Effects of Red Wine Polyphenols
on
Vascular Smooth Muscle Cell Function

-Molecular Mechanism of French Paradox-

赤ワインポリフェノールの
血管平滑筋細胞増殖・遊走に対する効果

－フレンチ・パラドックスの分子機序－

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ABBREVIATIONS

VSMC; vascular smooth muscle cells

RASMC; rat aortic smooth muscle cells

BCEC; bovine carotid endothelial cells

HASMC; human aortic smooth muscle cells

HUVEC; human umbilical vein endothelial cells

ATF; activating transcription factor

CRE; cyclic AMP-responsive element

CREB; cyclic AMP-responsive element binding protein

PDGF-BB; platelet-derived growth factor-BB

PI3-kinase;phosphatidylinositol 3'-kinase

MAPK; mitogen-activated protein kinase

ERK1/2; extracellular signal-regulated kinase 1/2

SAPK1/JNK; stress-activating protein kinase-1/c-Jun NH2-terminal kinase

$p38^{MAPK}$; p38 mitogen-activated protein kinase

MKK3/6; mitogen-activated protein kinase kinase 3/6

$p27^{Kip1}$; p27Kinase inhibitory protein 1

INTRODUCTION

Polyphenols are found in grapes and other fruits and vegetables, and are generated through the process of biological synthesis in these plants. Although polyphenols are widely distributed in plants, they are found in high concentrations in only a few foods and beverages, such as red wine and tea. Grapes are one of the most widely consumed fruits in the world, and are rich in polyphenols and 60-70% of grape polyphenols exist in grape seeds. Only 2-5% of grape polyphenols exists in grape pulp. In grape skin, there are different polyphenols, called anthocyanins or resveratrol, which usually have purple color and amount to approximately 30% of total polyphenols in grape. Some of the individual polyphenols in grape seeds are also present in tea, such as catechins, but most of the grape seed polyphenols are quite different from tea polyphenols. For example, polyphenols in grape seeds are mainly rich in polymers, whereas most tea polyphenols are monomers, such as catechins. The polyphenolic fraction of red wine includes phenolic acids such as p-cumaric, cinnamic, caffeic, gentisic, ferulic and vanillic acids; trihydroxy stilbenes such as resveratrol and polydatin or resveratrol-glucoside; and flavonoids such as catechin, epicatechin, and quercetin. The combined name for monomers of the derivatives of flavan-3-ol is catechins and for oligomers is procyanidins or proanthocyanidins. In red wine and grape juice, bitter taste (or astringent taste) is resulted from tannins and catechins, and source of purple color is due to anthocyanins. Although numerous investigations using polyphenols have recently progressed, the mechanisms on the absorption, metabolism and distribution of polyphenols after consumption of polyphenol-rich foods are unclear yet.

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High intake in saturated fat and cholesterol is usually associated with increased risk of coronary heart disease (CHD). In spite of high-cholesterol diet, epidemiological studies have demonstrated that French population has lower mortality from CHD compared to other countries.[1,2] Recently, regular consumption of red wine in the French population has been believed to contribute to the phenomenon "French paradox". Red wine has approximately 10-fold excess volume of polyphenols compared with other alcoholic beverages. Among these epidemiological data, naturally occurring polyphenolic antioxidants contained in red wine have received increasing attention in recent years. Recently, it has been shown that this paradoxical finding, "French paradox" is attributed to regular consumption of red wine, and the unique anti-atherogenic effects of red wine reside in the action of polyphenols which are sparse or absent in white wine and other alcoholic beverages.[2] It has been previously reported that polyphenolic substances in red wine inhibit the copper-catalysed oxidation of low-density lipoprotein (LDL) *in vitro*[3], and red wine consumption by healthy volunteers reduced the susceptibility of their LDL to lipid peroxidation.[4] Moreover, it has been recently reported that red wine polyphenols exert decreasing activity of lipoprotein (a), an independent atherogenic risk factor, and increasing levels of HDL-cholesterol.[5] Additionally, it has been also reported that red wine polyphenols have not only beneficial effects for lipid metabolism but also other various anti-atherogenic effects, such as inhibition of adhesion molecule expression in cytokine-stimulated vascular endothelial cells (EC)[6], induction of vaso-relaxation because of NO release[7], downregulation of tissue factor expression[8], and inhibition of platelet aggregation.[9]

Initial events of atherosclerotic plaque are characterized by dysfunction of vascular EC, LDL oxidation, and foam cell formation from macrophage.[10] Because LDL oxidation is known to be one of the initial events in atherogenesis[11, 12], the anti-oxidative effects of red wine polyphenols may explain in part the mechanism of French paradox.[1] In other aspects of atherogenesis, progressive intimal thickening in development of atherosclerotic plaque is characterized by migration of vascular smooth muscle cells (VSMC) from the arterial media into intima, excessive proliferation of VSMC in the neointima, and increased extracellular matrix deposition.[10] Although the proliferation and migration of VSMC play key roles in the pathogenesis of atherosclerotic lesions[10], the effects of red wine polyphenols on VSMC proliferation and migration have not been elucidated. The aim of this study is investigation of effects of red wine polyphenols on vascular smooth muscle cell function, such as proliferation and migration, and elucidation of molecular mechanisms for "French paradox".

STUDY 1.

Effects of Red Wine Polyphenols on Proliferation of Vascular Smooth Muscle Cells or Vascular Endothelial Cells

INTRODUCTION

VSMC proliferation in the neointima has been known to play a key role in the progressive development of atherosclerotic plaque.[10] Cell growth is regulated by the sequential expression of cyclins and the activation of their associated cyclin-dependent kinases (Cdks).[13-15] Cyclin A, one of these cyclins, is important in the G1/S transition, and in the S and G2/M phases of cell cycle[16, 17], and has a critical role in DNA replication during S phase of cell cycle.[18, 19] The cyclin A promoter contains activating transcription factor (ATF) site, also termed the cyclic AMP-responsive element (CRE), as a potential regulatory element.[20, 21] The ATF/CRE site in the cyclin A promoter is bound by cyclic AMP-responsive element binding protein (CREB) and activating transcription factor-1 (ATF-1) by heterodimer formation.[22-24] Their heterodimeric binding to the ATF site in the cyclin A promoter plays an important role in the induction of maximal cyclin A gene expression.[22-24] Previously, it has been reported that cyclin A is one of the key regulators of VSMC proliferation caused by mechanical balloon injury in rat and human arteries *in vivo*[25], and by homocysteine or serum *in vitro*.[26] The aim of this study is elucidation of effects of red wine polyphenols on the proliferation of VSMC stimulated by serum and molecular mechanism in relation to cell cycle regulation.

MATERIALS AND METHODS

Preparation of Red Wine Polyphenolic Compounds

As shown in Fig. 1, pulverized red wine "Tomi-no-Oka" (10 bottles, 7600 ml in total) (Suntory Co., Osaka, Japan) was fractionated by the adsorption chromatography using Diaion HP-20 column (40×40 cm; Mitsubishi Chemical Industries, Tokyo, Japan). The column was eluted with deionized water, and eluent which did not contain polyphenolic substances was discarded. Total polyphenolic fraction of red wine (24 gram as a dry weight), named RWP, was obtained by eluting the column with 100% ethanol. The fraction RWP was further separated into six fractions, Fraction 1 to 6, by successive column chromatography using Toyopearl HW-40ec (20×50 cm; Tosoh, Tokyo, Japan). Elution of RWP with 50% ethanol through this column resulted in the formation of four bands, which were collected as Fraction 1 to Fraction 4. The remaining compounds from RWP in the column were further eluted with 100% ethanol, and the eluent was named as Fraction 5. The last fraction, Fraction 6, was obtained by eluting the column with 70% acetone. To measure each content of polyphenolic substances, the quantitative estimation of total polyphenolic content in each fraction were was systematically analyzed by ultraviolet (UV) spectrophotometer according to the Folin-Denis method.[27-29] Polyphenolic content of each fraction was shown in the Figure 1. The content of proanthocyanidins (polymerized anthocyanidins) was measured according to the Butanol-HCl method.[30] All fractions were lyophilized and kept at -20℃. Just before the experiments, each compound was solved with 50% ethanol using ultrasonication.

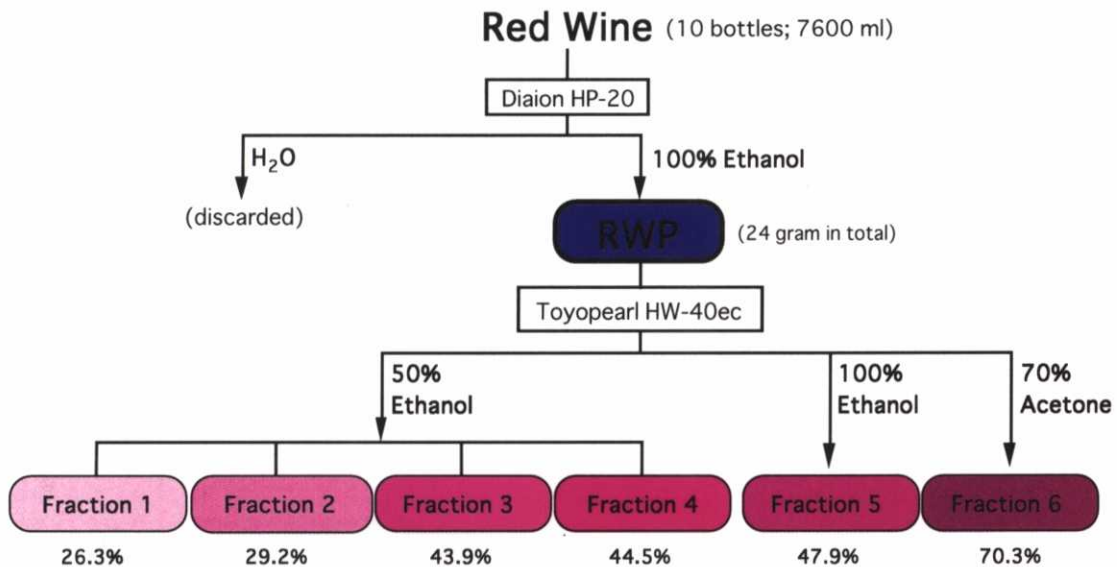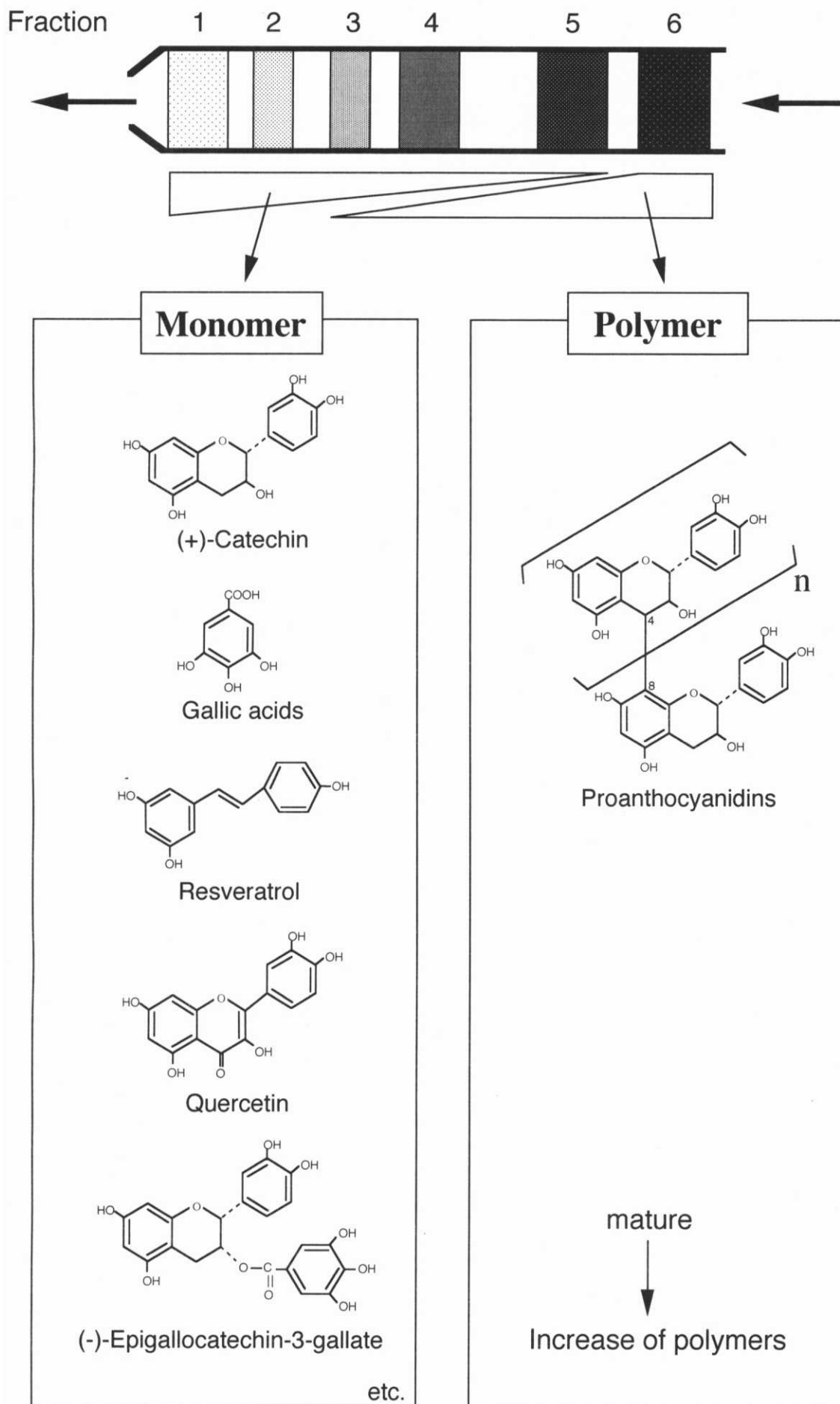## Preparation of Red Wine Polyphenolic Compounds

Figure 1. Preparation of red wine polyphenols. The total polyphenolic fraction, called RWP, from 10 bottles (7,600 ml) of red wine was extracted using Diaion HP-20 column chromatography. RWP was further separated into six polyphenolic fractions, Fraction 1 to 6, using a Toyopearl HW-40ec column. Polyphenolic content of each fraction by Folin-Denis method is underlined.

Cell Culture

Rat aortic smooth muscle cells (RASMC; passages 6 to 8) were prepared from Sprague-Dawley rats and cultured in Dulbecco's modified Eagle's medium (DMEM, Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY), 25mM HEPES (pH 7.4), and antibiotics as described.[31, 32] Bovine carotid endothelial cells (BCEC; passages 6 to 9) were cultured in the same medium without HEPES.[31] Human aortic smooth muscle cells (HASMC) or human umbilical vein endothelial cells (HUVEC) were cultured in Medium 199 or endothelial cell basal medium (EBM) supplemented with 10% FBS.

Fraction 1 2 3 4 5 6

Monomer

(+)-Catechin

Gallic acids

Resveratrol

Quercetin

(-)-Epigallocatechin-3-gallate

etc.

Polymer

Proanthocyanidins

mature

Increase of polymers

Cell Proliferation and [^{3}H] -Thymidine Incorporation

RASMC and BCEC were plated at a density of 10,000 cells per cm^2 in 24-well plate (surface area; 2 cm^2/well). After 48 hr, the cells were treated with RWP solved in 50% ethanol at final concentrations of 1, 3, 10, 30, and 100 μg/ml for 72 hr. Cell number was measured by Coulter cell counter (Coulter Co., Tokyo, Japan). For the thymidine incorporation study, cells were labeled with [methyl-^{3}H] thymidine (Du-Pont NEN, Tokyo, Japan) at 1 μCi/ml during the last 3 hr. After the cells had been labeled, they were washed with PBS, fixed in cold 10% trichloroacetic acid, and washed with 95% ethanol. Incorporated [^{3}H] thymidine was extracted in 0.2N NaOH and measured in a liquid scintillation counter. The effects of RWP on the proliferation and DNA synthesis in RASMC or BCEC were expressed as a percentage of control containing 0.5% ethanol as a vehicle. Moreover, to examine the effect of six fractions separated from RWP on the thymidine incorporation into RASMC, the cells were treated with or without each fraction at a final concentration of 30 μg/ml for 72 hr. Results were shown as mean±SEM from six wells. Statistical analysis was performed by ANOVA.

RNA Isolation and Northern Blot Analysis

RASMC were plated at the same density on a 10 cm-diameter dish (surface area, 55 cm^2) for 48 hr prior to the experiments. Total RNA was extracted from RASMC by guanidinium isothiocyanate extraction and centrifugation through cesium chloride.[33] The rat cyclin A cDNA fragment was amplified by the reverse transcription-polymerase chain reaction as described.[34] The RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nylon filters (Hybond-N,

Amersham Pharmacia, Bucks, UK). The filters were hybridized at 68°C for 2 hr with a random-primed, ^{32}P-labeled rat cyclin A cDNA probe in QuikHyb solution (Stratagene, La Jolla, CA). The hybridized filters were washed in 30 mM NaCl, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate at 56°C and autoradiographed on x-ray film (Kodak X-OMAT AR film) at −80°C for 24 to 48 hr. Moreover, after the hybridized filters were washed in a re-wash solution (50% formamide, 1mM EDTA, 10mM Tris) at 80°C, they were rehybridized using ^{32}P-labeled CREB and ATF-1 cDNA probes. The human CREB plasmid[35] and a human ATF-1 plasmid[36] were obtained from Drs. M. R. Green (University of Massachusetts, Amherst, MA) and M. R. Montminy (Harvard Medical School, Boston, MA). To confirm the equal loading of RNA, the filters were washed in a re-wash solution and rehybridized with a radiolabeled 18S rRNA oligonucleotide probe.[37] The filters were scanned, and radioactivity was measured on an Instant-Imager (Packard Instrument Company, Meriden, CT).[38]

Transfection and Luciferase Assay

To investigate the mechanisms mediating the regulation of cyclin A gene expression by treatment with RWP, we transfected into RASMC a luciferase reporter plasmid containing the human cyclin A promoter. Reporter constructs containing fragment of the human cyclin A 5'-flanking region (bp -266 to +205) were inserted into the promoterless firefly luciferase reporter plasmid pGL2-Basic (Promega) as described.[39] RASMC were transfected with 8 μg of luciferase constructs by the DEAE-dextran method followed by a 1-min DMSO shock.[31] The normal ATF consensus sequence (TGACGTCA) in the plasmid -266/+205 was mutated

to TGCCCCCA by polymerase chain reaction to generate the plasmid mut -266/+205 as described.[40] To correct for variability in transfection efficiency, we cotransfected 2 μg of pRL-SV40 control plasmids (containing the potent SV40 enhancer and promoter driving the structural gene coding for *Renilla* luciferase) in all experiments. Twenty-four hours after transfection, the cells were incubated with different concentrations of RWP for 72 hr, were then harvested using a lysis buffer. We measured two kinds of luciferase activity by Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's protocol, and the ratio of Firefly luciferase activity to *Renilla* luciferase activity in each sample was used as a measure of normalized luciferase activity. The results were expressed as a percentage of control containing 0.5% ethanol as a vehicle. Values were shown as mean±SEM. Statistical analysis was performed by ANOVA.

Gel Mobility Shift Assay

Nuclear extracts from RASMC treated with or without RWP were prepared as described.[40] Protein concentrations in nuclear extracts were measured by the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA), which is based on the Bradford method.[41, 42] A double-stranded oligonucleotide probes synthesized according to the sequence of the human cyclin A 5' flanking region containing a typical ATF site (bp -84 to -63; 5'-TGAA<u>TGACGTCA</u>AGGCCGCGAG-3') were radiolabeled. A typical binding reaction mixture contained DNA probe at 40,000 cpm, 1 μg of poly(dI-dC) · poly(dI-dC), 25 mM HEPES (pH 7.9), 40 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1mM dithiothreitol, 10% glycerol, and 3 μg of nuclear extract in a final volume of 25 μl. The reaction mixture was

incubated at room temperature for 20 min and analyzed by 5% native polyacrylamide gel electrophoresis (PAGE) in a 0.25 × TBE buffer (22 mM Tris base, 22 mM boric acid, and 0.5 mM EDTA). To determine the specificity of the DNA-protein binding complexes, we performed competition assay using a 100-fold molar excess of unlabeled double-stranded oligonucleotides encording the wild-type ATF sequence or the mutated ATF sequence (5'-TGAATGCCCCCAAGGCCGCGAG-3'). The gel was dried and autoradiographed at -80°C.

Cell DNA Staining

RASMC were plated in 2 ml medium on culture chamber slides (Becton Dickinson Lab., Franklin Lakes, NJ). After 24 hr incubation, the cells were treated with RWP at the highest concentration (100 μg/ml) for 12 hr. As a positive control, RASMC were also treated with pyrrolidine dithiocarbamate (PDTC) at a final concentration of 100 μM for 12 hr. Cells were washed once with PBS and were fixed with PBS containing 1% glutaraldehyde for 30 minutes at room temperature. The cells were re-wash with PBS and incubated with PBS containing 10 μM bis-benzimide (Hoechst 33258; Funakoshi Co. Tokyo, Japan) for 15 minutes in the dark and examined under fluorescence microscopy.

Statistical Analysis

The data were analyzed by ANOVA. When statistically significant effects were found, the Newman-Keuls test was used to isolate the differences between groups. A value of $P < 0.05$ was considered significant. All data in the text and figures are expressed as mean ± SEM.

RESULTS

Effects of RWP on Proliferation and DNA Synthesis

To investigate the effect of RWP on the proliferation of VSMC or vascular EC, we examined cell number and DNA synthesis with or without different concentrations of RWP. As shown in Figure 2, we found that RWP significantly inhibited growth and thymidine incorporation of 10 % serum-stimulated RASMC in a concentration-dependent manner. RWP decreased cell number of RASMC to 94%, 93%, 87%, 77%, and 61% of control, and inhibited thymidine incorporation into RASMC to 92%, 86%, 71%, 52%, and 18% of control at final concentrations of 1, 3, 10, 30, and 100 μg/ml, respectively (Figure 2A, 2C). The difference in either cell count or thymidine incorporation was not statistically significant between control medium containing 0.5% ethanol and ethanol-free medium. To rule out the possibility that this inhibitory effect of RWP on VSMC proliferation is a non-specific toxic effect, we examined the effect of RWP on the proliferation and DNA synthesis of BCEC. In contrast, RWP did not inhibit the cell growth and thymidine incorporation of BCEC stimulated by 10 % serum except at much higher concentrations, especially at 100 μg/ml (Figure 2B, 2D).

Effects of Six Fractions from RWP on DNA Synthesis in RASMC

Since the physiological concentration of red wine polyphenols is thought to be below 30 μg/ml, we decided to use RWP and its six fractions, Fraction 1 to Fraction 6, from RWP at final concentration of 30 μg/ml for later experiments. To investigate whether difference between low or high

molecular weight in polyphenolic fractions affect VSMC proliferation, the effect of six fractions (30 µg/ml) on the DNA synthesis in 10 % serum-stimulated RASMC was examined. In this experiment, effects of these fractions were compared to that of control containing 0.15% ethanol as a vehicle. These fractions, F1 to F6, decreased the thymidine incorporation into RASMC to 41%, 37%, 30%, 26%, 30%, and 22% of control (Figure 3A). Interestingly, all fractions potently inhibit the DNA synthesis of RASMC without relation to their molecular weight, and this inhibitory effect of each fraction was in proportion to total polyphenolic content of each fraction (Figure 3B).

Effects of RWP on Cyclin A Gene Expression

To elucidate the mechanisms of these anti-proliferative effects of RWP in RASMC, we investigated the expression of cyclin A gene that is known to be one of the key cell cycle regulators. We performed Northern analysis with RNA prepared from RASMC using a rat cyclin A probe amplified by PCR. At first, we harvested total RNA from RASMC at different incubation time points with RWP (30 µg/ml). The expression of cyclin A mRNA was slightly decreased at 4 hr after the addition of RWP, and completely suppressed at 48 hr to 72 hr (Figure 4A). Next, we examined the levels of cyclin A mRNA in RASMC treated with different concentrations of RWP for 72 hr. On the measurement of radioactivity, RWP significantly inhibited the levels of cyclin A mRNA to 80%, 43%, 16%, and 4% of control at final concentrations of 3, 10, 30, and 100 µg/ml, respectively (Figure 4B). In contrast, RWP did not inhibit cyclin A gene expression in BCEC, except at a concentration of 100 µg/ml (Figure 4C).

Effects of RWP on DNA Synthesis and Cyclin A Gene Expression in Human Cells

To rule out the possibility that the inhibitory effects of RWP on the serum-stimulated cell proliferation and cyclin A gene expression in RASMC but not in BCEC are due to difference of species, we examined the DNA synthesis and expression of cyclin A mRNA in human VSMC and vascular EC (Figure 5). RWP inhibited thymidine incorporation into HASMC to 70% and 30% of control at 10 and 30 µg/ml (Figure 5A) and downregulated the expression of cyclin A mRNA in HASMC in a dose-dependent manner (Figure 5B). In contrast, the inhibitory effects of RWP on the DNA synthesis and cyclin A gene expression in HUVEC were observed at much higher concentrations, especially at 100 µg/ml (Figure 5C, 5D).
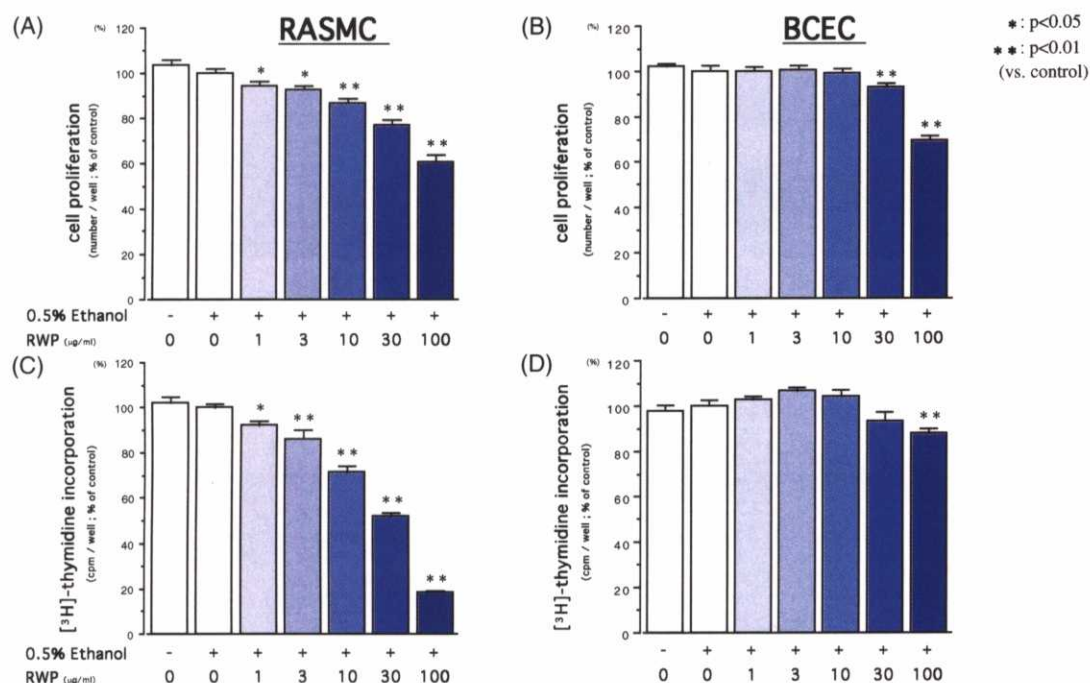
Figure 2. Effects of RWP on cell proliferation and DNA synthesis in RASMC or BCEC.
   RASMC or BCEC were plated at a density of 10,000 cells per cm ^{2} in 24-well plates. After 48 hr incubation, cells were treated with RWP (final concentration; 1 ∼ 100 µg/ml) for 72 hr. Effects of RWP on cell number and thymidine incorporation into RASMC after 72 hr treatment is expressed as percentage of the control containing 0.5% ethanol as vehicle. Similarly, the effects of RWP on cell number and thymidine incorporation into BCEC are shown.
   Data are expressed as mean ±SEM (n=6). (* P<0.05, * * P<0.01 vs. vehicle.)
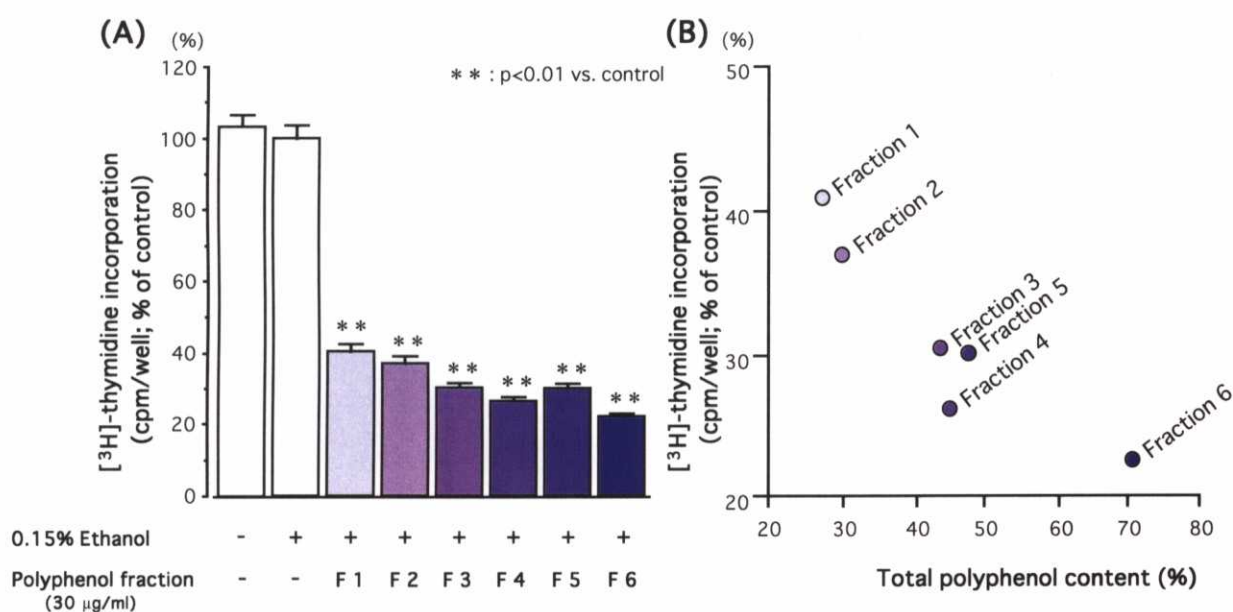

**Figure 3. Effects of six fractions from RWP on DNA synthesis in RASMC.**
   RASMC were treated with each fraction (30 µg/ml) for 72 hr in the same way as in Figure 2. (A) The effects of six fractions, Fraction 1 (F1) to Fraction 6 (F6), on thymidine incorporation into RASMC are expressed as percentage of the control containing 0.5% ethanol as vehicle. Data are expressed as mean ±SEM (n=6).
(B) A directly proportional relationship between the inhibitory effects of these fractions and total polyphenolic content was demonstrated, using the results shown in Figure 1 and in panel A of Figure 3.
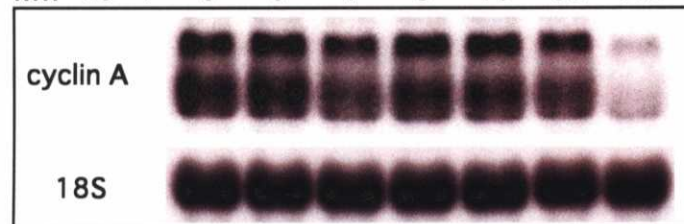
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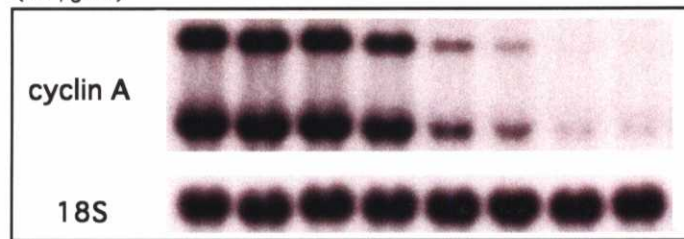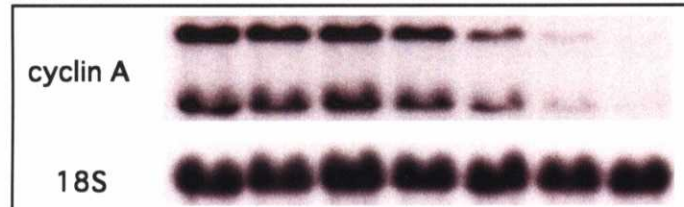
Figure 4. Effects of RWP on Cyclin A Gene Expression in RASMC and BCEC.
(A) Total RNA was extracted from RASMC treated with RWP (30 µg/ml) at different time. Northern analysis with cyclin A and 18S probes was performed. (B) Total RNA was extracted from RASMC treated with RWP (1 ~100 µg/ml) for 72 hr to show the concentration-dependent effect of RWP on the downregulation of cyclin A mRNA level. (C) Similarly, the effect of RWP (1~100 µg/ml) on the expression of cyclin A mRNA in BCEC was examined in the same way.
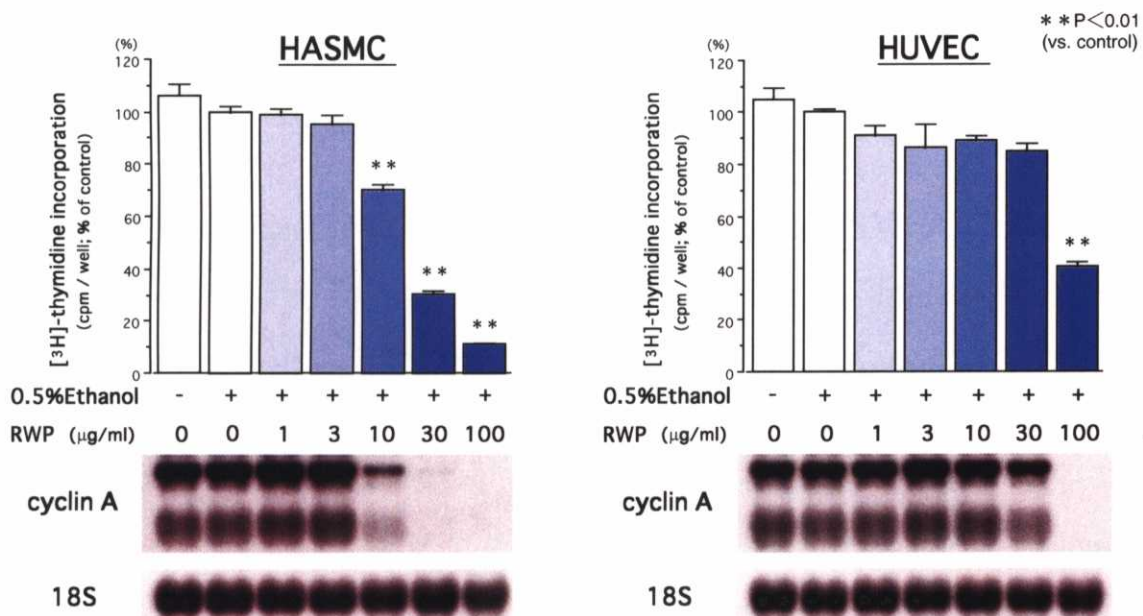
Figure 5. The effects of RWP on DNA synthesis and cyclin A gene expression in HASMC or HUVEC.
Serum-stimulated HASMC were treated with RWP (1 ~100 µg/ml) for 72 hr, and thymidine incorporation and expression of cyclin A mRNA were examined. Similarly, the effects of RWP on serum-stimulated HUVEC also are shown. Thymidine incorporation is expressed as percentage of the control containing 0.5% ethanol as vehicle. Data are expressed as mean ±SEM (n=6). (* *P<0.01 vs. vehicle.)

Effects of RWP on Cyclin A Promoter Activity

To further investigate the molecular mechanisms of cyclin A mRNA downregulation by RWP in VSMC, we examined effects of RWP on cyclin A promoter activity in RASMC. We transfected luciferase reporter plasmids containing the human cyclin A 5' flanking sequence into RASMC. This plasmid used in this experiment contained both the proximal Sp1 motifs and the one ATF motif, and directed full promoter activity.[39] RASMC were treated with different doses of RWP for 72 hr after transfection. RWP significantly and dose-dependently decreased the transcriptional activity to 87%, 71%, 69%, and 45% of control at final concentrations of 3, 10, 30, and 100 μg/ml, respectively (Figure 6). In contrast, the plasmid containing a mutated ATF site in cyclin A promoter had luciferase activities approximately 12-fold lower than that of plasmid containing a normal ATF site, and the luciferase activity was not affected by RWP. In case of VEC, RWP did not inhibit the transcriptional activity of the cyclin A promoter in BCEC. The transcriptional activity was 96% and 103% of control at 10 and 30 μg/ml, respectively.

Effects of RWP on ATF-Binding Nuclear Proteins in VSMC

Because the ATF site in the cyclin A promoter is important in the expression of cyclin A gene, we examined the binding of nuclear proteins extracted from 10 % serum-stimulated RASMC to this site using gel mobility shift assay. As shown in Figure 7, the reaction mixture, nuclear extracts and ^{32}P-labeled ATF site oligonucleotide were incubated at room temperature for 20 min and analyzed by 5% native PAGE. We found that RWP decreased the abundance of the ATF-binding nuclear proteins in a

concentration-dependent manner (lane; 2, 5-10). To determine the specificity of the DNA-protein binding complexes, nuclear extracts were added with a 100-fold molar excess of unlabeled oligonucleotides encording the ATF or mutated ATF sequence as competitors. Competition assay revealed that the binding complexes were completely competed by oligonucleotide with the wild-type ATF sequence (lane; 3), but not by oligonucleotide with mutated ATF sequence (lane; 4).

Since binding proteins to the ATF site have been reported to be important in regulating the activity of cyclin A promoter[41, 43], we performed a super-shift assay using specific antibodies, and confirmed the existence of CREB and ATF-1 in RASMC nuclear extracts (data not shown). After repeated independent experiments, we could not find any difference between RASMC in ethanol-free medium (lane 2) and RASMC with 0.5% ethanol (lane 5).
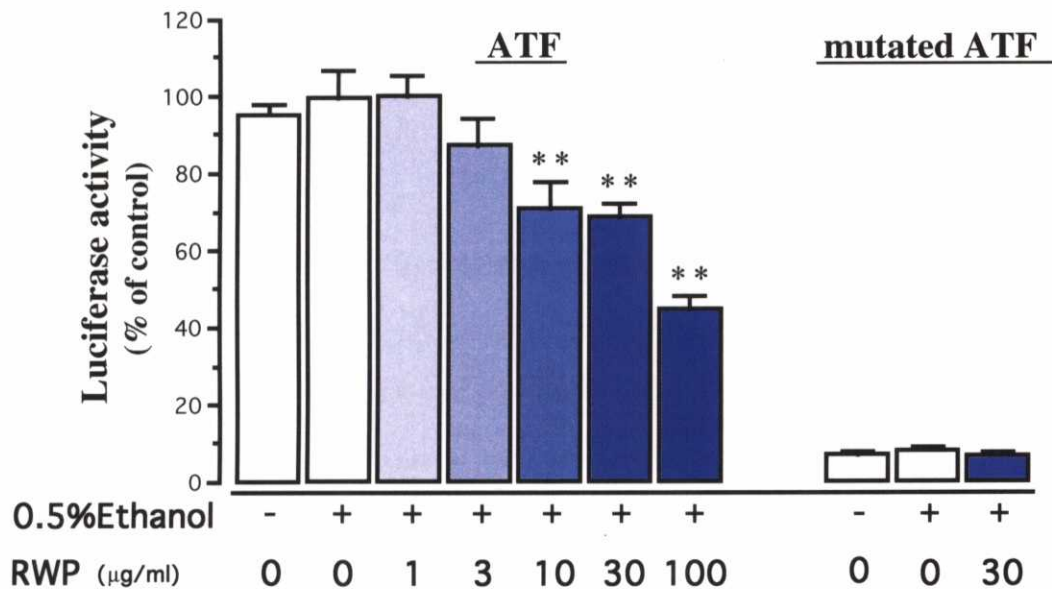
Figure 6. Effect of RWP on Transcriptional Activity of Cyclin A promoter in RASMC.
RASMC were transfected with luciferase reporter plasmids containing the human cyclin A promoter containing a normal ATF site (ATF) or a mutated ATF site (mut ATF). After transfection for 24 hr, the cells were treated with RWP (1 ∼100 μg/ml) for 72 hr. Luciferase activity was compared to that of control (defined as 100%) containing 0.5% ethanol as vehicle. Data are expressed as mean ±SEM (n=4). (* *P<0.01 vs. vehicle.)
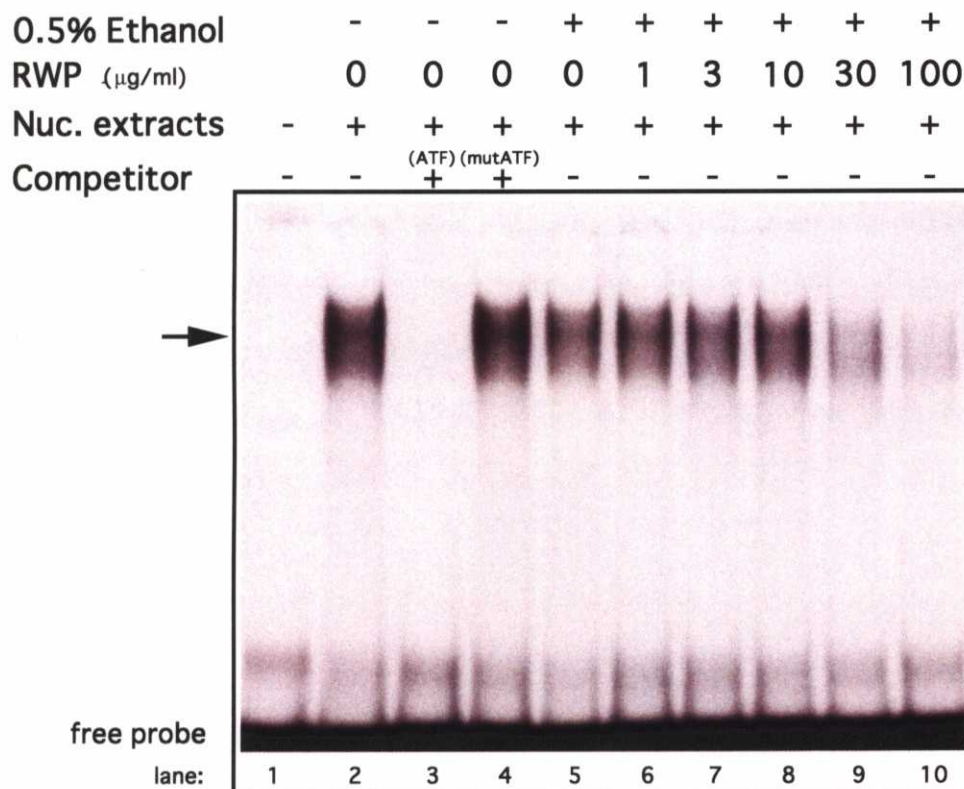
Figure 7. Effect of RWPon Binding Activity of Nuclear Proteins to ATF site in RASMC.
Nuclear extracts from RASMC treated with RWP (1 ∼100 μg/ml) for 72 hr were incubated with ^{32}P-labeled ATF probe and analyzed by 5% native PAGE. A 100-fold molar excess of unlabeled oligonucleotides encoding the ATF site in the cyclin A promoter (lane 3) or mutated ATF sequence (mut ATF, lane 4) were added to the nuclear extracts as competitors to determine the specificity of the DNA-protein complexes.

Effects of RWP on Transcription Factor Expression

We examined the expression of transcription factors, CREB and ATF-1, that are known to bind to the ATF site in the cyclin A promoter and exert potent promoter activity. RWP (30 µg/ml) downregulated the expression of CREB and ATF-1 mRNA in a similar time course to that of cyclin A mRNA (Figure 8A). Treatment with RWP for 72 hr downregulated these mRNA levels in a concentration-dependent manner (Figure 8B).

Examination of Apoptotic Cell Death

To rule out the possibility that RWP induce apoptosis in VSMC, we examined RASMC under fluorescent microscopy following nucleic acid staining with Hoechst 33258 dye. It has been reported that antioxidant agent PDTC induces apoptosis in rat and human VSMC.[44] In the fluorescence microscopic study following nucleic acid staining with Hoechst 33258 dye, we confirmed that PDTC induced morphological changes indicative of apoptotic cell death, such as condensation of chromatin and nucleoplasmic segmentation, in RASMC (Figure 9C). However, RWP even at the highest dose (100 µg/ml) did not cause any morphological changes in RASMC (Figure 9B) compared with RASMC under no-treatment (Figure 9A).

(A) RWP (30 µg/ml) pre 1 2 4 8 24 48 72 (hrs)

Cyclin A

CREB

ATF-1

18S

(B) 0.5% Ethanol - + + + + + +

RWP (µg/ml) 0 0 1 3 10 30 100

Cyclin A

CREB

ATF-1

18S
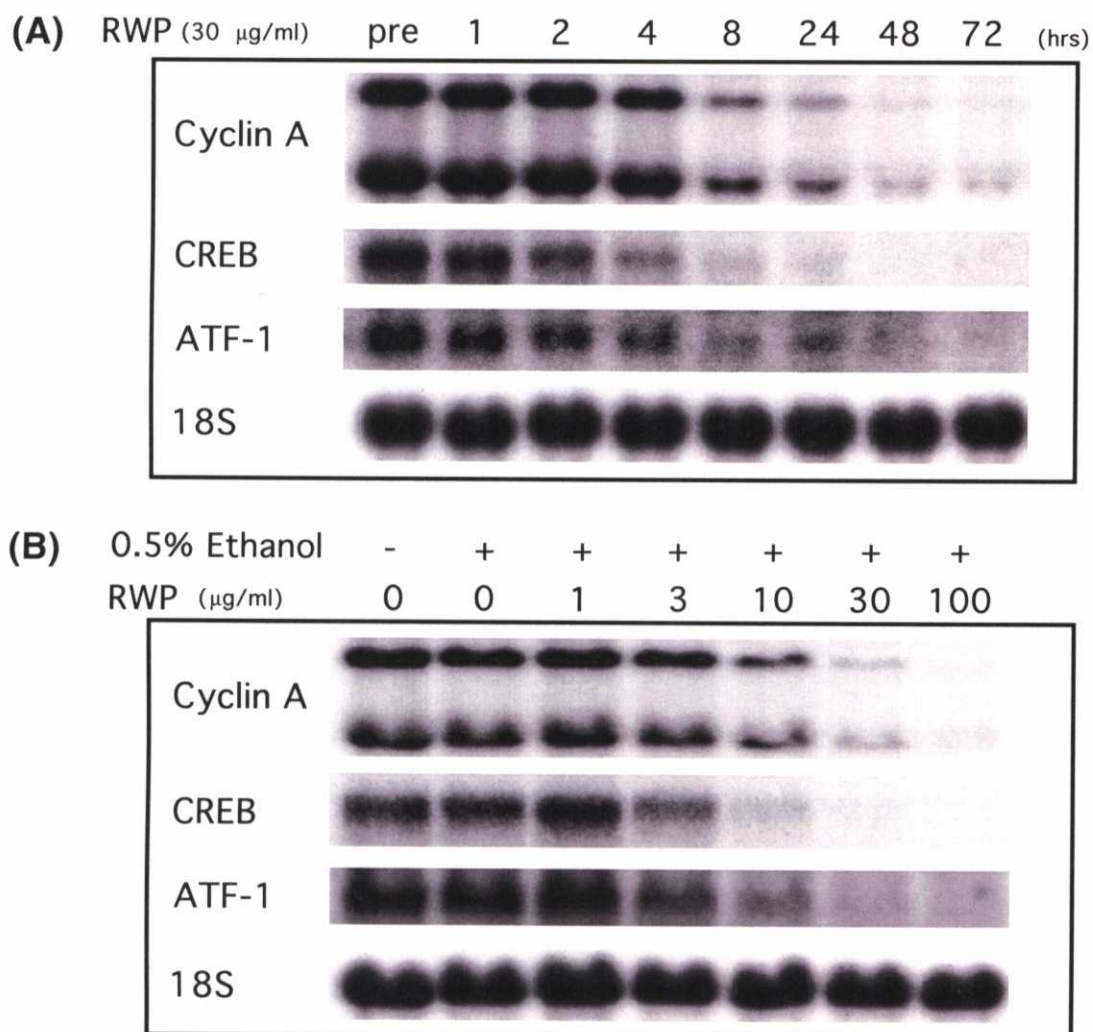
Figure 8. The effects of RWP on the transcription factor expression in RASMC.
Northern analysis with CREB and ATF-1 probes was performed using the same filters as in the Figure 4A (time course) and 4B (concentration-dependence).

(A) Control   **(B) RWP**   **(C) PDTC**
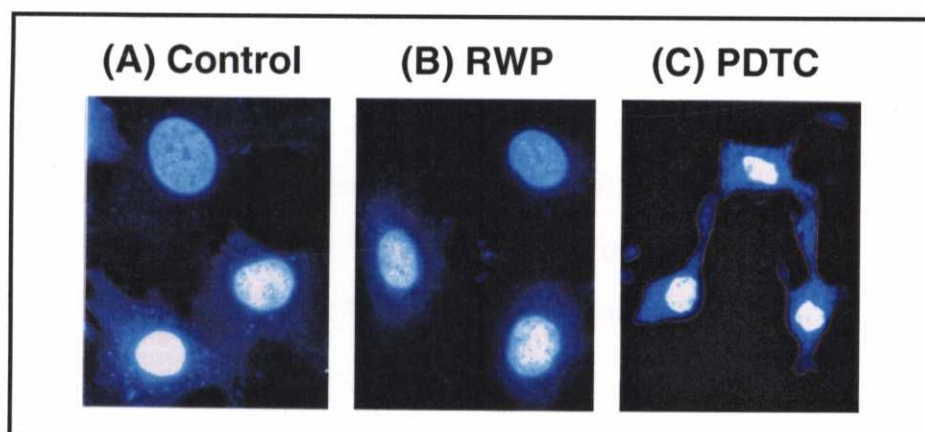
Figure 9. The effects of RWP on cell DNA staining of RASMC.
After 24 hr incubation of RASMC, the cells were treated with RWP (100 µg/ml) (B) or PDTC (100 µmol/L) (C) for 12 hr, as indicated. The cells were fixed with PBS containing 1% glutaraldehyde, and morphological changes were examined under fluorescence microscopy (magnification of objective lens, X20) after nucleic acid staining with Hoechst 33258 dye.

<center>STUDY 2.</center>

Effects of Red Wine Polyphenols on Migration of Vascular Smooth Muscle Cells or Vascular Endothelial Cells

<center>**INTRODUCTION**</center>

Progressive development of atherosclerotic plaque is characterized by not only VSMC proliferation in the neointima but also VSMC migration from the arterial media into intima.[10]   VSMC migration is shown to be induced by several growth factors, such as platelet-derived growth factor (PDGF) and insulin-like growth factor-I, in the injured rat carotid artery.[45, 46]   It has been reported that induction of VSMC migration stimulated by growth factors was reduced by tyrosine kinase inhibitors, suggesting that tyrosine phosphorylation by potent growth factors plays a key role in the process of cell migration.[47]   Among several factors that cause the VSMC migration, PDGF is one of the most potent chemoattractant for VSMC *in vitro* and *in vivo.*[48]   PDGF activates diverse intracellular signal transduction cascades through PDGF receptors and may be responsible for pathological processes in the vasculature.   It has been reported that activation of the phosphatidylinositol 3'-kinase (PI3-kinase) pathway is a rapid response to PDGF in several cell types and is implicated in cellular motility, such as migration, actin reorganization, and membrane ruffling.[49-51]   In the case of VSMC, activation of PI3-kinase has been known to play a key role in the migratory action of VSMC stimulated by growth factors.[46]   Moreover, it has been recently demonstrated that activation of mitogen-activated protein kinase (MAPK) pathways also are

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associated with cell migration. Especially, it has been reported that stress-activated protein kinase-2/ p38^{MAPK}, one of the MAPK pathways, may mediate the migratory action of tracheal smooth muscle cell in response to stimulation of PDGF.[52] The extracellular signal-regulated kinase (ERK)1/2, a classic MAPK, signaling pathway has been shown to be associated with VSMC migration stimulated by growth factors.[53-55] In contrast, ERK1/2 is unlikely to be involved in VSMC migration[56, 57], suggesting that the role of ERK1/2 in VSMC migration is controversial. The aim of this study is elucidation of effects of red wine polyphenols on the migration of VSMC stimulated by PDGF-BB or serum and intracellular signaling pathways that are known to mediate cell migration.

MATERIALS AND METHODS

Cell Migration Assay

Two types of migration assay were performed. In the first, confluent cells were scraped using a monolayer-wounding protocol, and cells that migrated from wound edge were counted. In the second, cell chemotactic action in response to short exposure to growth factors was determined using a modified Boyden chamber method.

1) Monolayer-Wounding Cell Migration Assay (Wounding Assay)

For the monolayer-wounding cell migration assay (wounding assay), RASMC or HASMC that had been grown to confluence in six-well culture plates were subjected to wounding as previously described.[58] The cells were pre-incubated with serum-free medium for 24 hr and pretreated with

RWP at final concentrations of 1-100 µg/ml for 10 hrs. Cell layers were scraped with a sterile single-edged razor blade and rinsed twice with PBS. The cells were re-incubated with serum-free medium and RWP at each concentration, and then stimulated with PDGF-BB (10 ng/ml) or 10% serum. Following a 30 hr incubation at 37°C, the cells were rinsed twice with PBS, fixed, and stained with Diff-Quick (Wright-Giemsa solution; International Reagents Corp.). Photographs of the fixed cells were taken under microscope, and the number of cells that migrated across the regions of the wound edge was counted. In addition, the effect of RWP on 10% serum-stimulated migration of vascular EC (BCEC or HUVEC) was examined.

2) Boyden-Chamber Assay

To examine the effect of RWP on VSMC migration briefly exposed to chemoattractant, cell migration was assayed using a modified Boyden chemotaxis chamber (96-well microchemotaxis chamber; Neuro Probe Inc.) as previously described.[59] Briefly, either RASMC or HASMC were rinsed with PBS and trypsinized, and then resuspended in serum-free DMEM or M199 medium (5×10^5 cells/ ml). A polycarbonate membrane (polyvinylpyrrolidione-free, pore size 8.0 µm) was immersed in acetic acid solution for 12 hr and then pre-coated with type V collagen (Funakoshi Inc., Tokyo) for 72 hr. To the lower chamber, serum-free medium (35 µl) containing PDGF-BB (10 ng/ml), as a chemoattractant, and RWP (1-100 µg/ml) were added. Under these experimental conditions, the duration of RWP treatment was the same as that of chemoattractant (PDGF-BB). A cell suspension (225 µl) was placed in the upper chamber. The assembled chambers were incubated in a CO_2 incubator at 37°C for 6 hr. After

incubation, unmigrated cells were removed from the upper side of the membrane and migrated cells were stained using Diff-Quick solution, and the number of cells that had migrated to the lower surface of the membrane was counted as the number of stained nuclei per high-power field (hpf) in a microscope ($\times 200$). The effect of RWP on cell migration induced by PDGF-BB (10 ng/ml) in both the upper and lower chambers, which represents chemokinesis, was also investigated. To examine whether the signaling pathway of PI3-kinase or MAPK plays a role in VSMC migration, the cells were pretreated with each specific inhibitor, wortmannin (PI3-kinase inhibitor, 100 nM; Sigma), SB203580 (p38^{MAPK} inhibitor, 25 μM; Sigma), or PD98059 (MEK1 inhibitor, 25 μM; New England BioLabs. Inc.) for 30 min, and the inhibitors were added again in upper and lower chambers.

Phosphatidylinositol 3'-kinase (PI3-kinase) activity

The activity of PI3-kinase was assayed by the method described previously with slight modifications.[60] Serum-starved sub-confluent VSMC in a 10-cm-diameter dish were pretreated with RWP (1-100 μg/ml) for 10 hr. After activation of the cells with PDGF-BB (10 ng/ml) for 10 min, the cells were lysed with 1 ml ice-cold buffer A (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 100 μM Na$_3$VO$_4$, 10% glycerol, 1% Nonidet P-40, 2 mM PMSF, 1 mM MgCl$_2$, and 1 mM CaCl$_2$) and centrifuged at 15,000 rpm for 15 min at 4 ℃. The supernatant was incubated with anti-phosphotyrosine monoclonal antibody (PY-20; Transduction Lab., KY, USA) for 2 hr and then coupled with protein A-Sepharose (Amersham Pharmacia Biotech AB, Sweden) for 1 hr at 4℃. The immunoprecipitate was washed three times with wash solution I (PBS containing 1% NP-40,

100 µM Na$_3$VO$_4$), solution II (0.5 M LiCl, 0.1 M Tris-HCl, pH 7.5, 100 µM Na$_3$VO$_4$), and solution III (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 100 µM Na$_3$VO$_4$) and suspended in 50 µl wash solution III, and 10 µl of 1 mg/ml sonicated phosphatidylinositol (PI) was then added. The reaction was started by the addition of 37 Kbq [γ-^{32}P] ATP, and the samples were incubated at 30℃ for 15 min. The reaction was stopped by the addition of 20 µl of 8 N HCl and 160 µl chloroform-methanol (1:1). The lower phase containing phospholipids was recovered and spotted on silica gel thin layer chromatography plates (TLC; Gel-60, Merck), impregnated with 1% (w/v) potassium oxalate and allowed to dry prior to sample application, and developed in chloroform/methanol/28% NH$_3$/water (120:94:4:23.2, numbers equal milliliters). The radioactivity on the dried plate was visualized and quantified by a Phospho-imaging analyzer.

MAPK Phosphorylation

HASMC were grown to sub-confluence on 10-cm-diameter dishes with M199 medium. HASMC were serum-starved and were pretreated with various concentrations of RWP for 10 hr. The cells were then stimulated with PDGF-BB (10 ng/ml) for 10 min. The cells were washed with PBS and immediately lysed with MAPK extraction buffer (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 0.5mM EGTA, 10 mM NaF, 20 mM β-glycerophosphate, 100 µM Na$_3$VO$_4$, 1 mM PMSF). Cellular extracts were centrifugated at 15,000×g for 15 min at 4℃, and the supernatants were obtained. Protein concentration was determined by Bio-Rad protein assay.[41, 42] Total protein extracts (20 µg/ lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose PVDF membrane in blotting solution (99 mM

Tris, 192 mM glycine, 20% methanol) using a Blotter (Hoefer semiphor, Pharmacia Biotech.), and then the blots were blocked with blocking solution. To assess the activation of three distinct MAPK pathways, the total or phosphorylated proteins were detected with each totak or anti-phosphorylated MAPK antibody (diluted 1:1000; New England BioLabs), such as ERK1/2, phospho-ERK1/2, stress activated protein kinase-1/ c-Jun NH2 terminal kinase (SAPK1/JNK), phospho-SAPK1/JNK, p38^{MAPK}, and phospho-p38^{MAPK}, and then goat anti-rabbit IgG secondary antibody (diluted 1:2000; Amersham Lifescience). Blots from same lysates were examined by using antibodies, which can recognize both phosphorylated and unphosphorylated form of each MAPK. Moreover, to examine the activation of upstream of p38^{MAPK} pathway, anti-phosphorylated mitogen activated protein kinase kinase (MKK) 3/6 antibody (diluted 1: 1000; New England BioLabs.) was used. Antigen-antibody complexes were detected by an enhanced chemiluminescence ECL detection system (Amersham). The phosphorylation level of each MAPK pathway was scanned and these activities were measured by NIH imaging system.

Cell Proliferation and DNA Synthesis in PDGF-BB Induced VSMC

HASMC were plated at a density of 10,000 cells per cm^2 in 24-well plate (surface area; 2 cm^2/well) and were grown to sub-confluent. Serum-starved HASMC were pretreated with RWP at final concentrations (1 to 100 μg/ml) for 10 hr. For cell proliferation assay, the cells were stimulated by PDGF-BB (10 ng/ml) for 60 hr and then cell number was measured. For thymidine incorporation assay, the cells were stimulated by PDGF-BB (10 ng/ml) for 22 hr, and then were labeled with [methyl-^{3}H] thymidine at 1 μCi/ml during the last 3 hr. The effects of RWP on the

proliferation and DNA synthesis in PDGF-BB-stimulated HASMC were expressed as a percentage of control containing 0.5% ethanol as a vehicle.

Expression of p27^{Kip1}

RASMC that were grown to sub-confluent on 10-cm-diameter dishes were serum-starved for 24 hr. The cells were stimulated with 10% serum, and then treated with PI3-kinase inhibitor, wortmannin (100 nM) or LY294002 (25 μM), or RWP (30 μg/ml) for 24 hr. The cells were washed with PBS and immediately lysed. Cellular extracts were centrifuged at 15,000 × g for 15 min at 4 ℃, and the supernatants were obtained. Protein concentration was determined by Bio-Rad protein assay. Total protein extracts (20 μg/ lane) were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose PVDF membrane, and the blots were blocked with blocking solution. To assess the expression of p27^{Kip1}, the proteins were detected with a specific p27^{Kip1} antibody (diluted 1:2500; Transduction Lab.), and then goat anti-mouse IgG secondary antibody (diluted 1:2000; Amersham Lifescience). Antigen-antibody complexes were detected by an enhanced chemiluminescence ECL detection system (Amersham).

Statistical Analysis

The data were analyzed by ANOVA. When statistically significant effects were found, the Newman-Keuls test was used to isolate the differences between groups. A value of $P < 0.05$ was considered significant. All data in the text and figures are expressed as mean ± SEM.

RESULTS

Effects of RWP on VSMC Migration in Wounding Assay

We first examined the effect of RWP on migration of RASMC in response to PDGF-BB or serum using a wounding assay. After pretreatment of RASMC with various concentrations of RWP, the cells were stimulated with PDGF-BB or serum for 30 hr. PDGF-BB as well as serum stimulated migration of RASMC, and the migration was inhibited by RWP in a concentration-dependent manner, as shown in Figure 10 (upper panel). The results of quantitative assessment in this assay were shown in lower panel. RWP inhibited the migration of PDGF-BB-induced of RASMC to 46%, 13%, and 8% of control at concentrations of 10, 30, and 100 μg/ml, respectively. Similarly, the migration of serum-stimulated RASMC was also inhibited by RWP to 68%, 21%, and 9% of control at concentrations of 10, 30, and 100 μg/ml, respectively. In contrast, RWP did not affect the migration of BCEC stimulated with 10% serum (Figure 10). RWP also inhibited the migration of HASMC stimulated with serum in a similar concentration-dependent manner, but did not inhibit the migration of serum-stimulated HUVEC (data not shown).

Effects of RWP on VSMC Migration in Boyden Chamber Assay

In the wounding assay, since the incubation time was 30 hr, newly divided cells may result in the increase in migrated cells. We further performed a modified Boyden chamber assay to examine the effect of RWP on cell migration for 6 hr. First, we examined the inhibitory effect of RWP on the migration of VSMC stimulated with various concentrations

of PDGF-BB in this assay. As shown in Figure 11, migration of RASMC was increased by PDGF-BB in a concentration-dependent manner. RWP exhibited potent inhibitory effects on the PDGF-BB (10 ng/ml)-induced migration of RASMC to 58%, 15%, and 4% of control at a RWP concentration of 10, 30, and 100 μg/ml, respectively.

Cell migration can be in general characterized by three forms: (1) random motion, which occurs in the absence of any stimulus, (2) chemokinesis, which is a random motion that is influenced by a stimulus, and (3) chemotaxis, which is directed motion toward a gradient of a stimulus. Growth factor-induced cell migration consists of chemokinesis and chemotaxis. As shown in Figure 12, PDGF-BB (10 ng/ml) in the lower chamber significantly increased the migration (chemotaxis) of RASMC by approximately 19-fold. RWP (30 μg/ml) in the lower chamber inhibited the PDGF-BB-induced VSMC migration. Next, we investigated the effect of RWP on not only chemotaxis but also chemokinesis under our experimental conditions. PDGF-BB in both the upper and lower chambers increased the migration of RASMC by 7.5-fold, which represents the chemokinesis. Interestingly, RWP significantly inhibited not only the chemotactic action of RASMC but also the chemokinetic action of RASMC, and the both chemotaxis and chemokinesis of RASMC were more potent inhibited by RWP in both chambers compared with in the lower chamber only. These findings suggest that RWP exert the potent inhibitory effects on the VSMC migration, including chemotaxis and chemokinesis, even in a short time exposure. In Boyden chamber assay, since the chemotactic action of cell account for the greater part of the cell migration, the effects of RWP on the chemotaxis of other cells were examined in following experiments.

Moreover, RWP inhibited the PDGF-BB-induced migration of HASMC to 84%, 49%, 22%, and 12% of control at a concentration of 3, 10, 30, and 100 μg/ml, respectively (Figure 13A; left panel). In contrast, RWP did not inhibit the migration of BCEC stimulated with 10% serum even at a highest concentration (100 μg/ml) of RWP (Figure 13B; right panel).

It has been previously reported that treatment with PI3-kinase inhibitors, wortmannin and LY294004, inhibited the migration of several cell types, including VSMC.[46, 53, 61] On the other hand, other laboratories recently reported that MAPK pathways might play a role in cell migration. Particularly, it has been demonstrated that p38^{MAPK} might mediate cell migration in vascular EC[57] and VSMC.[52] To elucidate which pathway, PI3-kinase or MAPK, mediates PDGF-BB-stimulated migration of RASMC, we investigated the effect of a specific inhibitor for each pathway. As shown in Figure 14, treatment with wortmannin, a PI3-kinase inhibitor, and SB203580, a p38^{MAPK} inhibitor, significantly decreased the migration of RASMC induced by PDGF-BB to approximately 39% and 45% of control, respectively. In contrast, the migration of RASMC was not significantly inhibited by PD98059, a MEK-1 inhibitor. Moreover, simultaneous treatment of RASMC with wortmannin and SB203580 showed an additional inhibition of VSMC migration in our experimental conditions. These findings suggest that the pathways of both PI3-kinase and p38^{MAPK}, but not of ERK1/2, are implicated in PDGF-BB-induced VSMC migration.
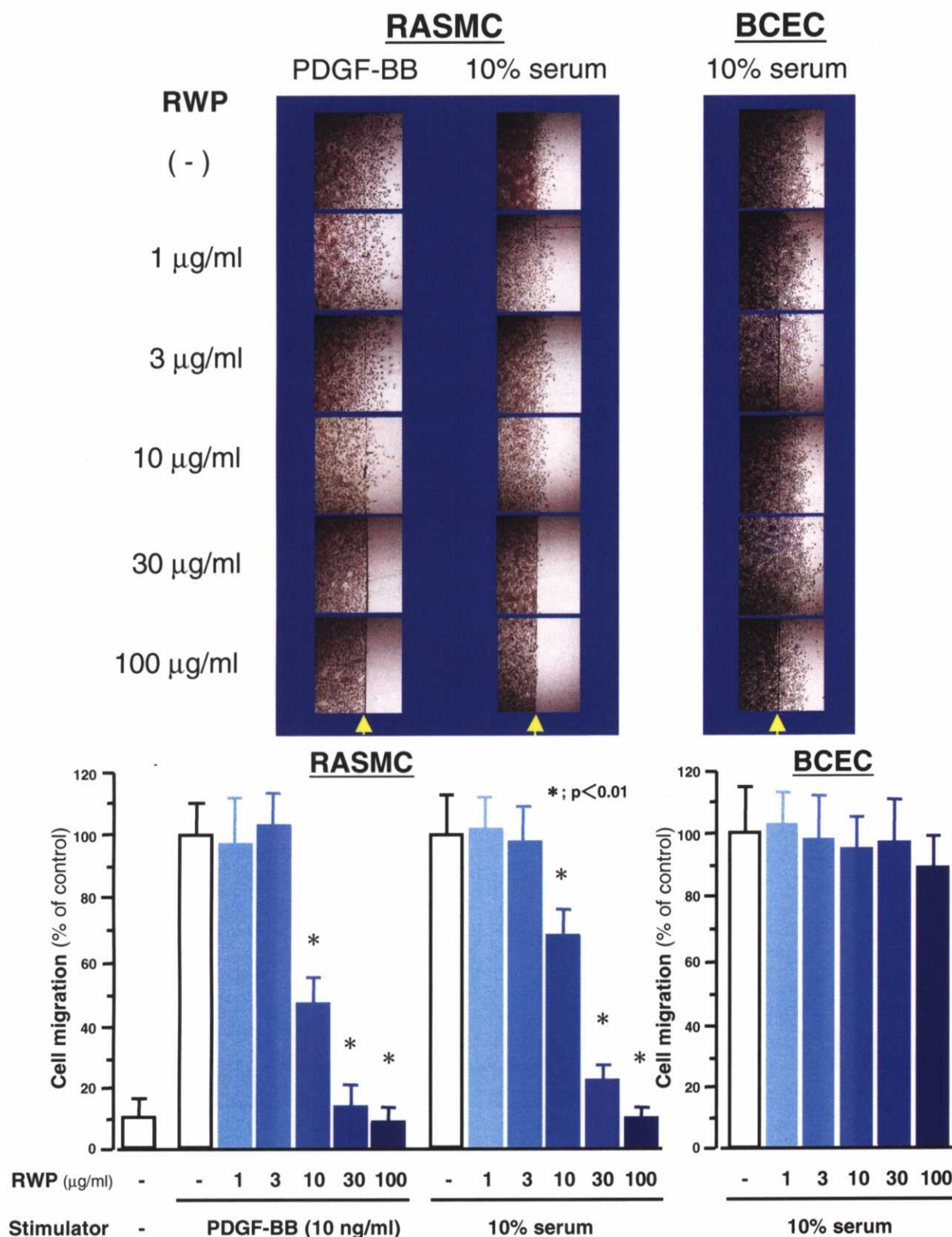
Figure 10. Effects of RWP on RASMC in monolayer-wounding cell migration assay.
Serum-starved RASMC and BCEC that had been grown to confluence in six-well culture plates were incubated with RWP at final concentrations (1-100 μg/ml) during 10 hrs. Cell layers were scraped with a sterile single-edged razor blade. The cells were re-incubated with serum-free medium and RWP of each concentration, then the cells were stimulated by PDGF-BB (10 ng/ml) or 10% serum. Following a 24 hr incubation at 37 ℃, the cells were fixed and stained. Fixed cells were underscoped and the number of cells migrated across the regions of the wound edge was counted.

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Figure 11. Effects of RWP on the migration of PDGF-BB-stimulated RASMC.

To examine the effect of RWP on migration of VSMC briefly exposed to RWP with chemoatractant, transwell cell migration was assay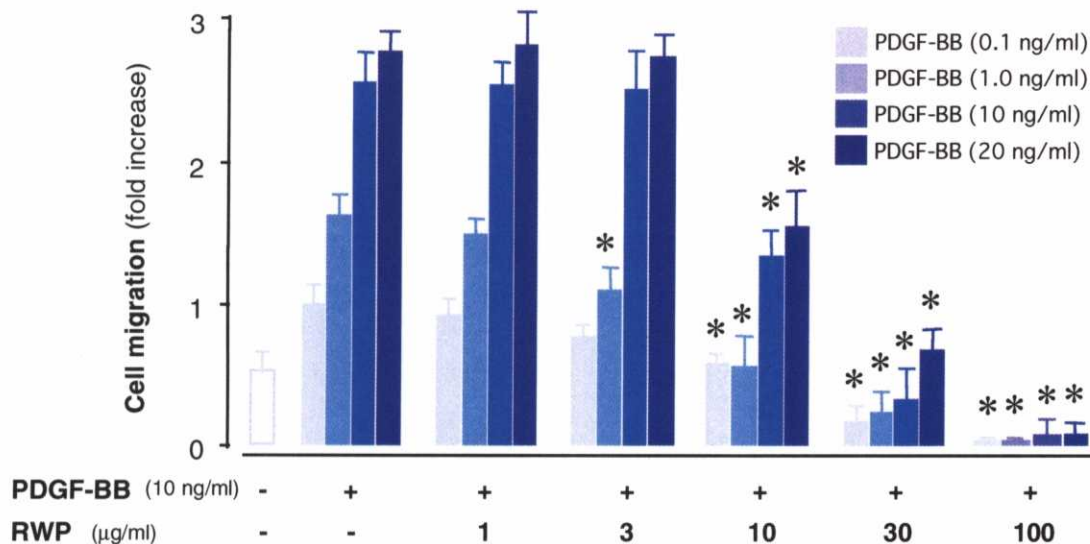ed using a modified Boyden chemotaxis chamber. RASMC were trypsinized and resuspended in serum-free medium to give a final concentration of 5×10^5 cells/ ml (225 µl; upper chamber). Chemoattractant solution (35 µl) containing various concentrations (0.1-20 ng/ml) of PDGF-BB was added with RWP (1-100 µg/ml) was placed in the lower chamber. After 6 hr incubation, the number of migrated cells to the lower surface of the membrane was measured.
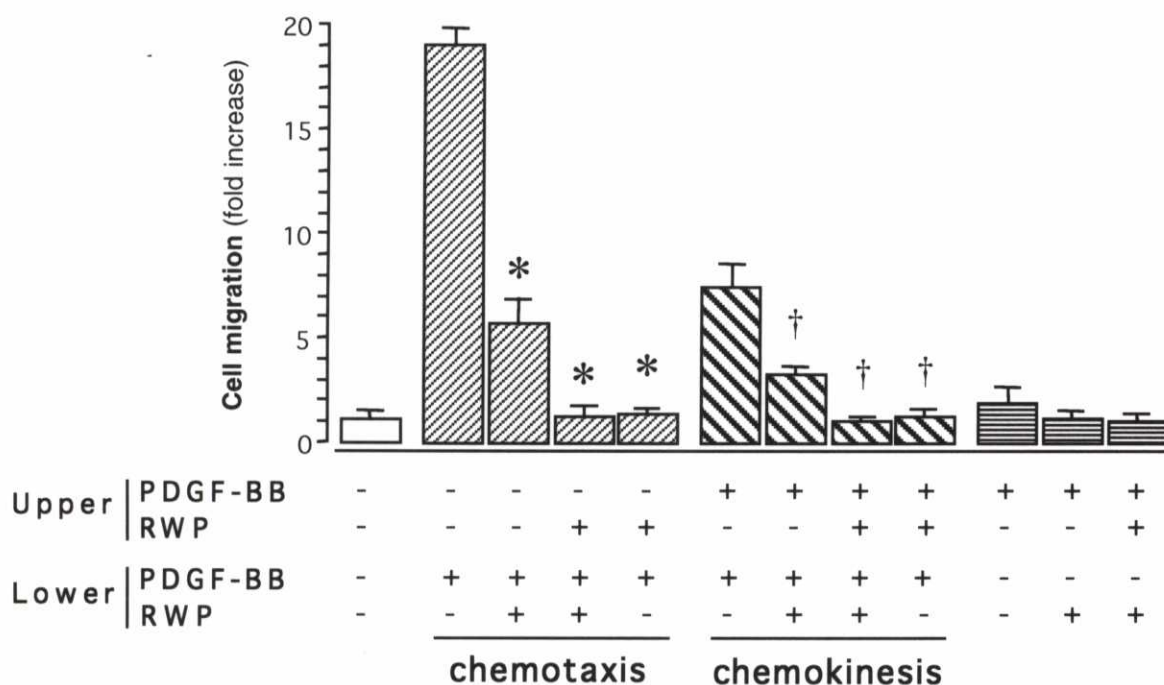
Figure 12. Effects of RWP on chemotaxis and chemokinesis of PDGF-BB-stimulated RASMC.

RASMC were stimulated by PDGF-BB (10 ng/ml) in the lower chamber only (chemotaxis) or in the upper and lower chambers (chemokinesis) for 6 hr, and the effects of RWP contained in the lower chamber only or in both chambers on the chemotaxis or chemokinesis of RASMC were examined. Value are mean±SE (n=4). (*;<0.05 vs. control of chemotaxis, †;<0.05 vs. control of chemokinesis)
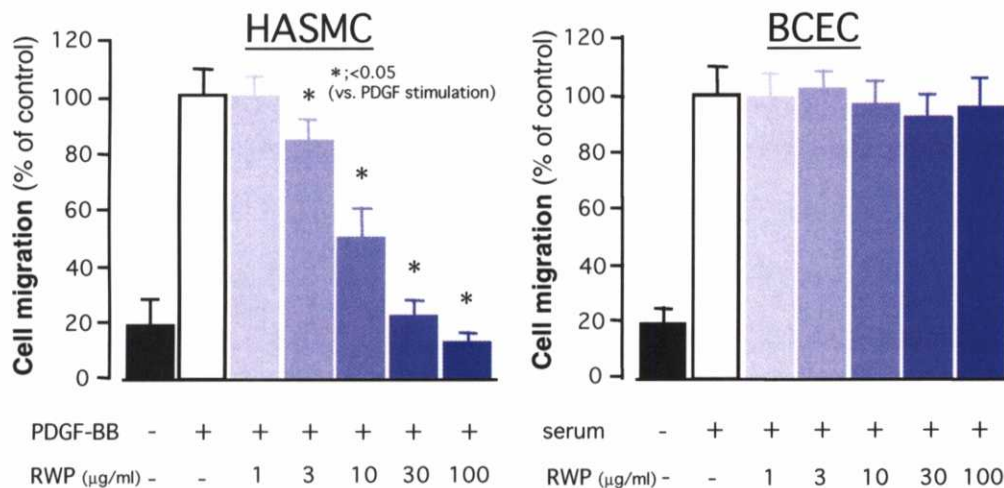
Figure 13. Effects of RWP on cell migration of HASMC or BCEC using Boyden-chamber assay.
(A) The effects of RWP on the migration (chemotaxis) of HASMC stimulated by PDGF-BB (10 ng/ml) for 6 hr were examined in the same way to the experiment of Fig. 11. (B) The effects of RWP on the migration of BCEC stimulated by 10% serum were examined in the same way to the experiment of Fig. 11. Value are mean±SE (n=4).
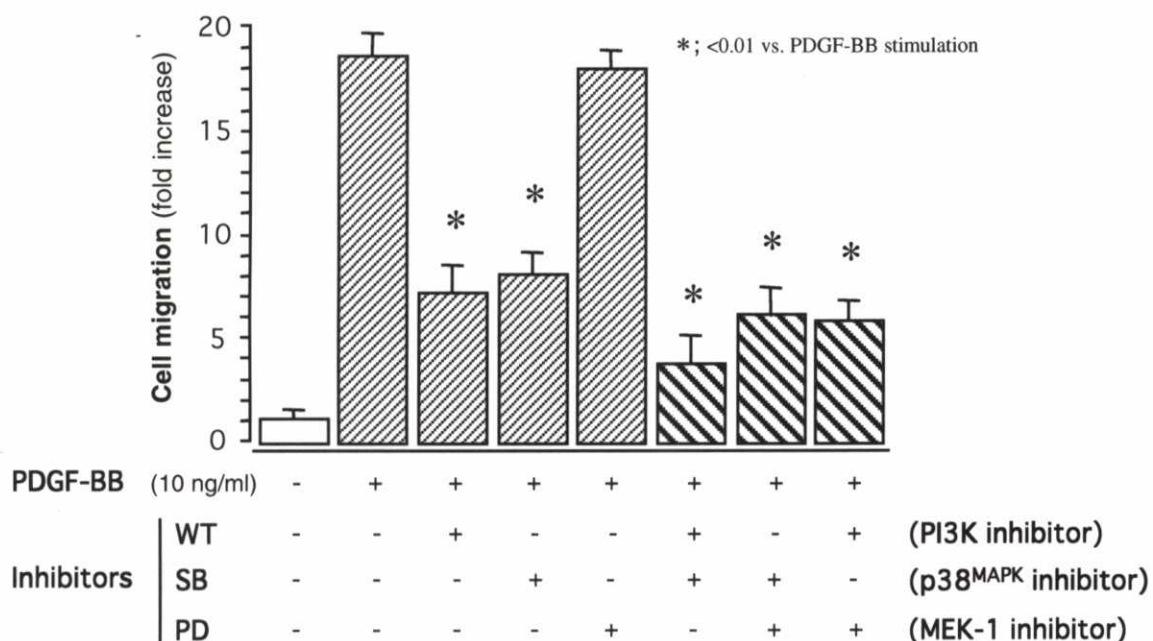
Figure 14. Effects of specific inhibitors on the VSMC migration.
The effects of each inhibitor, WT (wortmannin; PI3-kinase inhibitor; 100 nM), SB (SB203580; p38^{MAPK} inhibitor; 25 μM), and/or PD (PD98059; MEK-1 inhibitor; 25 μM), on the migration of RASMC stimulated by PDGF-BB (10 ng/ml) were examined. RASMC were pretreated with each inhibitor for 30 min, and the chemotactic action of PDGF-BB-stimulated RASMC using Boyden-chamber assay. Value are mean±SE (n=4).

Effects of RWP on PI3-kinase Activity in VSMC

PI3-kinase activity has been shown to be important in cell migration of various cell types[46, 49-51], including VSMC as we demonstrated in Figure 14. To elucidate whether the activity of PI3-kinase would be responsible for the anti-migratory effect of RWP on VSMC, we investigated the effect of RWP on the lipid kinase activity of PI3-kinase in PDGF-BB-stimulated VSMC. As shown in Figure 15, stimulation of RASMC or HASMC with PDGF-BB significantly increased PI3-kinase activity. Pretreatment of RASMC with RWP inhibited the PI3-kinase activity in a concentration-dependent manner, to 64%, 17%, and 6% of control at concentrations of 10, 30, and 100 μg/ml, respectively. The PI3-kinase activity in the PDGF-BB-stimulated HASMC was also decreased by RWP to 82%, 12%, 5%, and 2% of control at concentrations of 3, 10, 30, and 100 μg/ml, respectively.

Effects of RWP on MAPK Phosphorylation in VSMC

Because the activation of p38^{MAPK} has been also shown to regulate the VSMC migration[52], we investigated the effect of RWP on the phosphorylation status of three MAPK pathways. After pretreatment of serum-starved HASMC with various concentrations of RWP, the cells were stimulated with PDGF-BB (10 ng/ml). Total or phosphorylated MAPK was detected by Western blotting and quantified by densitometry. After stimulation with PDGF-BB for 10 min, ERK1/2, SAPK1/JNK, and p38^{MAPK} were maximally phosphorylated, resulting in approximately 11-fold (measured for ERK2), 14-fold, and 25-fold phosphorylation of the baseline value, respectively (Figure 16). RWP inhibited the activation of

$p38^{MAPK}$ to 28%, 5%, and 3% of control at concentrations of 10, 30, and 100 μg/ml, respectively. To our surprise, the phosphorylation of other pathways, ERK1/2 and SAPK1/JNK, was not inhibited by RWP except at the highest concentration (100 μg/ml). This result suggests that the inhibitory effect of RWP on the activation of MAPK pathways could be specific for the $p38^{MAPK}$ pathway. To clarify the mechanism of the inhibition of $p38^{MAPK}$ phosphorylation by RWP, we examined the effect of RWP on the phosphorylation of MKK3/6, an upstream kinese of $p38^{MAPK}$. The phosphorylation of MKK3/6 was also inhibited by RWP treatment in a concentration-dependent manner, suggesting that the inhibitory effect of RWP on the $p38^{MAPK}$ pathway works at the upstream level of MKK3/6. It is noteworthy that the concentrations of RWP necessary for the inhibition of PI3-kinase and $p38^{MAPK}$ pathway were similar to those of RWP for the inhibition of VSMC migration.

Effects of RWP on Cell Growth in PDGF-BB-Stimulated VSMC

We have previously reported that RWP inhibited the serum-stimulated proliferation and DNA synthesis of VSMC as shown in Figure 2. It is well known that the ERK1/2 pathway is important in growth factor-induced cell proliferation. However, PDGF-BB-induced activation of ERK1/2 in HASMC was not significantly inhibited by RWP, as shown in Figure 16. To examine whether RWP could inhibit PDGF-BB-stimulated VSMC proliferation, the effect of RWP on cell proliferation and DNA synthesis in VSMC was examined. As shown in Figure 17, RWP potently inhibited the cell number and [^{3}H]-thymidine incorporation of HASMC stimulated with PDGF-BB, in a concentration-dependent manner. These

data suggest that the anti-proliferative effect of RWP on VSMC might be independent of the classic MAPK pathway, ERK1/2.

Effects of RWP on Expression of p27^{Kip1} in Serum-Stimulated VSMC

Recently, it has been reported that the activation of PI3-kinase is associated with cell cycle regulation in several cells, such as vascular cells and tumor cells. One report has demonstrated that treatment of contact-inhibited vascular EC with growth stimulator induces cell cycle progression, and inhibition of PI3-kinase by a specific inhibitor induces G1 arrest through upregulation of p27^{Kip1}, one of the CDK inhibitors.[62] Other reports have demonstrated that inhibitors of PI3-kinase reduce tumorgenesis through induction of cell cycle arrest of tumor cells, and the inhibition is mediated by the induction of p27^{Kip1} expression.[63, 64] We investigated the effect of RWP on the expression of p27^{Kip1} in RASMC. Serum-starvation of RAMC induced p27^{Kip1} at the protein level, and stimulation with 10% serum suppressed the induction of p27^{Kip1}. In our experimental conditions, treatment of serum-stimulated RASMC with PI3-kinase inhibitor, wortmannin or LY294002, significantly induced the expression of p27^{Kip1}, and RWP (30 μg/ml) also induced the expression of p27^{Kip1} in RASMC stimulated with serum (Figure 18).

(A)

RASMC **HASMC**

-	+	+	+	+	+	+	PDGF-BB	-	+	+	+	+	+	+
-	-	1	3	10	30	100	RWP	-	-	1	3	10	30	100

(B)

-	+	+	+	+	+	+	PDGF-BB	-	+	+	+	+	+	+
-	-	1	3	10	30	100	RWP	-	-	1	3	10	30	100
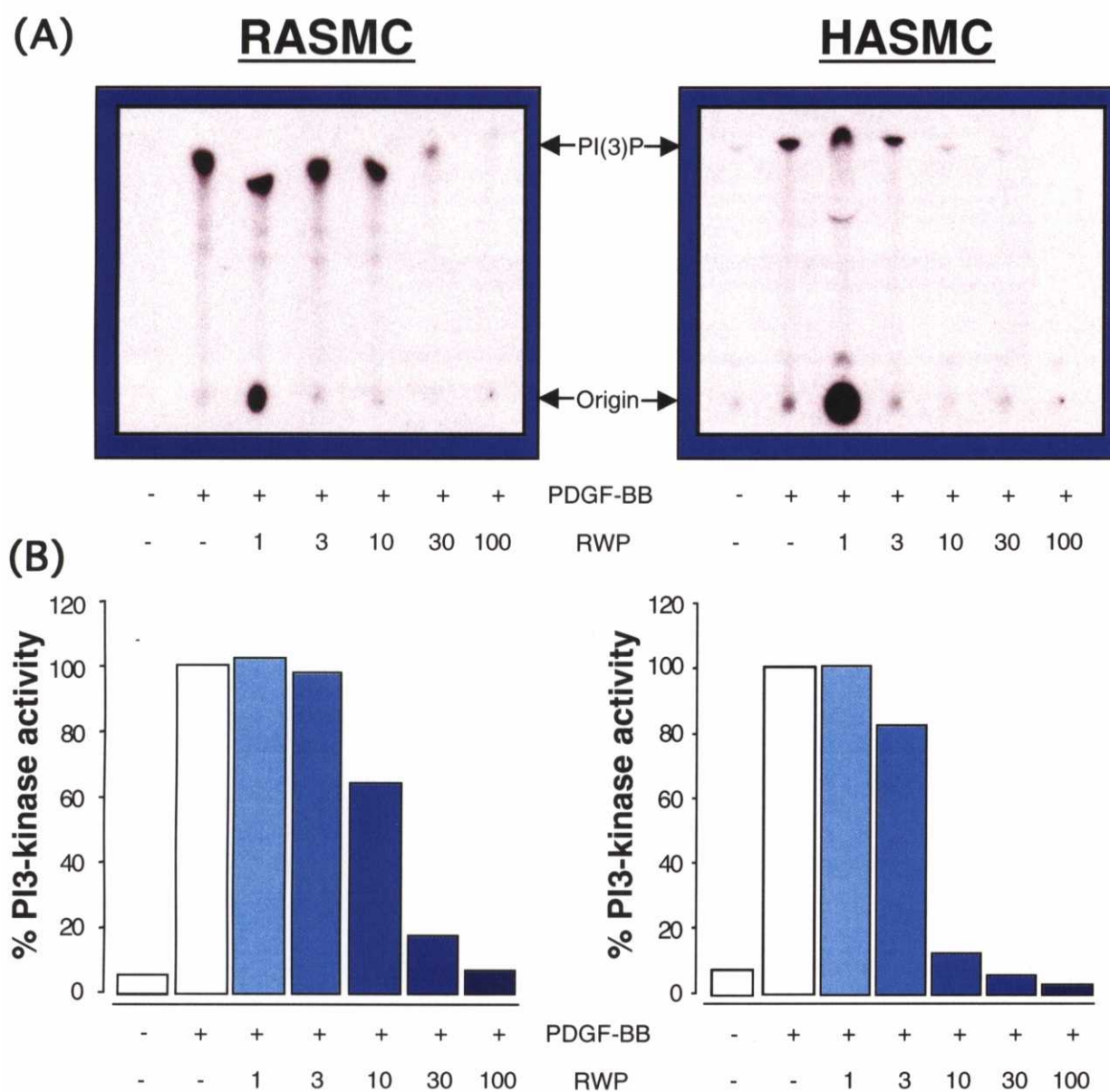
Figure 15. Effects of RWP on PI3-kinase activity in PDGF-BB-induced VSMC.

(A) Effects of RWP on the tyrosine phosphorylated protein-associated PI3-kinase activity in rat or human VSMC. After treatment of serum-starved cells with each concentration (μg/ml) of RWP for 10 hr, the cells were stimulated by PDGF-BB (10 ng/ml) for 10 minutes. Cell lysates were immunoprecipitated with the anti-phosphotyrosine antibody PY20, and assayed for PI3-kinase activity using thin layer chromatography.   (B) The radioactivities of PI3-kinase product, phosphatidylinositol-3' phosphate (PI(3)P), were quantified by imaging analyzer.

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| PDGF-BB (10 ng/ml) | - | + | + | + | + | + | + |
| RWP (μg/ml) | - | - | 1 | 3 | 10 | 30 | 100 |

| ERK 1/2 |
| p-ERK 1/2 |

| SAPK1/JNK |
| p-SAPK1/JNK |

| p38 MAPK |
| p-p38 MAPK |

| p-MKK3/6 |

Figure 16. Effects of RWP on phosphorylation of MAPK pathways in PDGF-BB-induced VSMC.
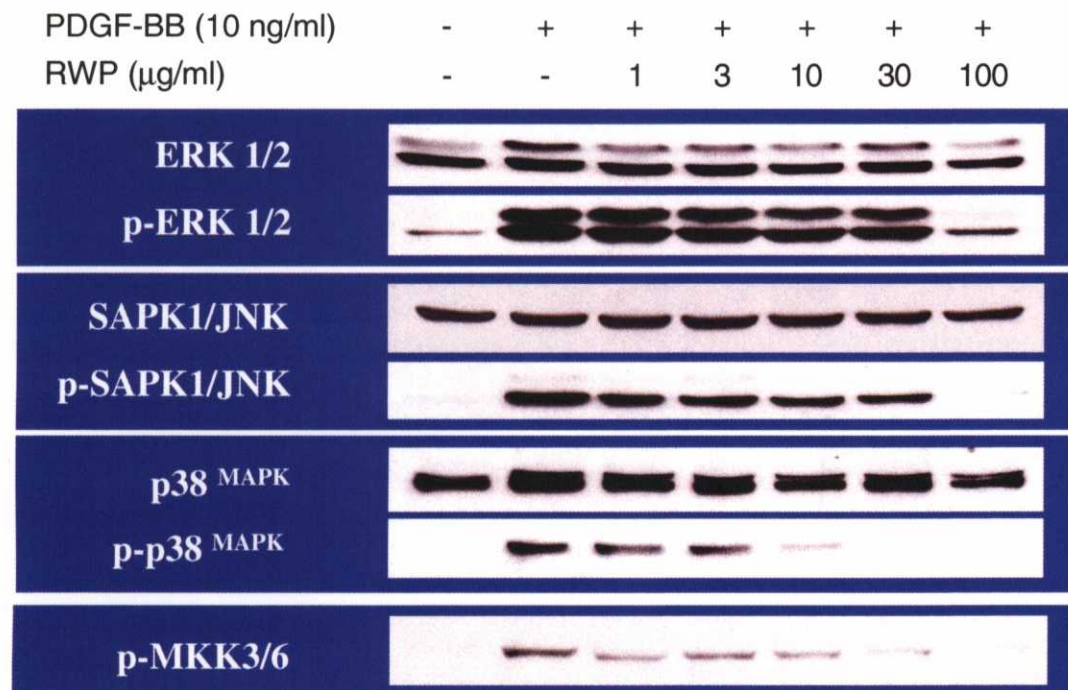After pretreatment of serum-starved HASMC with each concentration (1-100 μg/ml) of RWP for 10 hr, the cells were stimulated by PDGF-BB (10 ng/ml) for 10 minutes.   The cells were lysed and subjected to SDS-PAGE on a 12% gel. Phosphorylated proteins were detected with Western immunoblotting with total or anti-phosphorylation antibodies of each MAPK pathway.
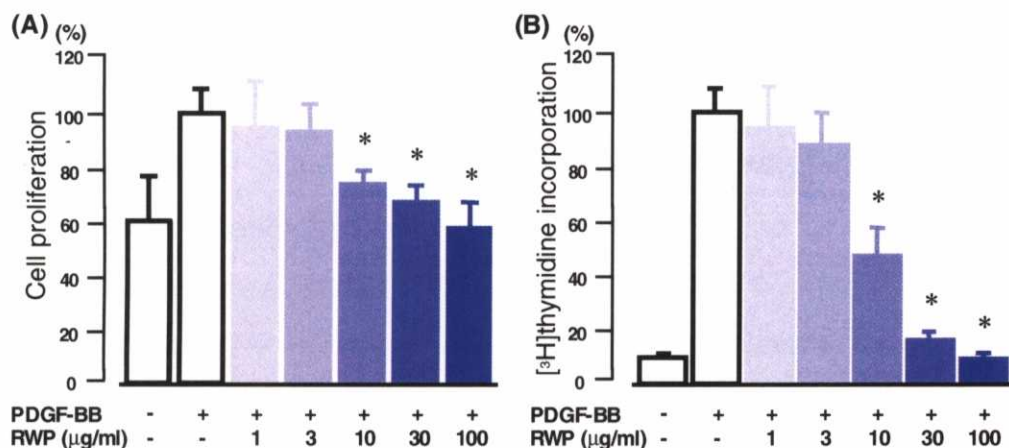
(A) (%)

Cell proliferation

| PDGF-BB | - | + | + | + | + | + | + |
| RWP (μg/ml) | - | - | 1 | 3 | 10 | 30 | 100 |

(B) (%)

$[^3H]$thymidine incorporation

| PDGF-BB | - | + | + | + | + | + | + |
| RWP (μg/ml) | - | - | 1 | 3 | 10 | 30 | 100 |

Figure 17. Effects of RWP on cell proliferation and DNA synthesis in PDGF-BB-stimulated RASMC.

After pretreatment of serum-starved RASMC with each concentration (1-100 μg/ml) of RWP for 10 hr, the cells were stimulated with or without PDGF-BB (10 ng/ml) for 60 hr (A; cell proliferation assay) or 22 hr (B; thymidine incorporation assay). Value are mean±SE (n=4). (*; <0.05 vs. PDGF-BB stimulation.)

Serum	-	+	+	+	+
Inhibitor	-	-	WT	LY	-
RWP (μg/ml)	-	-	-	-	30

| p27 |

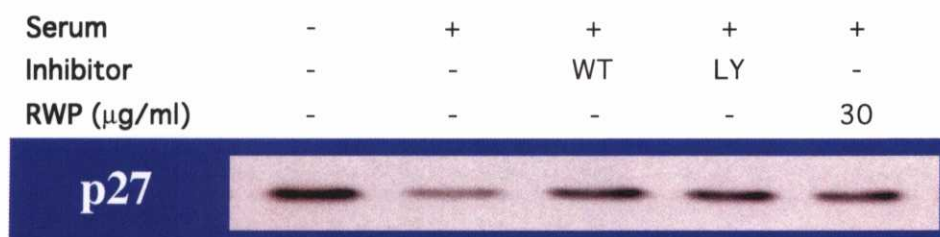**Figure 18. Effects of RWP on expression of p27^{Kip1} in serum-stimulated RASMC.**

After serum-starvation of RASMC for 24 hr, the cells were treated with 10% serum and PI3-kinase inhibitor (wortmannin; 100 nM or LY294002; 25 μM) or RWP (30 μg/ml) for 24 hr. The cell lysates were sujected to SDS-PAGE, and the expression of p27^{Kip1} was determined by a specific antibody for p27^{Kip1}.

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DISCUSSION

The anti-atherogenic effects of red wine consumption have been attributed in part to its anti-oxidant property, especially anti-oxidation for LDL cholesterol.[2-4] This potent anti-oxidative activity of RWP has been proposed as one of explanations for "French paradox".[2-4] However, it has been recently reported that RWP exert several anti-atherogenic effects not only by this anti-oxidative property but also by the direct effects on vascular cells.

In the first study, our results demonstrated that RWP had anti-proliferative effects on VSMC, and it might be associated with the downregulation of cyclin A gene expression through the decreased expression of transcription factors, CREB and ATF-1. In contrast, RWP did not significantly inhibit the proliferation of vascular EC. As long as we know, there is no detailed report on the possible beneficial effects of RWP in relation to cell cycle regulation of VSMC, and this is the first report that polyphenols in red wine inhibit the proliferation of VSMC through the inhibition of their cell cycle.

RWP, total polyphenolic fraction extracted from red wine, contained wide variety of polyphenols including phenolic monomers and polymers, which have different molecular weights. RWP was divided broadly into two groups. One group characterized by lower average molecular weight consisted of four fractions, Fraction 1 to 4, eluted first by 50% ethanol, and another group characterized by higher average molecular weight consisted of two fractions, Fraction 5 and 6, eluted by 100% ethanol and 70% acetone, respectively. First four fractions (Fraction 1-4) contained various polyphenolic monomer components, such as anthocyanidins, catechins, and

flavonoids. Although the percentage of each monomer may be quite different among four fractions, we have not found any specific polyphenolic substance, which is dominant in a specific fraction. Fraction 5 and 6 contained significant amount of specific polyphenolic compound, proanthocyanidins, which are polymerized anthocyanidins. Especially, Fraction 6 contained almost 100% proanthocyanidins. Average molecular weight of Fraction 1-4 and Fraction 6 (mainly proanthocyanidins) are believed to be approximately 200-400 and 1600-2000, respectively. It is noteworthy that all fractions of different molecular weight have shown basically similar potent anti-proliferative effects on VSMC.

The concentration of RWP in blood after red wine intake has not been determined, because RWP consist of polymers as well as monomers, and it is impossible to measure the concentration of polymers in blood so far. Several researchers have reported that the absorption efficiency is approximately 1-5% for monomers.[65] If the absorption efficiency of polymers is similar to this value, the calculated final concentration of RWP in blood could be 1 to 10 μg/ml after intake of 30-300 ml red wine. If this concentration of RWP in blood is maintained long term by the regular consumption of red wine, it might be possible that RWP would exert various anti-atherosclerotic effects, including the anti-proliferative and anti-migratory effects, on VSMC *in vivo*.

RWP have been shown to have anti-oxidative effects in terms of LDL oxidation *in vitro*.[3] Moreover, in *in vivo* studies, it has been recently shown that RWP contribute to the inhibition of LDL oxidation in healthy volunteers[4], and in Apo-E knock out mice.[66] Although anti-oxidant agents have been known to induce apoptosis in VSMC, despite prolonged treatment of RASMC with RWP, we have not observed any evidence of

apoptosis. It suggested that the inhibitory effects of RWP on RASMC might not be attributed to an apoptotic process.

It has been recently reported that resveratrol, one of polyphenolic compounds, is an agonist for the estrogen receptor.[67] The agent is similar, in structure, to synthetic estrogen-like compounds. Estrogen, which is a ligand for estrogen receptor expressed in VSMC, has been shown to have an anti-atherogenic effect by inhibiting the proliferation of VSMC.[68] This estrogen-like agonistic function of resveratrol may be one possible explanation for the anti-proliferative effects of RWP on VSMC.

In regard to the downregulation of cyclin A gene, it has been demonstrated that in mink lung epithelial cells transforming growth factor-β1 (TGF-β1) treatment decreased cyclin A mRNA and promoter activity but not the abundance of the ATF-binding proteins CREB and ATF-1 at 24 hr.[39] In an experiment using vascular EC, contact inhibition decreased the level of cyclin A mRNA almost completely, however, the level of ATF-1 mRNA was partially decreased and that of CREB mRNA was not changed.[69] In this study, RWP treatment completely decreased the levels of CREB and ATF-1 mRNA. It has been shown that CREB promoter contains ATF/CRE site, which might be regulated by CRE-binding proteins, including CREB itself.[70] Since the transcription of CREB gene may be controlled by positive autoregulation through the ATF/CRE site, it may be postulated that RWP may downregulate the transcription of CREB gene through some unknown mechanism first, and may start to decrease the cyclin A promoter activity. The downregulation of CREB may also work on the ATF/CRE site of its promoter, and finally CREB may become completely downregulated. Same cases might be true in the regulation of ATF-1 gene.

Although activation of cellular motility has been demonstrated in response to a number of growth factors, such as PDGF-BB[60], the intracellular signaling pathways involved in cellular motility are poorly understood compared with those of mitogenesis. In the second study, we demonstrated that RWP potently inhibited the PDGF-BB- and serum-stimulated VSMC migration in a concentration-dependent manner (3-30 μg/ml). Moreover, RWP inhibited the activities of two distinct signaling pathways, PI3-kinase and p38^{MAPK}, but not the activities of ERK1/2 and SAPK1/JNK. The concentrations of RWP necessary for the downregulation of these pathways were similar to those for the inhibition of VSMC migration.

RWP inhibited PDGF-BB-stimulated PI3-kinase activity in VSMC. Previously, several laboratories have reported that PI3-kinase is indispensable for cell chemotaxis induced not only by PDGF-BB but also by other growth factors.[46, 49] One report has shown that replacement of two tyrosine residues within the PI3-kinase binding sites of the PDGF β-receptor causes a loss of chemotactic response to PDGF-BB.[60] Our results showed that inhibition of PI3-kinase by a specific inhibitor, wortmannin, partially reduced the PDGF-BB-induced VSMC migration. Other reports have also shown that PI3-kinase plays a central role in the migratory action of several types of cells[52], including VSMC.[46] Contrary to these reports, Higaki et al. have reported that two different PI3-kinase inhibitors, wortmannin and LY294002, did not inhibit PDGF-induced chemotaxis in VSMC and Swiss 3T3 cells.[71] In our study, the inhibition of PI3-kinase by a specific inhibitor, wortmannin, partially reduced PDGF-BB-induced VSMC migration in Boyden chamber assay. The underlying mechanism of the discrepant observations concerning the

functional role of PI3-kinase in PDGF-induced chemotaxis of VSMC remains to be elucidated. It also has been demonstrated that the cytoskeletal organization changes dynamically when cells increase their motility[49-51], and PI3-kinase activity associates with an increase of cell motility. Small G proteins downstream of the PI3-kinase pathway, Rac and Rho, are known to be involved in PDGF-stimulated cellular responses, such as Rac-mediated lamellipodia and Rho-mediated stress fiber and focal adhesion formation.[51] The potent inhibitory effect of RWP on VSMC migration might be associated with, at least in part, the downregulation of these signaling pathways downstream of PI3-kinase.

It has been reported that Protein kinase B (PKB)/ Akt, a serine-threonine kinase, is a member of downstream signalings of PI3-kinase and is a major mediator for growth factors in promoting cell survival by inhibiting cell apoptosis in vascular EC. Under our experimental conditions, phosphorylation of PKB/Akt might not be important for cell survival because RWP at the highest concentration, 100 μg/ml, significantly downregulated the phosphorylation of PKB/Akt, however, RWP did not induce apoptosis in PDGF-BB- or serum-stimulated VSMC (data not shown).

Recently it has been reported that p38^{MAPK} activation plays a role in the migration of not only smooth muscle cells but also several other cell types.[52, 57, 59] Several reports have shown that p38^{MAPK} is important for formation of the cytoskeleton, including actin reorganization, and phosphorylation of heat shock protein 27 (hsp27). Hsp27 , which is downstream of p38^{MAPK}, has been shown to play a role in membrane ruffling.[72, 73] In the present study, we showed that treatment of PDGF-BB-stimulated VSMC with RWP resulted in downregulation of p38^{MAPK}

phosphorylation, but did not affect other MAPK pathways, and inhibition of p38^{MAPK} by a specific inhibitor (SB203580) showed a potent inhibitory effect on VSMC migration, but inhibition of ERK1/2 by a MEK-1 inhibitor (PD98059) did not inhibit the VSMC migration, in good agreement with previous reports.[52, 57] These results suggest that RWP would exert the anti-migratory effect on VSMC via the inhibition of cytoskeletal formation in part.

Matsumoto *et al.* have reported that expression of dominant-negative Ras inhibited PDGF-BB-induced migration in vascular EC expressing wild-type PDGF β-receptor.[57] They demonstrated that the dominant-negative Ras inhibited PDGF-BB-induced activation of p38^{MAPK} as well as that of ERK1/2, and inhibition of p38^{MAPK} by SB203580 significantly decreased PDGF-BB-induced cell migration, but inhibition of ERK1/2 by PD98059 did not. Since RWP (3-30 μg/ml) did not downregulate PDGF-BB-induced phosphorylation of ERK1/2, it is very likely that RWP did not interfere with the pathway from the PDGF receptor, via Ras, to ERK1/2. These findings suggest that the site of RWP action might be supposed to be between Ras and p38^{MAPK}. We examined the effect of RWP on the activation of MKK3/6, upstream of p38^{MAPK}. The phosphorylation of MKK3/6 was also inhibited by RWP in a concentration-dependent manner, suggesting that RWP work upstream of p38^{MAPK}. Regarding the inhibitory effect of RWP on the p38^{MAPK} pathway, further investigation will be necessary to identify the exact point where RWP work.

In the study 1, we showed that RWP had inhibitory effects on proliferation and DNA synthesis in serum-stimulated VSMC. Moreover, RWP strongly inhibited PDGF-BB-stimulated proliferation and DNA synthesis of HASMC in a concentration-dependent manner; however, the

phosphorylation of ERK1/2, which is known to relate to cell growth[74], was not inhibited by RWP (up to 30 µg/ml). These results suggest that the inhibitory effects of RWP on cell cycle progression in VSMC do not depend upon the ERK1/2 pathway. On the other hand, it has been recently reported that the activation of PI3-kinase is associated with cell cycle regulation. Several reports have demonstrated that inhibition of PI3-kinase activity by specific inhibitors upregulates $p27^{Kip1}$, one of the CDK inhibitors, in several cells.[62-64] To clarify the mechanism of the anti-proliferative effects of RWP on VSMC, we investigated the effect of RWP on the expression of $p27^{Kip1}$. In our experimental conditions, stimulation of VSMC with 10% serum suppressed the induction of $p27^{Kip1}$ by serum-starvation, and treatment of serum-stimulated VSMC with PI3-kinase inhibitor, wortmannin or LY294002, significantly induced the expression of $p27^{Kip1}$. Interestingly, RWP (30 µg/ml) also induced the expression of $p27^{Kip1}$ in VSMC, stimulated with serum. This result suggests that RWP suppress cell cycle progression not only through the downregulation of cyclin A expression but also through the induction of a CDK inhibitor, $p27^{Kip1}$, and RWP may inhibit intracellular signaling cascades activated by stimulators of cell growth and migration at an upstream site. Moreover, we investigated effect of RWP on expression of cyclin D_1, one of G_1 cyclins. The expression of cyclin D_1 was also inhibited by treatment of RASMC with RWP. This result suggests that it may be possible that the downregulation of cyclin A mRNA in the presence of RWP may be induced by the cell cycle arrest at G_1 phase. Although the downregulation of cyclin A gene by RWP is mediated by G_1 arrest in part, RWP may have a direct effect on the cyclin A expression because the

reduction of cyclin A mRNA by RWP was induced even at early phase (4 hr) of treatment with RWP and the downregulation of cyclin D_1 mRNA by RWP was weaker than that of cyclin A mRNA.

When detachment of the endothelial monolayer is induced by several injuries, including mechanical intervention, chemical substances, and inflammatory processes, EC migration is mainly responsible for the wound repair of damaged vessels.   In the present study, we showed that RWP had no inhibitory effect on proliferation, DNA synthesis, and cyclin A gene expression in serum-stimulated vascular EC.   Moreover, RWP did not inhibit serum-induced migration of vascular EC in the wounding assay or in the Boyden chamber assay.   In our experimental conditions, RWP had no significant effects on vascular EC.   However, we found that RWP inhibited expression of vascular cell adhesion molecule-1 (VCAM-1) in TNF-α-stimulated HUVEC in a concentration-dependent manner (data not shown).   Moreover, recently it has been shown that gallates, one of the contents of RWP, inhibited cytokine-induced adhesion molecule expression in vascular EC through a reduction of NF-κB activity.[6]   These beneficial effects of RWP on vascular EC suggest that RWP may play a role in not only maintenance of vascular structural integrity, but also in the restoration of EC function.   In the future, it will be necessary to elucidate the effects of RWP on EC, including membrane sensitivity, intracellular signaling, and target molecules.

SUMMARY AND CONCLUSION

The anti-atherogenic effects of red wine consumption have been attributed in part to its anti-oxidant property, especially anti-oxidation for LDL cholesterol. This potent anti-oxidant activity of red wine polyphenols has been proposed as one of explanations for "French paradox". Our results demonstrated that red wine polyphenols inhibited the proliferation of VSMC, but not of vascular EC, and it might be associated with the downregulation of cyclin A gene expression through the decreased expression of transcription factors, CREB and ATF-1. Moreover, red wine polyphenols inhibited the migration of VSMC, but not of vascular EC, through the inhibition of two distinct signaling pathways, PI3-kinase and $p38^{MAPK}$.

Taken together, red wine polyphenols had potent anti-proliferative and anti-migratory effects on VSMC in the context of atherogenesis, though red wine polyphenols may have benefical effects on vascular EC in atherogenic process. As long as we know, there is no detailed report on the possible beneficial effects of red wine polyphenols in relation to cell cycle regulation and intracellular signaling transduction on VSMC. Our findings suggest that the inhibitory effects of red wine polyphenols may be one of possible mechanisms for the anti-atherogenic effects of red wine, other than the anti-oxidative effects of red wine polyphenols on LDL. Further investigation of the intracellular signaling pathways modulating proliferation and migration of VSMC may clarify the mechanism of the "French paradox", and may enable us to innovate a therapeutic approach for the prevention of atherosclerosis progression.

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