called gIAP), and *Akp-ps1* encodes

the inactive pseudo-type ALP (*5*, *7*).

Previously, we reported the enhanced effects of vitamin K on IAP activity in rats (*12*). Sprague-Dawley rats (6 wk old) were divided into three groups: a control (AIN-93M diet), phylloquinone (PK: 600 mg/kg diet), and menaquinone-4 (MK-4: 600 mg/kg diet) diet group. After 3 mo of feeding of vitamin K, the animals were fasted overnight. The small intestine was removed and divided into five segments. In each segment, both PK and MK-4 increased IAP activity (*12*).

Vitamin K acts as a cofactor for γ-glutamyl carboxylase (GGCX), and is well-known to participate in the activation of blood coagulation factors and bone mineralization (*13*). All forms of vitamin K have 1,4-naphthoquinone as a common ring structure, and natural vitamin K exists in two molecular forms, vitamin K_1 (phylloquinone: PK) and vitamin K_2 (menaquinone: MK-*n*). PK is abundant in green vegetables in a compound with a phytyl side chain. Vitamin K_2 is classified into MK-1–14 due to the repeat structure of the side chain, with isopren comprising the side chain. MK-4 shows marked physiological activities as a vitamin K, and is included in many animal-based foods such as meat. Recent studies have demonstrated the possibility that vitamin K regulates the expression of bone-related genes such as ALP through steroid X receptor (SXR, also termed pregnane X receptor: PXR) (*14*).

In the present study, we examined whether the enhancing effect of PK or MK-4 administration on IAP activity occurs via the intestinal mucosa directly, and we revealed the effects of the oral administration of PK or MK-4 on the expression of IAPs (*Akp3* and *Akp6*) and PXR in the mouse intestine.

MATERIALS AND METHODS

Experimental animals. The care and use of mice in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals, and the study protocol was approved by the Institutional Review Board of Japan Women's University. A total of 21 male ICR strain mice (7 wk old) were used (31.1±0.2 g). They were fasted overnight with free access to water. On the following day, the animals were given 0.1 mL of solution via an intragastric tube: vehicle (physiological saline) for the control group (Cont.), PK (3 mg/kg mouse) for the PK group, and MK-4 (3 mg/kg mouse) for the MK group. The molecular weights of PK ($C_{31}H_{46}O_2$: MW=450.7) and MK-4 ($C_{31}H_{40}O_2$: MW=444.7) are very similar. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan).

Serum and tissue sampling. Four hours after administration, blood was collected from the abdominal aorta under ether anesthesia, and perfusion with saline was performed until the liver was blanched, in order to minimize the blood contamination of tissue samples. The small intestine was removed and divided into three regions. From the pylorus, we took the first 1 cm as the duodenum, and then separated the remaining part into the jejunum and ileum. The segments were slit longitudinally, rinsed with ice-cold saline, and scraped from the mice just after dissection. Each sample was homogenized using a Polytron homogenizer (Kinematica, Switzerland) with 10 mM Tris-buffered saline containing 1% Triton X-100 (pH 7.3) and 1 mM phenylmethylsulfonylfluoride (PMSF). The supernatant obtained after centrifugation at 7,000 ×*g* for 15 min was used as the enzyme extract.

Enzyme assay. ALP activity was determined with 10 mM *p*-nitro-phenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediole HCl buffer containg 5 mM $MgCl_2$, pH 10.0, at 37°C, as previously reported (*15*). To analyze the biochemical properties of ALP, an inhibitory assay using levamisole (Lev) and L-phenylalanine (L-Phe) and a thermostability assay were performed, as previously described (*15*).

The enzyme activity was defined as the rate of hydrolysis of *p*-nitro-phenylphosphate and expressed in units (U=μmol *p*-nitro-phenol formed/min).

Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber et al. (*16*). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling of β-naphthyl-phosphoric acid monosodium salt with Fast Violet B salt (*17*).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the intestinal mucosa was extracted employing the acid guanidinium thiocyanate-phenol-chloroform method (*18*). As a template for PCR, single-strand cDNA was prepared from 1 μg of total RNA using Ready-to-go You-Prime First-Strand Beads (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). PCR primers were used for *Akp3* (*19*), *Akp2* (*20*), *Akp5* (*21*), and PXR (*22*). In order to detect *Akp6*, sense (*Akp6*-up) and anti-sense (*Akp6*-down) primers were designed on the basis of the *Akp6* nucleotide sequence (GenBank: NCBI sequence data: AK008000). *Akp6*-up spans nucleotide positions 882–902 and *Akp6*-down spans 1,333–1,354 (*23*). The PCR conditions were as follows: 5 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles at 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by 10 min at 72°C. Negative controls were performed with each RT-PCR reaction, omitting the template. The efficiency of reverse transcription was verified by the detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′), as previously described (*23*).

The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide and observed under UV light. The band intensity on PCR photographs was quantified by densitometry (AE6920M, ATTO, Tokyo, Japan). The PCR product was normalized to the intensity of the band for the house-keeping gene GAPDH, and is expressed as a ratio of the relative band intensity.

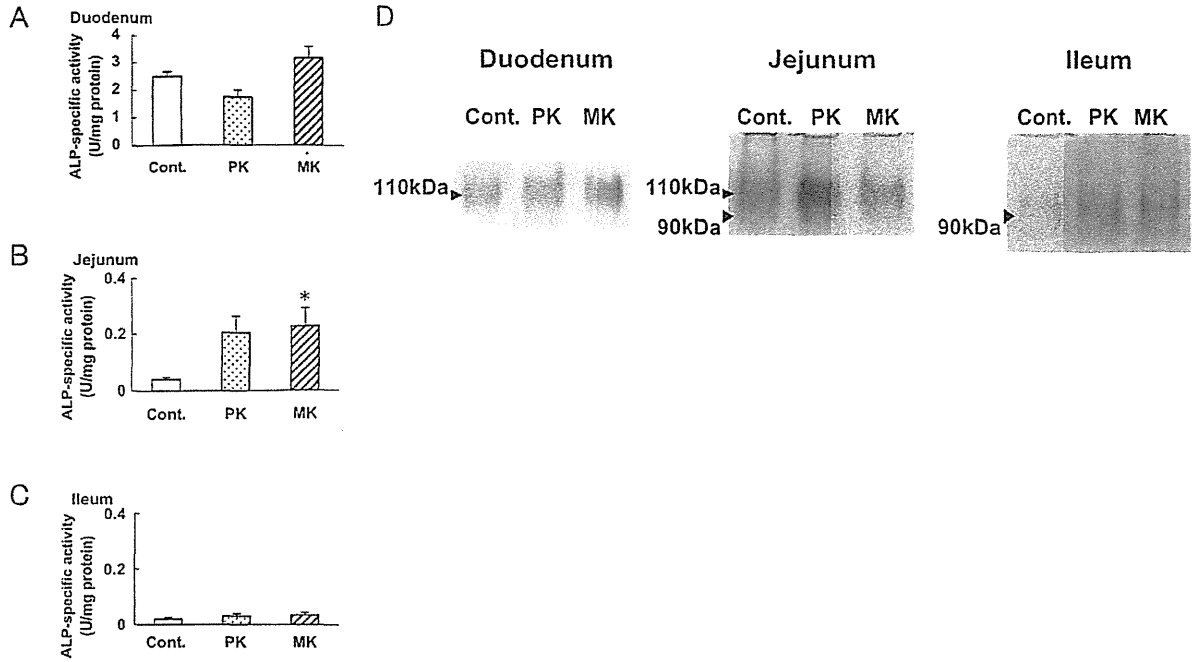

Fig. 1. ALP-specific activities of the duodenum (A), jejunum (B), and ileum (C). Results are the mean±SE of 7 animals. Significant difference between the MK and control groups (*$p<0.05$). D: Mouse intestinal ALP isozymes separated by polyacrylamide gel electrophoresis. The gels was stained for ALP activity with a β-naphtyl-phosphoric acid monosodium salt, Fast violet B salt. Cont.: control, PK: phylloquione, MK: menaquinone-4.

Statistical analyses. Values are shown as the mean± standard error (SE).

Dunnet's multiple comparison test was used after ANOVA to compare the significance of differences among the control and PK or MK. Differences were considered significant at $p<0.05$. Analysis was conducted using SPSS 18.0J (SPSS, Inc., Chicago, IL, USA).

RESULTS

ALP activity

To examine whether IAP was secreted from the intestinal mucosa on the oral administration of PK or MK, we measured the levels of serum ALP activity (mU/mL) in the control, PK, and MK groups, being 13.7±1.2, 10.3±2.0, and 14.1±1.8 (mean±SE), respectively, showing no significant differences among these groups.

ALP-specific activities in the intestine are shown in Fig. 1. There were no significant differences in ALP activities among these groups in the duodenum (Fig. 1A) and ileum (Fig. 1C). As presented in Fig. 1B, ALP activity of the MK group in the jejunum was significantly higher compared with the control group ($p<0.05$).

Molecular weight determination by SDS-PAGE

The molecular weights of ALPs of each intestinal segment were estimated employing SDS-PAGE analysis. As shown in Fig. 1D, the 110-kDa band of the major ALP isozyme was detected in the duodenum among these groups. In the jejunum, ALP enzymes were separated into two bands of 110 and 90 kDa, and the intensity of their enzymatic activity increased markedly in both PK and MK groups, similarly to the results regarding the specific ALP activity in the jejunum. In the distal part of the intestine (ileum), the ALP isozyme showed a main band of 90 kDa among these groups. No additional band was observed in any intestinal samples among the groups.

Table 1. Inhibitory effects of levamisole, L-phenylalanine and heat inactivation of ALP preparations of the jejunum.

Groups	Relative activity(%)		
	Levamisole (1 mM)	L-Phenylalanine (20 mM)	Heat inactivation (60°C 10 min)
Cont.	95.2±1.6	23.0±1.0	42.4±3.4
PK	94.2±2.1	23.5±1.4	46.1±2.3
MK	95.5±1.6	24.2±2.0	45.9±2.5

Each value represents mean±SE ($n=7$).
The ALP activity was assayed based on the rate of *p*-NPP hydrolysis. The effect of the inhibitor was determined in the presence of 5 mM $MgCl_2$ in the assay mixture. Remaining ALP activity with inhibitors or after heat treatment is expressed as a percent of non-treated controls. Results are the mean±SE of 7 animals.

Properties of ALP in the intestine

The enzymatic properties of ALP preparations of the jejunum which increased significantly on PK or MK administration were investigated employing an inhibition experiment with levamisole (Lev) and L-phenylalanine (L-Phe) and through a thermo-stability test. It is well known that IAP activity is not inhibited by Lev and is more stable to L-Phe and more heat-stable than TNSALP. As shown in Table 1, there was no significant

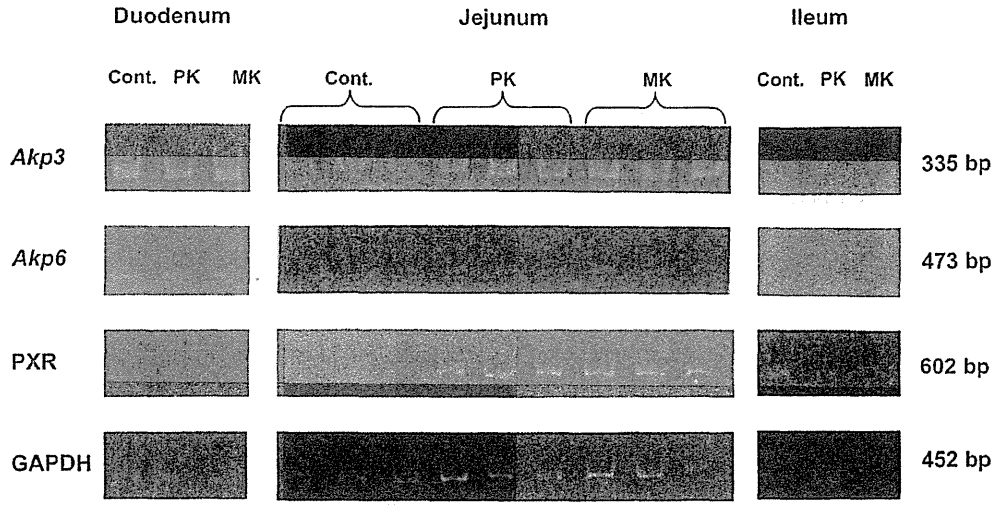

Fig. 2. Detection by RT-PCR of mRNAs for *Akp3*, *Akp6*, PXR, and GAPDH in the duodenum, jejunum, and ileum. PCR products were electrophoresed in a 5.25% polyacrylamide gel. Cont.: control, PK: phylloquinone, MK: menaquinone-4.

difference in the relative activities among these groups. We confirmed that these jejunum ALP preparations were effectively inhibited by L-Phe but not by Lev, and were heat-stable (60°C, 10 min), corresponding to the property of other mammalian intestinal type ALPs.

RT-PCR analysis of ALP mRNA expression

RT-PCR-based detections of mRNA for *Akp3*, *Akp6*, and PXR in the duodenum, jejunum, and ileum are shown in Fig. 2. The PCR products of *Akp3* (335 bp) were detected in the duodenum and jejunum. The PCR products of *Akp6* (473 bp) were detected in the duodenum, jejunum, and ileum. The PCR products of PXR (602 bp) were detected in the duodenum, jejunum, and ileum. The intensities of mRNA expression of *Akp3* and *Akp6* were very similar both in the duodenum and ileum among the three groups (Cont., PK, and MK groups). The intensities of mRNA expression of *Akp3*, *Akp6*, and PXR were enhanced in both the PK and MK groups compared with the control group in the jejunum.

PCR products of *Akp2* (198 bp) and *Akp5* (500 bp) were not detected in any of these intestinal samples.

In order to compare these intensities of mRNA expression in the jejunum, we determined the relative density of the PCR products of the mouse jejunum. As shown in Fig. 3A, the intensities of *Akp3* expression increased significantly in the PK group compared with the control group ($p<0.01$). Furthermore, the intensities of *Akp6* expression were also increased in both the PK and MK groups compared with the control group ($p<0.01$, $p<0.05$, respectively) (Fig. 3B). Interestingly, the intensities of PXR expression in both the PK and MK groups were significantly higher than in the control group ($p<0.05$, $p<0.01$, respectively) (Fig. 3C).

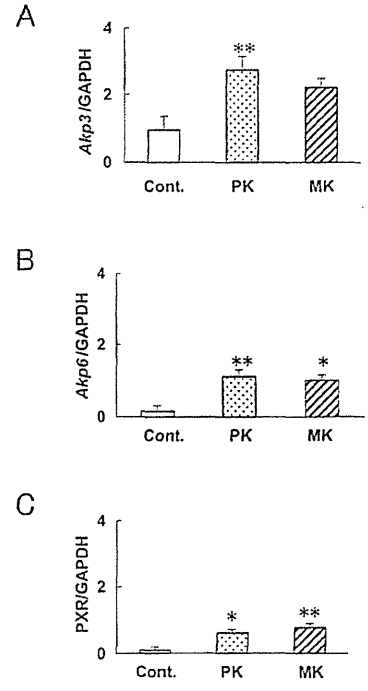

Fig. 3. The relative density of PCR products in the mouse jejunum. The diagrams show the relative density of the PCR products. A: The relative density of PCR products for *Akp3*. B: The relative density of PCR products for *Akp6*. C: The relative density of PCR products for PXR. Results are the mean±SE of 4 animals. Significant difference between the PK or MK and control groups (*$p<0.05$, **$p<0.01$). Cont.: control, PK: phylloquinone, MK: menaquinone-4.

DISCUSSION

Previously, we reported that several dietary factors such as fat-feeding, vitamin K, and lactose increased IAP activities in rats (*12*, *15*, *24*). The high-level activity of IAP, which localizes at the brush border of intestinal epithelium cells, suggests the participation of this enzyme in the transport of nutrients.

Recently, we reported that both long-term dietary PK and MK-4 supplementations enhance IAP activity in rats (*12*). After 3 mo of feeding, we measured IAP activity by dividing it into five segments. In each segment, both PK and MK-4 increased IAP, and the level of IAP activity in the proximal jejunum was significantly

higher than that in the control group ($p<0.05$) (*12*). To examine whether the enhancing effect of PK or MK-4 on IAP activity occurs via the intestinal mucosa directly, we performed an oral administration of PK or MK-4 using mice. In the present study, we discovered that the levels of mouse jejunum ALP activity were also significantly increased by the oral administration of PK or MK compared with the control group (Fig. 1B). In addition, we confirmed that the increased ALP isozymes induced by the oral administration of PK or MK showed similar biochemical properties to the typical intestinal type ALP, with no significant differences among these groups (Table 1).

By SDS-PAGE analysis, we detected a 110-kDa ALP enzyme in the duodenum and 90-kDa ALP enzyme in the ileum (Fig. 1D). Both the 110- and 90-kDa ALP enzymes were detected in the jejunum, and the enzymatic activities of these bands were enhanced by the oral administration of PK or MK. The product of the *Akp3* gene was expressed specifically in the duodenum, and the product of *Akp6* was expressed through the small intestine (5). Therefore, we considered that the 110- and 90-kDa ALP enzymes may correspond to the IAPs encoding *Akp3* and *Akp6*, respectively.

We then performed RT-PCR analysis in order to examine the expression of IAPs (*Akp3* and *Akp6*) in the mouse jejunum. PCR products for *Akp3* and *Akp6* mRNAs in the jejunum were detected, and a significant increase in the PCR products of *Akp3* due to the oral administration of PK was observed (Fig. 3A). Moreover, a significant increase in the PCR products of *Akp6* due to the oral administration of PK or MK-4 was also observed (Fig. 3B). These results suggest that the induction of *Akp3* and *Akp6* may be regulated by PK or MK-4.

As the results of RT-PCR, the expression of mRNA for PXR was detected in the duodenum, jejunum and ileum, and it was enhanced significantly in both the PK and MK groups in the jejunum compared with the control group (Fig. 3C). Interestingly, the intestinal segment where the expression of mRNA for PXR by vitamin K had been enhanced corresponded to a similar segment where the expression of mRNA for IAP was enhanced.

Recent studies have revealed that vitamin K functions as a ligand for nuclear steroid and xenobiotic receptor (SXR), as well as a cofactor for γ-carboxylase (*25*). SXR is expressed predominantly in the liver and intestine, and it regulates transcription such as of cytochrome P450 (CYP) 3A4, which is an enzyme involved in drug metabolism, and MDR1 (multidrug resistance protein 1) which is activated by a diverse array of pharmaceutical agents including taxol, rifampisin, and clotrimazole (*26*, *27*). Ichikawa et al. identified novel SXR target bone-related genes that were regulated by MK-4 in osteoblastic cells using microarray analysis (*28*). Among extracellular matrix-related genes, they demonstrated that a small leucin-rich repeat proteoglycan, tsukushi, contributes to collagen accumulation (*28*).

We demonstrated for the first time that the oral administration of vitamin K (both PK and MK-4) enhanced the level of IAP mRNA expression in the mouse intestine, and PXR mRNA expression also increased. Further studies on the physiological functions of ALP and transcriptional regulation of ALP induction will provide useful data on the novel effect of vitamin K.

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Ⅱ. 歯

2. 歯周病と炎症

Periodontitis and inflammation

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key words

Porphyromonas gingivalis
gingipain
HMGB1
血管炎症
E-selectin

歯周病の病態形成には，細菌感染による炎症反応と非感染性の慢性炎症が複雑に絡み合っている．歯周ポケットに存在する各種の歯周病関連細菌は，Toll 様受容体を活性化し，歯周組織の慢性炎症形成に寄与している．加えて，HMGB1などの内因性リガンドが恒常的な炎症反応を誘導する．また，歯周病関連細菌 *P. gingivalis* は血管内皮との相互作用により，血管炎症を進展させる．

歯周病は細菌感染を伴った慢性炎症性疾患である

歯周病は感染症であり，歯周病関連細菌によって惹起される炎症性疾患である．また，その発症と進行にはそれらの細菌の病原性だけではなく，歯周組織における宿主側の各種細胞の応答が深く関与する[1]．歯周ポケットには，グラム陰性桿菌をはじめとした数多くの細菌が生息し，競合・共生関係を維持しながら細菌叢を形成している．そのなかで，特定の細菌が歯周病の発症や進行に関与しており，特に関連性が高いと考えている細菌として，*Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Tannerella forsythia, Treponema Denticola* などが知られている[2]．それらの菌は，内毒素，蛋白分解酵素，線毛などの病原因子を有しており，それらの菌が歯周組織に感染する際の重要な因子となる．また，同因子は歯周組織において宿主細胞を活性化し，強力に炎症反応および免疫応答を惹起する．歯周ポケットに形成される細菌叢は時にグリコカリックス様の構造物で被覆され，バイオフィルムの形態を呈する．このようなバイオフィルムを形成した歯周病関連細菌は，抗体，食細胞，抗生物質に対して抵抗性を示すことから，歯周組織に細菌が持続感染しやすい状態となる[3]．このような細菌と宿主の相互作用が持続することと，さまざまな環境因子や遺伝因子が複雑に絡み合って，歯周病の慢性炎症病態が形成されるものと考えられる(図1)．

生活習慣病は慢性炎症性疾患である

近年，肥満，糖尿病，動脈硬化性疾患などの生活習慣病，アルツハイマー病，パーキンソン病などの神経変性疾患，関節リウマチなどの自己免疫疾患，癌などに共通する分子基盤として慢性炎症が注目されている．慢性炎症では長期にわたるストレス応答によって炎症反応が遷延化するとともに，宿主の恒常性が十分維持されず破綻をきたし，不可逆的な組織のリモデリングを生じ，組織・臓器の機能不全をきたすようになる[4]．たとえば，動脈硬化では血管壁に対して，物理的，科学的刺激により内皮細胞や血管平滑筋細胞の機能が変化する．この内皮細胞の機能障害は脂質などの血管壁への浸透・蓄積，血

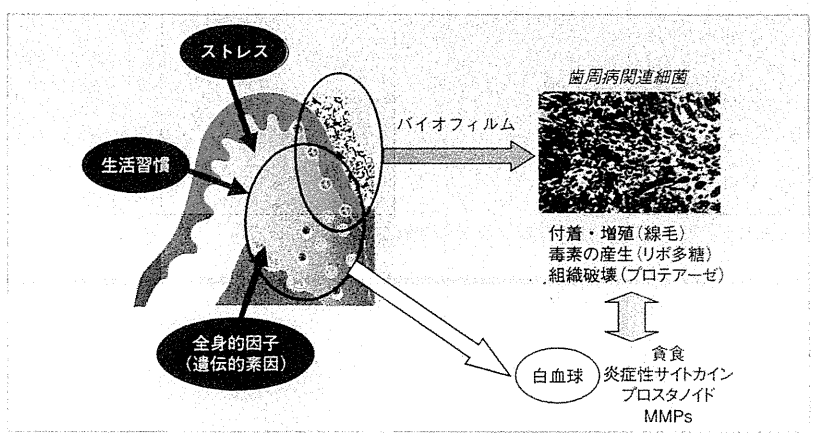

図1　歯周病はバイオフィルム感染症である

歯周病においては，歯周ポケットに生息する細菌群が歯周組織を直接傷害するとともに，各種菌体毒素によって宿主細胞を活性化し，炎症反応が惹起される．加えて，それらの細菌はバイオフィルムを形成し，宿主あるいは抗菌薬に対して抵抗性を示す．

管内皮への白血球の浸潤を引き起こす．このような細胞応答が長期に持続することにより，血管壁の破壊とそれに伴う修復反応が同時に進行し，その結果，組織構築が改変されることになる[5]．以上のような，複雑な細胞・組織応答が他の慢性炎症性疾患にも共通して認められる現象である．近年，歯周病と動脈硬化性疾患や糖尿病などの生活習慣病との関連性が注目されているが，両者の間には単に相関関係が認められるというだけではなく，その発症機序に共通の分子基盤が存在していることが考えられる．歯周病を生活習慣病として捉え，生活習慣病の共通分子基盤である慢性炎症といった視点から考えることは，両者の関係をより深く理解するために極めて重要である．

慢性炎症には臓器特異性が認められる一方，臓器を超えた共通の分子機構が存在する可能性が高い．慢性炎症は，微生物感染により誘導されるものと感染が関連しないものとに大別される．歯周病は前者に属し，糖尿病や動脈硬化性疾患などはおおむね後者に属するものと考えられる．ただし，歯周病の病態形成には細菌感染による炎症反応と非感染性の慢性炎症が複雑に絡み合っているものと考えられる[6]（図2）．

自然炎症としての歯周病

感染症における免疫反応の機序における重要な分子として，Toll 様受容体（Toll-like receptor ： TLR）をはじめとする病原体センサーが重要な役割を担うことが知られている．免疫システムにおいて，病原体などの外来抗原をいかに認識するかは極めて重要であり，TLR はそれらを認識し，自然免疫応答を誘導して炎症反応を惹起する重要な分子である[7]．歯周ポケットに存在する各種の歯周病関連細菌は，これらの受容体を活性化し，歯周組織の慢性炎症形成に寄与しているものと考えられる．歯周病関連細菌の一種である *P.gingivalis* はこれらのリガンドを数多く有し，樹状細胞やマクロファージなどを活性化し，自然免疫応答とともに炎症反応を強力に惹起する．*P.gingivalis* のリポ多糖（LPS）は，他のグラム陰性菌のそれとは異なり，細胞壁のリポ蛋白，リポタイコ酸やペプチドグリカンと同様に TLR2のリガンドとなり得ることも報告されている．そして，同受容体の活性化を介して，マクロファージや単球の活性化に寄与する．また，同菌の菌体表層に存在する線毛は歯周局所への付着・定着に関与するだけでなく，同受容体を介して，炎症性サイトカイン産生を誘導することも報告されている[8,9]．TLR からのシグナルは，MyD88などのシグナル伝達物質を介して，NF-κB（nuclear factor-κB）や IRF（interferon regulatory factor）などの転写因子を活性化し，TNF-α や IL-6（interlenkin-6）といった炎症性サイトカインの産生を誘導する[10]．また，同菌の産生するシステインプロテアーゼ gingipain は，間質のマトリックス蛋白質を分解し，歯周組織を直接破壊するだけでなく，好中球受容体や補体を分解し宿主の免疫反応を抑制する．他方で，マトリックスメタロプロテアーゼ（MMP）や炎症性サイトカインの産生を誘導し，炎症反応を促進する（図3）．さらに，血液凝固因子である Factor X や プロトロンビンなどを活性化し，血液凝固反応

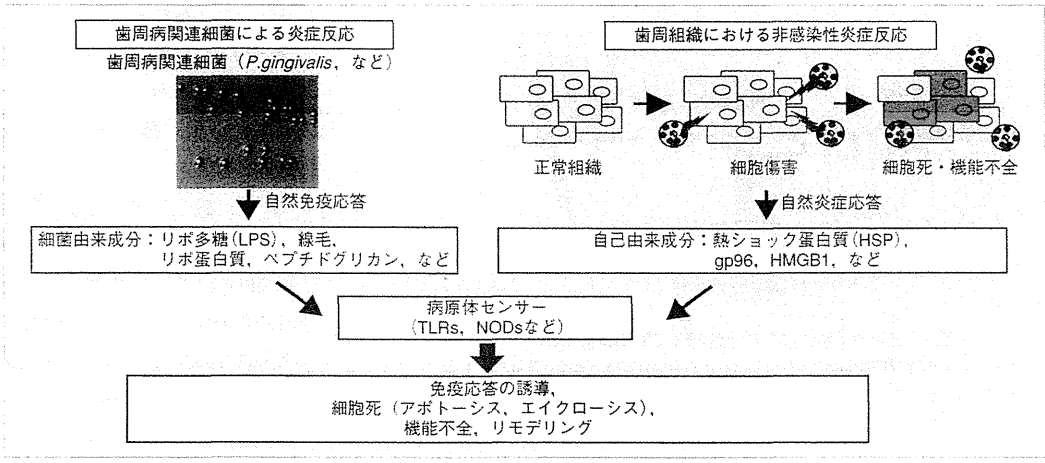

図2　慢性炎症としての歯周病の発症機序

歯周病関連細菌の毒素は，宿主の病原センサーに補足され，宿主細胞の免疫応答を促す．加えて，傷害を受けた細胞は細胞から放出される自己由来成分も同受容体を介して炎症反応を惹起する．これらが複合して慢性炎症としての歯周病の病態が形成される．NOD：nucleotide oligomerization domain

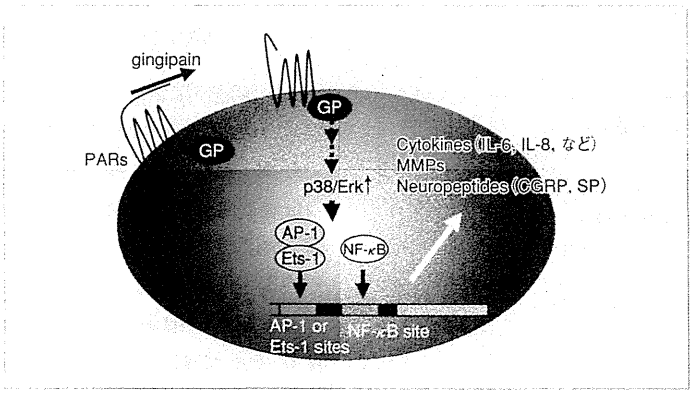

図3　Gingipain が炎症反応を惹起する仕組み

P.gingivalis が産生するトリプシン様システインプロテアーゼ gingipain は，PAR の活性化を介して，サイトカイン，マトリックスメタロプロテアーゼ，神経ペプチドなどの発現を誘導して炎症反応を惹起する．

を誘導したりもする[11]．我々は，gingipain が血管内皮細胞に発現する PAR（protease-activated receptor）を活性化し，同細胞における血管形成調節因子 angiopoietin 2を含む分泌顆粒のエキソサイトーシスを誘導し，LPS に対する感受性を高めて血管の炎症応答を亢進することを明らかにした[12]（図4）．このように，歯周病関連細菌は歯周組織に侵入・定着し，さまざまな病原因子を産生して，歯周組織の炎症反応を惹起している．加えて，組織障害に伴って放出される内因性リガンドが TLR などの病原体センサーを活性化し，外因性リガンドの刺激と相まって感染症における慢性炎症の病態形成に寄与していることが考えられている．傷害を受けたり，壊死したりした細胞はそこに潜む危険を排除するために，免疫系の細胞の活性化を促すようなシグナル（danger signal）を発し，炎症反応を引き起こすことが知られている．このような分子は，DAMPs（danger-associated molecular patterns）とも呼ばれ，ATP，尿酸結晶，HSP（heat shock protein），HMGB1（high mobility group box protein-1）などが知られている．DAMPs は，宿主が自分自身を守り自らを再生させるために生ずると考えられるが，一方こうした

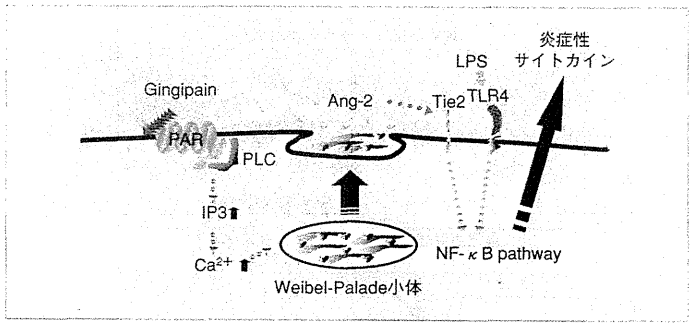

図4　Gingipainによる血管炎症の増強機序
gingipainは，血管内皮細胞からのWeibel-Palade小体のエキソサイトーシスを誘導しangiopoietin-2（Ang-2）の放出を促す．放出されたAng-2は血管内皮のLPSに対する感受性を高める．

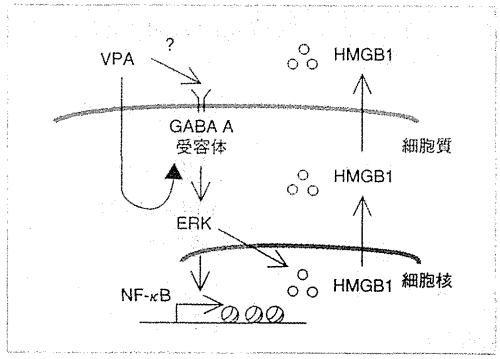

図5　バルプロ酸によるHMGB1の放出機序
バルプロ酸（VPA）はGABA受容体とERKの活性化を介して，HMGB1の放出を誘導する．
ERK：extracellular signal-regulated kinase

免疫応答が過剰に働き，炎症反応が必要以上に生ずるとさまざまな慢性疾患の発症や進行へとつながってゆく[13]．そのような分子の一つであるHMGB1は，細胞核に存在するDNA結合蛋白質の一種で遺伝子の転写を制御する因子である．HMGB1の受容体として，RAGE（receptor for advanced glycation end products），TLR2/4などが知られている[14]．同分子は細胞死によって細胞から漏出するが，活性化した単核球からもHMGB1は放出され炎症反応を惹起することが知られており，多くの炎症性疾患へのHMGB1の関与が示されている．具体的には敗血症の致死因子として，あるいは腎炎，肝炎，肺炎などの慢性炎症病態の形成に関与する因子として注目されている．歯周病との関与もいくつか報告されている．歯肉上皮細胞や歯肉由来線維芽細胞において，炎症性サイトカインTNF-αやLPSなどの刺激によるHMGB1の産生・放出が認められている[15]．また，慢性歯周炎の歯肉溝浸出液中や歯肉上皮にHMGB1の産生が増加している[16][17]．最近，我々は抗てんかん薬の一種であるバルプロ酸（VPA）がLPSと協同して，マクロファージからのHMGB1の産生・放出を増強し，炎症反応を強力に進展することを，*in vitro*および*in vivo*の実験系で明らかにした（図5）．VPAの長期投与の副作用として，歯肉増殖症と歯周炎の増悪が知られているが，VPAは歯周組織の炎症の増悪因子である可能性が示唆された．

血管炎症と歯周病

炎症反応の進展において，白血球が血管内から組織へ浸潤する過程が重要である．また，それらの細胞の血管への付着と血管外への浸潤は，細胞接着因子およびモノカインなどで制御されている．白血球はまず，活性化した血管内皮細胞に補足（capture）され，血管にそって転がり（rolling），付着（adhesion）する．その後，血管内皮細胞を通り抜け血管外へと移動し，組織へ浸潤する．特に，captureからrollingの過程で重要になってくる分子がselectinである[18]．血管内皮細胞には，E-selectinという同細胞に特異的に発現しているselectinが存在し白血球と同細胞との相互作用に寄与している．E-selectinは，TNF-α，IL-1β，LPSなどにより活性化した血管内皮細

胞に発現する．E-selectin は白血球上のリガンドである sialyl lewis X と相互作用し，白血球の rolling を誘発する．また，E-selectin の一部は，細胞外領域で切断され可溶性 E-selectin (sE-selectin) として血中に遊離される．敗血症や膠原病などの炎症性疾患，血栓性血小板減少性紫斑病のような微小血管障害および冠動脈疾患，脳血管障害，閉塞性末梢動脈疾患などの動脈硬化性疾患の患者において血中 sE-selectin 濃度が高値を示すことが知られている．重度の歯肉炎患者の血漿中あるいは歯肉溝浸出液中にも高濃度の sE-selectin が検出される．近年，閉塞性血栓性血管炎と歯周病との関連性が報告され，血管病変部に歯周病関連細菌が高頻度に検出されることが示されている．我々は，歯周病関連細菌，特に *P.gingivalis* が血管病変部に定着する機序の一つとして E-selectin に着目し，同分子と *P.gingivalis* との相互作用について検討した．その結果，TNF-α で刺激した血管内皮細胞上では，*P.gingivalis* の付着が著しく亢進すること，またそれは E-selectin 抗体および sialyl lewis X の添加によって有意に抑制されることを明らかにした (図6)．また従来，血管内皮への *P.gingivalis* の付着には，同菌の線毛が重要であることが報告されてきたが，E-selectin との相互作用には OmpA 様外膜蛋白質が重要であることが明らかになった．これらの結果は，炎症血管においては，*P.gingivalis* が付着しやすいこと，またその付着様式は上皮や正常血管内皮とのそれと異なる可能性が考えられた．このように血管炎症部位に付着した *P.gingivalis* は，その後，細胞あるいは組織に侵入し持続感染を成立させるとともに，血管炎症を増強する可能性が考えられる (図7)．

歯周病関連細菌の血管炎症への関与は，歯周病の病態形成に極めて重要である．その分子機構と病態生理学的意

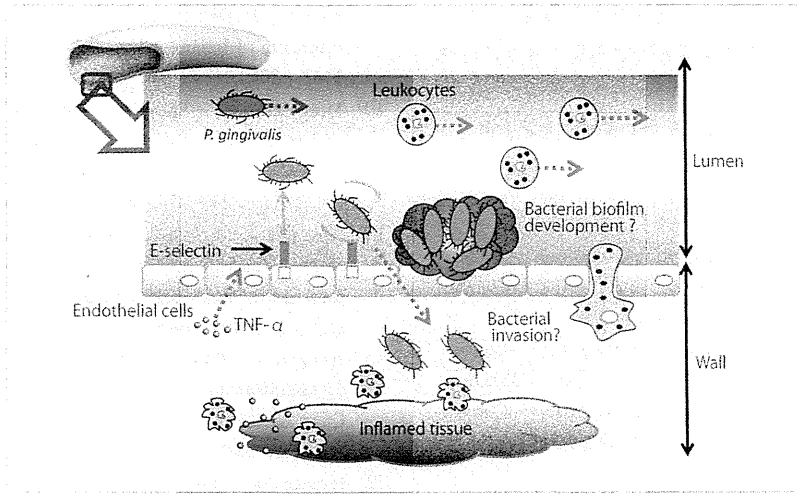

図6　E-selectin を介した *P.gingivalis* の血管付着
P.gingivalis は，E-selectin を介して活性化した血管内皮と付着する．その後，同菌は血管内皮に侵入，あるいは内皮上にバイオフィルムを形成し，血管炎症を増強する可能性がある．

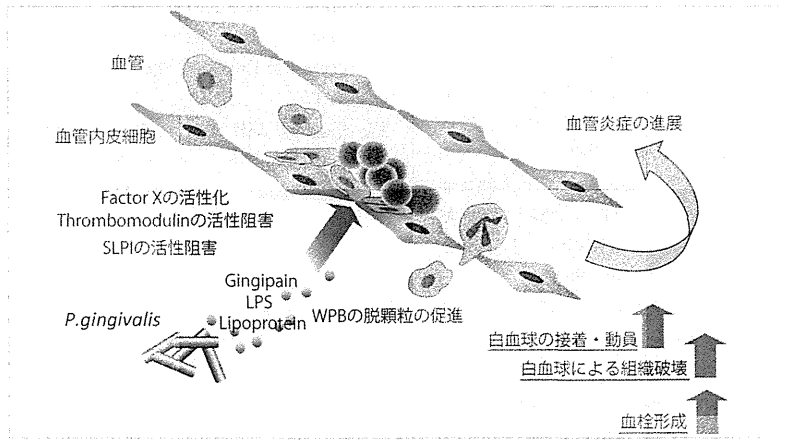

図7　*P.gingivalis* が血管炎症を増悪する機序
P.gingivalis が産生する種々の毒素は，白血球の活性化および血管内皮への接着・動員とそれによる血管傷害，さらには血管内における血栓形成を促進し，血管炎症を進展させる可能性がある．

義の解明は，慢性炎症を基盤とした歯周病の理解に必須であるとともに，歯周病の新しい治療戦略や診断法を確立するためにも極めて重要であると考える．

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VALPROIC ACID INCREASES SUSCEPTIBILITY TO ENDOTOXIN SHOCK THROUGH ENHANCED RELEASE OF HIGH-MOBILITY GROUP BOX 1

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ABSTRACT—High-mobility group box 1 (HMGB1) is a nuclear factor and a secreted protein. During inflammation, HMGB1 is secreted into the extracellular space where it can interact with the receptor for advanced glycation end products and trigger proinflammatory signals. Extracellular HMGB1 plays a critical role in several inflammatory diseases such as sepsis and rheumatoid arthritis. Valproic acid (VPA) is one of the most frequently prescribed antiepileptic drugs. The present study was undertaken to investigate the effect of VPA on secretion of HMGB1 in systemic inflammatory responses induced by lipopolysaccharide. Pretreatment with VPA increased the susceptibility of mice to lipopolysaccharide in endotoxemia. Valproic acid induced HMGB1 release and nuclear factor κB activation in RAW-blue cells. Valproic acid promoted the phosphorylation of ERK1/2 but not that of p38 or JNK. The MEK1/2 inhibitor PD98059 also suppressed HMGB1 release and activation of nuclear factor κB induced by VPA. Valproic acid induced expression of γ-aminobutyric acid receptors in macrophages, and picrotoxin, a γ-aminobutyric acid A receptor antagonist, inhibited the VPA-activated phosphorylation of ERK and VPA-induced HMGB1 release. These results suggest that VPA may exacerbate innate immune responses to endotoxin through enhanced release of HMGB1.

KEYWORDS—Sepsis, alarmin, lipopolysaccharide, histone deacetylases, inflammation, immunomodulation, cytokines

INTRODUCTION

Sequential cytokine induction by cells of the mononuclear phagocyte system has been found *in vitro* and *in vivo* following endotoxin stimulation. These cytokines, such as tumor necrosis factor (TNF), interleukin 6 (IL-6) and IL-1, are thought to be important as regulators of the immune system under physiologic conditions. However, excessive amounts of cytokines play a role in the pathologic manifestations of endotoxemia. High-mobility group box 1 (HMGB1), a protein previously known as a nuclear transcription factor, is a critical mediator of lethality in endotoxemia and sepsis (1). During a systemic inflammatory response, HMGB1 is released into the circulation by activated monocytes and by damaged cells. Administration of antibodies to HMGB1 attenuates endotoxin lethality in mice. Therefore, it is thought that HMGB1 is an important mediator of lethal systemic inflammation (1).

Inhibitors of nuclear histone deacetylases (HDACs) have been shown to suppress cancer cell proliferation *in vitro* (2, 3) and reduce experimental tumor growth *in vivo* (4, 5). Histone deacetylase inhibitors include short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides. Suberoylanilide hydroxamid acid (SAHA)—the classic member of the class of hydroxamic acids—has potent anti-inflammatory activities, both *in vitro* and *in vivo* (6, 7). Histone deacetylase inhibition was shown to be associated with significant suppression of the production of proinflammatory cytokines (8, 9). These anti-inflammatory properties were confirmed by studies demonstrating that treatment with SAHA resulted in a significant reduction of disease severity in a murine model of systemic lupus erythematosus, the MLR-*lpr/lpr* mouse (10). The anti-inflammatory effects described for HDAC inhibitors have so far been limited to SAHA (11) and trichostatin A (TSA) (8, 12), both members of the class of hydroxamic acids. Thus, it remains to be clarified whether the anti-inflammatory effects of HDAC inhibition are restricted to this class or whether inhibition of HDACs in general results in suppression of cytokine production.

Many studies show that various HDAC inhibitors suppress inflammation. A few studies show that select HDAC inhibitors can increase inflammation. The differences between these results are probably explained by differences in the specificity of HDAC inhibitors and the various animal models used to uncover the mechanisms of inflammation.

Valproic acid (VPA) is an HDAC inhibitor of the class of short-chain fatty acids (13), and it is an established drug in the treatment of epileptic seizures and bipolar disorder (14). Accumulated experimental and clinical data also show that VPA might be a potent anticancer drug (15). Valproic acid also has anti-inflammatory effects (16). On the other hand, in some cases, VPA augments inflammatory responses; patients

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receiving VPA may well develop hemorrhagic pancreatitis, bone marrow suppression, and hepatotoxicity (17, 18). Gingival overgrowth and progression of gingival inflammation are sometimes observed in patients who are taking VPA chronically (19). However, the mechanisms underlying the inflammatory effects of VPA are not known. In the present study, we found that VPA enhanced systemic inflammatory responses induced by lipopolysaccharide (LPS) by promoting the secretion of HMGB1 in murine macrophage cultures and in a murine model of endotoxic shock.

MATERIALS AND METHODS

Materials

Valproic acid and SAHA were purchased from Sigma-Aldrich (St Louis, Mo). Trichostatin A was purchased from Calbiochem (San Diego, Calif). *Escherichia coli* O18 LPS and TNF-α were purchased from Sigma-Aldrich. Purified rabbit polyclonal antibodies to HMGB1 and chicken polyclonal antibodies to HMGB1 were purchased from SHINO-TEST (Tokyo, Japan). Mouse monoclonal antibodies to phospho-ERK1/2, p38, and JNK were purchased from Cell Signaling Technology (Beverly, Mass). Mouse monoclonal antibodies to ERK1/2, p38, and JNK were purchased from BD Bioscience (Franklin Lakes, NJ). Mouse monoclonal antibodies to β-actin were purchased from BioVision (San Francisco, Calif).

Cell culture

RAW-blue cells were established from RAW 264.7 macrophage cells (InvivoGen, San Diego, Calif). They stably express a secreted embryonic alkaline phosphatase gene inducible by nuclear factor κB (NF-κB) and AP-1 transcription factors. RAW-blue cells were precultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Analysis of HMGB1 release

To determine the effect of VPA on HMGB1 release, RAW-blue cells were stimulated with various concentrations of VPA, *E. coli* LPS, TNF-α, TSA, and SAHA, and the amount of HMGB1 released into the medium was measured by enzyme-linked immunosorbent assay (ELISA) (SHINO-TEST).

Measurement of NF-κB activity

RAW-blue cells were incubated with VPA. Culture supernatants of the cells were mixed with QUANTI-blue (InvivoGen), an alkaline phosphatase substrate, and then incubated for 30 min at 37°C. The absorbance (λ = 595 nm) was measured by using Appliskan (Thermo Fisher Scientific, Waltham, Mass) to detect relative NF-κB activity.

Western blotting

RAW-blue cells were incubated with various specimens for 0.5 to 24 h. Cells were lysed in Celytic M (Sigma-Aldrich) with protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitor mixture (Nacalai Tesque). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blotted by the SNAP i.d. Protein Detection System (Millipore, Billerica, Mass) following the manufacturer's protocol. The membranes were blocked with 0.02% skim milk (BD Bioscience) in Tris-buffered saline (pH 7.4) containing 0.05% Tween 20 (TBST) and then incubated with the appropriate antibodies in TBST containing 0.02% skim milk. After washing, the membranes were incubated with horseradish peroxidase–conjugated anti–rabbit or mouse IgG antibodies (IBL, Takasaki, Japan) in TBST containing 0.02% skim milk. The membranes were washed twice with TBST, and then immunoreactive bands were visualized using an ECL Plus Detection System (GE Healthcare, Uppsala, Sweden).

Cytotoxicity assay

Cell viability was determined by using Cytotoxity Detection Kit PULS (LDH) (Roche Diagnostics, Basel, Switzerland) following the manufacturer's protocol. As an apoptosis marker, caspase 3/7 activity was determined by the Caspase-Glo 3/7 assay (Promega, Madison, Wis) following the manufacturer's protocol.

Fluorescence immunostaining

To investigate the translocation of HMGB1, RAW-blue cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Cambridge, Mass) and incubated with each specimen for 24 h. Cells were then fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min. After washing with phosphate-buffered saline three times, cells were blocked in 5% sheep serum albumin in TBST for 1 h and incubated with rabbit anti-HMGB1 polyclonal antibody (SHINO-TEST) for 1 h at room temperature. The slides were then washed with TBST and incubated with anti–rabbit IgG conjugated with green Alexa Fluor 488 (Invitrogen, Carlsbad, Calif). Finally, the slides were covered by ProLong Gold with DAPI (Invitrogen). Images were captured using a fluorescence microscope (Keyence, Osaka, Japan).

Quantitative polymerase chain reaction

RAW-blue cells were stimulated with 5 mM of VPA for 24 h, and mRNA expression of γ-aminobutyric acid (GABA) receptors was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA from cells was purified with an RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) and DNdigest (Qiagen), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) following the manufacturer's protocol. Real-time PCR was performed on a 7300 Real-time PCR System (Applied Biosystems, Carlsbad, Calif) using SYBR MIX Plus (Toyobo). The mouse HMGB1 transcript was amplified using the following primers: forward, 5′-CCA AAG GGG AGA CCA AAA AG-3′; reverse, 5′-TCA TAG GGC TGC TTG TCA TCT-3′. The mouse TNF-α transcript was amplified using the following primers: forward, 5′-AAG CCT GTA GCC CAC GTC GTA-3′; reverse, 5′-GGC ACC ACT AGT TGG TTG TCT TTG-3′. The mouse $GABA_A$ receptor subunit α1 transcript was amplified using the following primers: forward, 5′-CGA AGG TGG CTT ATG CAA CA-3′; reverse, 5′-CCC ACG CAT ACC CTC TCT TG-3′. The mouse $GABA_A$ receptor subunit α3 transcript was amplified using the following primers: forward, 5′-CTC CAA CAG CGA TTG CTT CA-3′; reverse, 5′-TGA TGC GGG AAA TTT TGT CA-3′. The mouse $GABA_A$ receptor subunit β2 transcript was amplified using the following primers: forward, 5′-AAC CGA GTG GCA GAC CAA CT-3′; reverse, 5′-TCG GGA TGC AAT CGA ATC AT-3′. Mouse β-actin transcript was used as an internal control: forward primer, 5′-CAT CCG TAA AGA CCT CTA TGC CAA-3′; reverse, 5′-ATG GAG CCA CCG ATC CAC-3′. The housekeeping gene, β-actin, was used to normalize all test genes, and data quantification was performed using the ΔΔCT method.

Murine model of endotoxemia

This study was approved and performed in accordance with the guidelines of the School of Dentistry, Aichi Gakuin University at Nagoya, Aichi, Japan. Endotoxemia was induced in Balb/c mice (male, 7–8 weeks old) by i.p. injection of *E. coli* LPS (1). Briefly, mice were injected s.c. with VPA (600 mg/kg) 12 h before LPS administration. The mice were monitored for survival over a period of 192 h after LPS administration. In some cases, VPA (600 mg/kg) with or without a neutralizing antibody for HMGB1 (2 or 4 mg/kg) was given to mice 12 h before LPS (25 mg/kg) administration. The mice were monitored for survival over a period of 192 h after LPS administration. In parallel experiments, blood was collected from mice at 24 h after LPS administration, and levels of serum IL-6 (kit purchased from IBL) and HMGB1 (kit purchased from SHINO-TEST) were determined by ELISA.

Statistics

Results are expressed as means ± SD. Mortality studies were analyzed using log-rank test. Statistical evaluation of the continuous data was performed by one-way analysis of variance, followed by Tukey test for between-group comparisons. These statistical analyses were done using the statistical software Statcel 2 (OMS Ltd., Saitama, Japan). The level of significance was considered to be $P < 0.05$.

RESULTS

VPA enhances systemic inflammatory responses induced by LPS in vivo and in vitro

To determine the effect of VPA on systemic inflammatory response *in vivo*, we used a mouse model of endotoxic shock induced by injecting purified *E. coli* LPS according to the modified method of Galanos et al. (20). Valproic acid was injected i.p. 12 h before administering saline or LPS, and the survival rate of the mice was determined. Fifty percent of the mice died within 3 days after administration of LPS (Fig. 1A). Valproic acid alone did not cause lethality in mice (Fig. 1A). However, pretreatment with VPA reduced the survival rate of

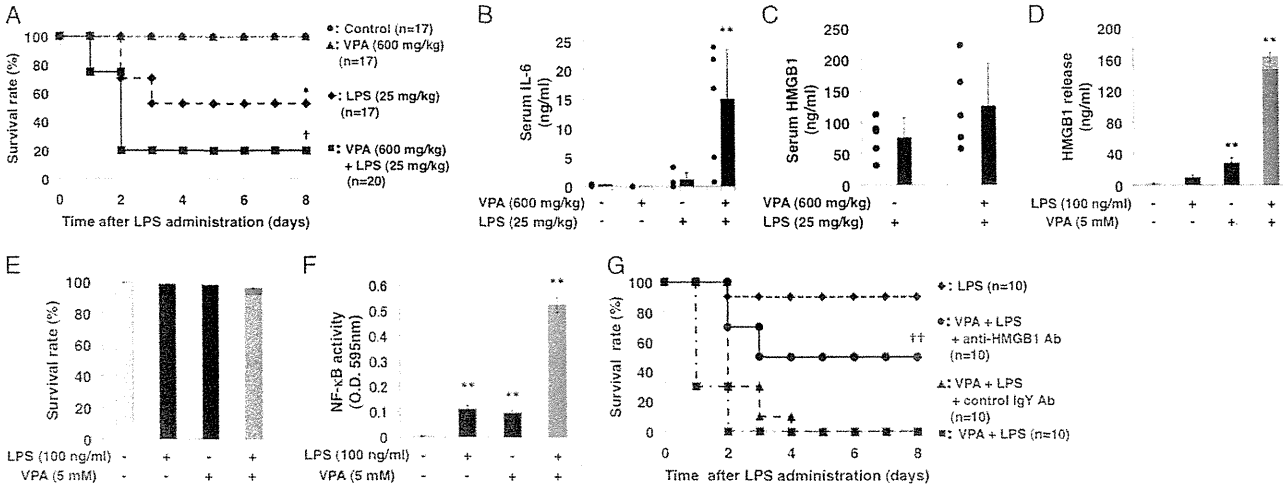

FIG. 1. **Valproic acid enhanced systemic inflammatory responses induced by LPS.** Valproic acid (600 mg/kg; s.c. injection) was given to mice 12 h before LPS administration (25 mg/kg; i.p. injection). Mice were monitored for survival over a period of 192 h after LPS administration (A) ($^{*}P < 0.05$ vs. control, $^{\dagger}P < 0.05$ vs. LPS). Blood in mice was collected at 24 h after LPS administration, and levels of serum IL-6 and HMGB1 were determined by ELISA (B and C) ($^{**}P < 0.01$ vs. control). RAW-blue cells were preincubated with VPA (5 mM) for 6 h and were then treated with LPS (100 ng/mL) for 24 h. The levels of HMGB1 in culture media were determined by ELISA (D) ($^{**}P < 0.01$ vs. control). Cell viability was evaluated by LDH assay (E). Nuclear factor κB activity in RAW-blue cells was analyzed by a reporter assay, as described in Materials and Methods (F) ($^{**}P < 0.01$ vs. control). Valproic acid (600 mg/kg) with or without a neutralizing chicken antibody for HMGB1 (4 mg/kg) and a control IgY antibody was given to mice 12 h before LPS (25 mg/kg) administration. Mice were monitored for survival over a period of 192 h after LPS administration (G) ($^{\dagger\dagger}P < 0.05$ vs. VPA + LPS).

mice (20%) (Fig. 1A). We also determined the concentrations of IL-6 and HMGB1 in serum of the mice. Serum was collected at 24 h after LPS administration, and levels of IL-6 and HMGB1 in the serum were determined by ELISA. Interleukin 6 was induced in serum in LPS-administered mice (Fig. 1B). Pretreatment with VPA significantly increased the concentration of IL-6 in serum in LPS-administered mice (Fig. 1B). Furthermore, serum levels of HMGB1 were increased in VPA-pretreated mice (Fig. 1C).

High-mobility group box 1 is a key mediator of systemic inflammation and sepsis (1). Therefore, we investigated whether VPA affects LPS-induced HMGB1 production in macrophages in culture. RAW-blue cells were preincubated with VPA (5 mM) for 6 h and were then treated with LPS (100 ng/mL) for 24 h. The levels of HMGB1 in culture media were detected by ELISA. Valproic acid as well as LPS induced HMGB1 release in macrophage cultures (Fig. 1D). However, release of HMGB1 significantly increased in cultures pretreated with VPA before stimulation with LPS (Fig. 1D). To exclude the possibility that HMGB1 in media leaks out from necrotic cells, we examined the effect of pretreatment of VPA and LPS stimuli on viability of macrophages. RAW-blue cells were preincubated with VPA (5 mM) for 6 h and were then treated with LPS (100 ng/mL) for 24 h. Cell viability was evaluated by LDH assay. Lipopolysaccharide or VPA alone or in combination did not affect the viability of macrophages (Fig. 1E). We next examined VPA activation of NF-κB in macrophage cultures. Pretreatment with VPA significantly enhanced LPS-induced NF-κB activation (Fig. 1F). To test the effect of HMGB1 blockade in the endotoxin shock mouse model, anti-HMGB1 chicken polyclonal antibodies or control IgY antibodies were injected into the peritoneal cavity simultaneously and after injection of VPA. All 10 mice in the group administered VPA and LPS were dead 2 days after LPS administration. However, when mice subjected to LPS administration were pretreated with anti-HMGB1 antibodies (total 4 mg/kg), 5 (50%) of the 10 mice survived for 8 days (Fig. 1G). In contrast, none of the 10 mice survived after pretreatment with VPA and control antibodies and then administration of LPS (Fig. 1G). These results suggest that VPA primes macrophages to respond to LPS and to produce inflammatory mediators such as HMGB1 and enhances systemic inflammatory responses in mice.

VPA induces HMGB1 release from macrophages

To confirm that VPA activates HMGB1 release, RAW-blue cells were incubated with VPA (5 mM), LPS (100 ng/mL), and TNF-α (20 ng/mL) for 24 h, and the amount of HMGB1 released into the medium was measured by ELISA. Valproic acid strongly induced HMGB1 in macrophage cultures, and the amounts of the protein released were comparable to those induced by *E. coli* LPS (Fig. 2A). Valproic acid–induced HMGB1 release increased time dependently and continued for up to 24 h (Fig. 2B). We next compared the localizations of HMGB1 in VPA-stimulated and nonstimulated macrophages. Strong nuclear localization of HMGB1 was observed in untreated macrophages, whereas nuclear-cytoplasmic translocation of HMGB1 was observed in macrophages stimulated with VPA, LPS, and TNF-α for 24 h (Fig. 2C). To determine whether VPA induces active HMGB1 release in the absence of cell death, the effects of VPA on cell viability were investigated by LDH assay. Valproic acid as well as LPS and TNF-α was not toxic to macrophages in culture (Fig. 2D). To determine the effect of VPA on the apoptosis pathway, we examined the activity of caspase 3/7 in VPA-treated macrophage cultures. Activity of caspase 3/7 was not increased by stimulation with VPA in macrophages (Fig. 2E). Moreover, VPA did not induce mRNA expression of HMGB1 in macrophages (Fig. 2F). We also examined mRNA expressions of HMGB1