British Journal of Pharmacology (1998) 124, 1227–1237
http://www.stockton-press.co.uk/bjp

Epigallocatechin suppression of proliferation of vascular smooth muscle cells: correlation with *c-jun* and JNK

[1]Liang-Huei Lu, [2]Shoei-Sheng Lee & [1,3]Huei-Chen Huang

[1]Department of Pharmacology and [2]School of Pharmacy, College of Medicine, National Taiwan University, Taipei, Taiwan

1 The mechanisms of the antiproliferative effect of epigallocatechin, one of the catechin derivatives found in green tea, in vascular smooth muscle cells were studied. The proliferative response was determined from the uptake of tritiated thymidine.

2 In the concentration range of 10^{-6} to 10^{-4} M, catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin, epigallocatechin gallate, concentration-dependently inhibited the proliferative response stimulated by serum in rabbit cultured vascular smooth muscle cells. Catechin and epicatechin were less effective in inhibiting the serum-stimulated smooth muscle cell proliferation, indicating that the galloyl group may be important for full inhibitory activity.

3 Epigallocatechin (EGC) inhibited the proliferative responses in different cells including rat aortic smooth muscle cells (A7r5 cells), rabbit cultured aortic smooth muscle cells, human coronary artery smooth muscle cells, and human CEM lymphocytes in a concentration-dependent manner. The possible mechanisms of the antiproliferative effect of EGC were further studied in A7r5 cells.

4 The membranous protein tyrosine kinase activity stimulated by serum in A7r5 cells was significantly reduced by 10^{-5} M EGC. In contrast, the cytosolic protein kinase C activity stimulated by phorbol ester was unaffected by directly incubating with EGC ($10^{-6}-10^{-4}$ M).

5 We also performed Western blot analysis using the anti-phosphotyrosine monoclonal antibody PY-20. EGC (10^{-5} M) reduced the levels of tyrosine phosphorylated proteins with different molecular weights, indicating that EGC may inhibit the protein tyrosine kinase activity or stimulate the protein phosphatase activity.

6 Reverse transcription-polymerase chain reaction analysis of *c-fos*, *c-jun* and *c-myc* mRNA levels demonstrated that *c-jun* mRNA level after serum-stimulation was significantly reduced by 10^{-5} M EGC. However, the reduction of *c-fos* and *c-myc* mRNA levels by 10^{-5} M EGC did not achieve significance.

7 Western blot analysis using the antibody against JNK (*c-jun* N-terminal kinase) and ERK (extracellular signal-regulated kinase) demonstrated that the level of phosphorylated JNK1, but not phosphorylated ERK1 and ERK2, was reduced by 10^{-5} M EGC. Direct measurement of kinase activity by immune complex kinase assay confirmed that JNK1 activity was inhibited by EGC treatment. These results demonstrate that EGC preferentially reduced the activation of JNK/SAPK (stress-activated protein kinase) signal transduction pathway.

8 It is suggested that the antiproliferative effect of epigallocatechin on vascular smooth muscle cells may partly be mediated through inhibition of protein tyrosine kinase activity, reducing *c-jun* mRNA expression and inhibiting JNK1 activation. Tea catechins may be useful as a template for the development of drugs to prevent the pathological changes of atherosclerosis and post-angioplasty restenosis.

Keywords: Antiproliferative effect; epigallocatechin; protein tyrosine kinase; *c-jun*; JNK; smooth muscle cells (vascular)

Introduction

Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells and extracellular matrix proteins (Lundergan *et al*., 1991; Ross, 1993). The proliferative responses of both vascular smooth muscle cells (SMC) and mononuclear cells are important in the pathogenesis of atherosclerosis and restenosis. We have previously found that some plant polyphenols reduced the proliferative responses of vascular SMC (Huang *et al*., 1992a,b; 1993; 1994a,b). Recently, we found that tea catechins reduced the proliferative responses of vascular SMC and human lymphoblastoid leukaemia (CEM) lymphocytes. Tea is traditionally and widely consumed as a beverage in the Orient. Green tea leaves contain a large amount (30% dry weight) of catechins (Graham, 1992). These catechins have many phenolic hydroxyl groups in their structures and are often called tea polyphenols. The term green tea refers to the product manufactured from fresh leaves while preventing oxidation of the polyphenolic components. Catechins contained in green tea consist mainly of epicatechin, epigallocatechin (EGC), epicatechin gallate and epigallocatechin gallate (Graham, 1992). These catechins have been shown to exert pharmacological effects including antioxidative, hypolipidaemic, antihypertensive and antimutagenic effects (Ikeda *et al*., 1992). Many laboratory studies have demonstrated inhibitory effects of tea preparations and tea polyphenols on tumour formation and growth. This inhibitory activity is believed to be mainly due to the antioxidative and possible antiproliferative effects of polyphenolic compounds in green tea (Yang & Wang, 1993). In the present study, the possible mechanisms of action of the antiproliferative effect of EGC were investigated. We have previously shown that

[3]Author for correspondence at: Department of Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan.

esculetin, a plant phenol, inhibits protein tyrosine kinase activity, thereby reducing the proliferative response of vascular smooth muscle cells (Huang *et al*., 1993). Therefore, it seemed of interest to investigate the effect of catechins on signal transduction pathways. The induction of *c-fos*, *c-jun*, and *c-myc* protoonocogenes expression, one of the earliest events associated with growth factor stimulation, can be detected in rat aortic smooth muscle cells following endothelial removal (Miano *et al*., 1990). In this study, the molecular mechanisms of the antiproliferative effect of EGC were investigated to determine whether the transduction signals and protooncogenes expression were affected.

Methods

Measurement of cell proliferation

A7r5 cells (rat aortic smooth muscle cells) and rabbit cultured vascular smooth muscle cells (rabbit VSMC) were grown in Dulbecco's modified Eagle's medium (supplemented with 10% v/v foetal calf serum, 100 u ml^{-1} penicillin, and 10^{-6} g ml^{-1} streptomycin). Human coronary artery smooth muscle cells (human CASMC) were grown in medium containing SmBM (Clonetics Corporation's BulletKit containing growth factors and antimicrobials). The proliferative responses of A7r5 cells, rabbit cultured VSMC, and human CASMC were determined by the uptake of tritiated thymidine (Huang *et al*., 1992b). Before all the experiments, confluent smooth muscle cells were rendered quiescent by culturing for 48 h (with one medium change after 24 h) in 0.5% v/v foetal calf serum (FCS) instead of 10%. In rabbit VSMC (2.5×10^4 cells/well) and human CASMC (2.5×10^4 cells/well), 5% v/v FCS together with the test compound were then added to the medium 24 h before [^{3}H]-thymidine (0.2 μCi/well) was included in the medium. Twenty-four hours after the addition of [^{3}H]-thymidine, the cells were harvested and the amount of [^{3}H]-thymidine incorporated into DNA was counted in a liquid scintillation counter. In A7r5 cells (10^4 cells/well), 5% v/v FCS and the test compound were added to the medium 16 h before [^{3}H]-thymidine (1 μCi/well) was included in the medium. Four hours after addition of [^{3}H]-thymidine, the cells were harvested and the [^{3}H]-thymidine incorporated was counted. CEM cells (human CEM lymphocytes) were cultured in RPMI-1640 medium containing 10% v/v FCS. Assessment of CEM cells proliferation was performed by [^{3}H]-thymidine incorporation assay as described previously (Watabe *et al*., 1984; Huang *et al*., 1994a). The test compound was added to the CEM cells suspension (5×10^3 cells/well) in the presence of 10% v/v FCS. Reaction mixtures were incubated in a CO_2 incubator at 37°C for 0.5 h before 1 μCi [^{3}H]-thymidine was added to each well. After an additional 4 h of incubation, cells were harvested and the [^{3}H]-thymidine incorporated to cells was counted.

Each experiment was performed in triplicate and repeated 5 or 6 times. The inhibitory activity of the test compound is expressed as percentage of the untreated control value. The concentration evoking 50% maximal inhibition (IC_{50}) was calculated for each experiment.

Cell viability was determined with the trypan blue dye exclusion method. After addition of the test compound for 20 h, cells were harvested from the dishes using a 0.1% w/v trypsin solution, and the viability was examined by the trypan blue dye exclusion test. The number of viable cells was estimated by microscopic cell counting using a haemocytometer.

Measurement of protein tyrosine kinase activity

The membranous protein tyrosine kinase activity of A7r5 cells was measured as previously described (Huang *et al*., 1993). A7r5 cells were lysed in ice-cold extraction buffer, containing 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 50 kiu aprotinin and 1 mM phenylmethylsulphonylfluoride, by a sonicator. The suspension was centrifuged first at 800 *g* for 10 min and then at 100,000 *g* for 1 h. The final pellet was resuspended in solubilizing buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $Mg(C_2H_3O_2)_2$ 5 mM NaF, 0.2 mM EDTA, 0.8 mM EGTA, 1 mM dithiothreitol, and 30 μM Na_3VO_4, and resonicated. After a 1 h incubation on ice, the homogenate was centrifuged at 100,000 *g* for 1 h. The supernatant thus obtained was the membrane fraction. The protein tyrosine kinase activity in the resultant supernatant was assayed with a protein tyrosine kinase assay kit (Gibco) by incubating with the synthetic peptide substrate, RR-SRC (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly), in the presence of [γ-^{32}P]-ATP, and activating enzyme with 5% v/v FCS. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by a scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}.

Measurement of protein kinase C activity

The protein kinase C activity of A7r5 cells was measured as previously described (Huang *et al*., 1993). A7r5 cells were lysed in ice-cold buffer A containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 300 mM sucrose, 2 mM phenylmethylsulphonylfluoride and 23 μM leupeptin by a sonicator. After centrifugation at 2,500 *g* for 5 min, the supernatant was centrifuged at 100,000 *g* for 1 h, and the resulting supernatant was retained as the cytosol fraction. After ultracentrifugation, the pellets were washed with buffer B (buffer A without sucrose) by centrifugation (12,000 *g*, 15 min), resuspended in buffer B with 1% v/v Triton X-100, and resonicated. After a 1 h incubation at 4°C, the homogenate was centrifuged at 100,000 *g* for 1 h, and the supernatant thus obtained was the membrane fraction. Protein kinase C activity was measured using a protein kinase C assay kit (Amersham) by a modification of a mixed micelle assay (Hannun *et al*., 1985), activating the enzyme with 39 μM phorbol-12-myristate-13-acetate. After removal of protein by acid precipitation, labelled peptide was bound to a phosphocellulose disc and phosphorylation of the peptide was measured by scintillation counter. The protein content was determined according to the method of Lowry *et al*. (1951). The results are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}.

RT-PCR analysis of c-fos, c-jun *and* c-myc *mRNA expression*

The expression of *c-fos*, *c-jun* and *c-myc* mRNA was analysed by the reverse transcription-polymerase chain reaction (RT-PCR) technique (Wang *et al*., 1989; Huang *et al*., 1994a). Before the RNA extraction, confluent smooth muscle cells were rendered quiescent by culturing for 48 h in 0.5% v/v FCS instead of 10%. Then, test compound together with 5% v/v FCS were added to the medium and incubated for the indicated time period. Total cellular RNA was prepared by

acid guanidinium thiocyanate extraction (Chomczynski & Sacchi, 1987). And, RNA was then reverse transcribed into cDNA. A 20 μl reverse transcription reaction mixture containing 1 μg of total cellular RNA, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.3 mM deoxynucleoside triphosphates (dNTPs), 1 unit of RNase inhibitor, 2.5 μM of oligo(dT)$_{16}$, and 10 units of M-MLV reverse transcriptase was incubated at 42°C for 1 h, heated to 95°C for 5 min, and then quick-chilled at 5°C for for 5 min, PCR was performed at a final concentration of 1 × PCR buffer/0.5 μM each 3′ and 5′ primers/2.5 unit of *AmpliTaq* DNA polymerase (Perkin-Elmer) in a total volume of 100 μl. The mixture was amplified for 35 cycles with the Perkin-Elmer thermal cycler (GeneAmp PCR System 9600). The amplification profile involved denaturation at 95°C for 15 s, annealing and extension at 65°C for 15 s. Primers for *c-fos*, *c-jun* and *c-myc* (Clontech) were used to generate 612, 409, and 479 bp fragments, respectively. The GAPDH primer (452 bp) was used as the internal standard. In each experiment, the negative control without reverse transcriptase was performed. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The gel was photographed with Polaroid type 667 film, and the digitized images were analysed by an image analyser (Winstar LV1, Taiwan). The protooncogenes signal intensity was normalized to its respective GAPDH signal intensity and expressed in arbitrary units. Samples were further confirmed by Southern blotting using standard methods (Sambrook *et al*., 1989).

In a preliminary experiment, various aliquots taken from the RT reaction were used in PCR for analysis of the test transcripts. The amounts of cDNA produced were proportional to the inputs of the RT-PCR products in the range 0.5 to 5 μl. Therefore, in subsequent experiments, the amounts of reverse-transcriptase products taken for PCR amplification of the test transcripts were within the linear ranges, in order to ensure that the amounts of cDNA produced truly reflect the levels of mRNA in the original samples.

Western blot analysis

Before the experiments, confluent smooth muscle cells were rendered quiescent by culturing for 48 h in 0.5% v/v FCS instead of 10%. The cells were then treated with stimulator (serum or tumour necrosis factor-α (TNF-α)) and test compound. At the end of the various treatments, cells were lysed in lysis buffer (1% Triton X-100, 20 mM HEPES pH 7.4, 50 mM β-glycerophosphate, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin, 1 mM phenylmethylsulphonylfluoride) for 4 min on ice. Soluble extracts were prepared by centrifugation at 16,000 *g* for 10 min at 4°C. An equal volume of 2 × sampling buffer (0.25 M Tris, pH 6.8, 4 mM EDTA disodium, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue) was added, and the cell extract was boiled for 4 min. Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gradient polyacrylamide gel. After electrophoresis, proteins were electrotransferred to a polyvinylidene difluoride membrane. Nonspecific binding sites on the membrane were blocked by incubation with 3% BSA in TBST buffer (20 mM Tris, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1 mM sodium orthovanadate, 0.05% Tween-20) for 90 min at room temperature. For Western blot assay of the levels of tyrosine phophorylated proteins, the membrane was then incubated with antiphosphotyrosine monoclonal antibody (PY-20, 1:1000 dilution) at 4°C for overnight at rotating table. After 10 min washes, the membrane was incubated with secondary antibody (goat anti-mouse IgG antibody, alkaline phosphatase conjugated) for 2 h at room temperature. The specific bands were visualized with the NBT/BCIP substrate of alkaline phosphatase.

For Western blot analysis of expression and phosphorylation of JNK or ERK, antibodies against JNK1 (46-kDa), ERK1 (44-kDa) and ERK2 (42-kDa) were used instead of PY-20. The signal intensity was quantified by an image analyser (Winstar LV1, Taiwan) and expressed in arbitrary units as a fold increase of the signal obtained with untreated cells.

Immunoprecipitation and kinase activity assays of JNK1

Quiescent A7r5 cells were treated with stimulator (serum or TNF-α) and test compound, and then lysed in lysis buffer. Soluble extracts were prepared by centrifugation at 16,000 *g* for 10 min at 4°C. Following normalization of protein content, endogenous c-Jun N-terminal kinase-1 (JNK1) was immunoprecipitated from the cell extracts using rabbit polyclonal antibodies against JNK1 and protein A-agarose. Kinase activity was then assayed for 20 min at 30°C in the presence of 6 μg substrate, 30 μM ATP, and 20 μCi of [γ-^{32}P]-ATP in 55 μl assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 20 mM $MgCl_2$ 0.1% Triton X-100, 1 mM dithiothreitol). GST-c-Jun (1 – 169) was used as a substrate for JNK1 activity. After completion of kinase activity assay, the protein was resolved by SDS-polyacrylamide gel electrophoresis, and the gels were dried and subjected to autoradiography. The incorporation of ^{32}P was quantified using a densitometer and expressed in arbitrary units as a fold increase of the signal obtained with untreated cells.

Materials

(+)-Catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate were purchased from Kurita Co. (Tokyo, Japan) (Figure 1). Rat aortic smooth muscle cells (A7r5 cells) and human CEM lymphocytes (CEM cells; human lymphoblastoid leukaemia cells) were purchased from the American Type Culture Collection (Rockville, MD). Human coronary artery smooth muscle cells (human CASMC) and SmBM smooth muscle growth medium (BulletKit containing growth factors and antimicrobials) were purchased from Clonetics Corporation (San Diego, CA). Rabbit cultured smooth muscle cells were isolated from rabbit thoracic aorta and cultured as previously described (Huang *et al*., 1992b). Foetal calf serum, penicillin, streptomycin, Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, and goat anti-mouse IgG antibody (alkaline phosphatase conjugated) were purchased from Gibco Life Technologies (Grand Island, NY). Thymidine [methyl-^{3}H] (5 Ci mmol^{-1}) and [γ-^{32}P]-ATP (5000 Ci mmol^{-1}) were purchased from Amersham Co. (Buckinghamshire, U.K.). Aprotinin, leupeptin, phenylmethylsulphonylfluoride, dithiothreitol, genistein (4′,5,7-trihydroxy-isoflavone), H-7 (1-(5-isoquinoline-sulphonyl)-2-methyl-piperazine 2HCl), Tris (tris [hydroxymethyl] aminomethane, HEPES hydroxyethylpiperazine-N′-2-ethanesulphonic acid), MOPS (3-[N-morpholino] propanesulphonic acid) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, substrate of alkaline phosphatase) were purchased from Sigma Chemical Co. (St. Louis, MO). Antiphosphotyrosine monoclonal antibody (PY 20), antibody against ERK1 or ERK2, and protein A-agarose were purchased from Transduction Lab., Inc. (Lexington, KY). Antibodies against JNK1 was purchased from Santa

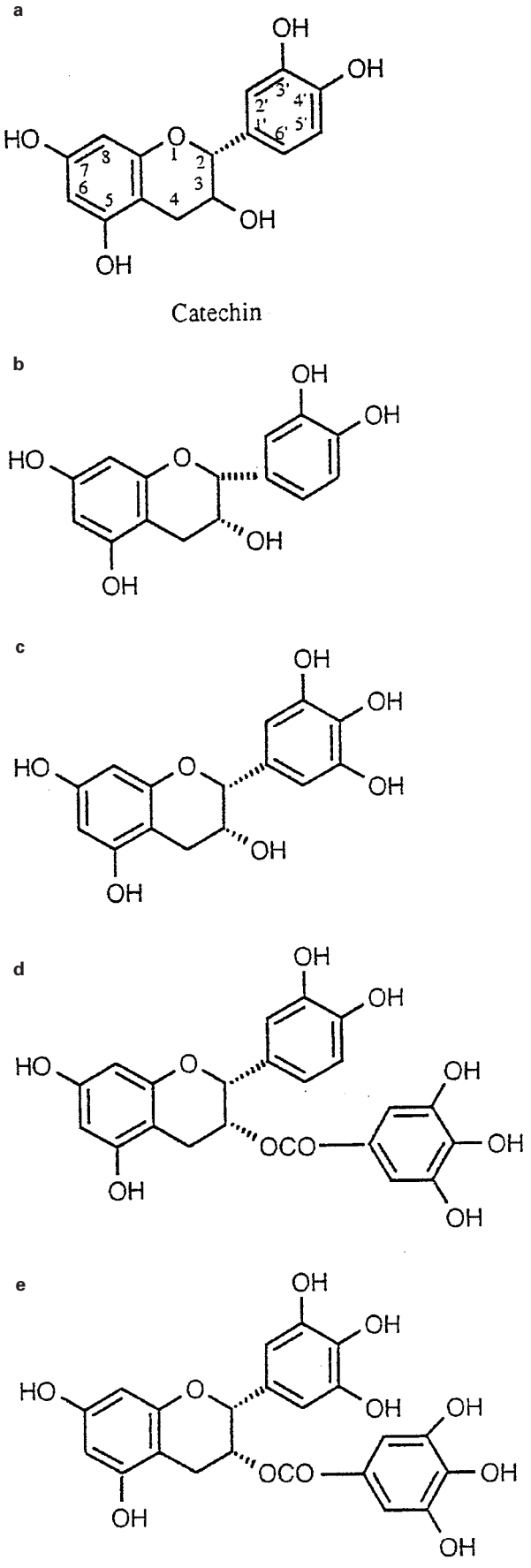

Figure 1 Structures of catechin and catechin derivatives contained in green tea. (a) Catechin, (b) epicatechin, (c) epigallocatechin (EGC), (d) epicatechin gallate and (e) epigallocatechin gallate.

Cruz Biotechnology, Inc. (Santa Cruz, CA). Glutathione-S-transferase fusion protein of c-Jun (1–169)(GST-c-Jun) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PVDF-PLUS transfer membrane (polyvinylidene difluoride) was purchased from Micron Separations, Inc., (Westborough, MA). Moloney murine leukaemia virus (M-MLV) reverse transcriptase and *AmpliTaq* DNA polymerase were purchased from Perkin-Elmer (Norwalk, CT). Primers for *c-fos* (612 bp), *c-jun* (409 bp), *c-myc* (479 bp), and *GAPDH* (452 bp) for PCR were purchased from Clontech Lab., Inc. (Palo Alto, CA).

Statistical analysis

The data are expressed as mean ± s.e.mean. P values less than 0.05 were considered to be statistically significant (ANOVA and Student's t test).

Results

Antiproliferative effects

Cell proliferation was studied by measuring the incorporation of [^{3}H]-thymidine into DNA in different cell types. The control values of [^{3}H]-thymidine incorporation in A7r5 cells (10^4 cells/well), rabbit vascular smooth muscle cells (2.5×10^4 cells/well), human coronary artery smooth muscle cells (2.5×10^4 cells/well), and human CEM lymphocytes (5×10^3 cells/well) stimulated by FCS without test compound were 7266 ± 755, 12,283 ± 879, 3250 ± 837, and 7031 ± 322 c.p.m./well, respectively. The effects of catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate on the serum-stimulated proliferative response of vascular smooth muscle cells were studied (Table 1). Exposure of rabbit cultured vascular smooth muscle cells to all catechin derivatives in the concentration range of 10^{-6} to 10^{-4} M, significantly inhibited the [^{3}H]-thymidine incorporation in a concentration-dependent manner. The inhibitory activities of catechins are shown in Table 1 ($n = 5$). Catechin and epicatechin were less effective inhibitors than epigallocatechin, epicatechin gallate and epigallocatechin gallate.

Exposure of different cell types to epigallocatechin (EGC) inhibited serum-stimulated proliferation in a concentration-dependent manner. The IC_{50} and maximal suppression values of EGC in various cell types are shown in Table 2 ($n = 5$). As shown in Figure 2, EGC (10^{-6}–10^{-4} M) concentration-

Table 1 Antiproliferative activities of catechin derivatives on rabbit cultured vascular smooth muscle cells

	n	IC_{50} (10^{-6} M)	*Max. suppression at* 10^{-4} M (%)
Catechin	5	0.5 ± 0.3	61.8 ± 6.9
Epicatechin	5	13.7 ± 7.5	51.3 ± 6.2
Epigallocatechin	5	3.3 ± 1.0	92.3 ± 1.5
Epicatechin gallate	5	7.0 ± 2.0	98.3 ± 0.7
Epigallocatechin gallate	5	3.1 ± 1.1	93.5 ± 5.6

The inhibitory effects of catehcin derivatives were tested on [^{3}H]-thymidine incorporation of vascular smooth muscle cells stimulated by serum. The inhibitory activities are expressed as percentages of control values (% of control). The serum-stimulated control value in the absence of test compound was 11,283 ± 879 c.p.m./well. Values are means ± s.e.mean for five triplicate experiments of cultured vascular smooth muscle cells from more than three rabbits.

dependently inhibited the proliferative responses of A7r5 and CEM cells ($n=5$).

Cell viability, as determined by the trypan blue dye exclusion method, was not affected by optimal concentrations of EGC ($<10^{-3}$M) (Figure 3) ($n=5$). The numbers of viable cells after EGC treatment ($10^{-7}-10^{-4}$ M) were more than the basal value (10^5 cells).

Table 2 Antiproliferative activity of epigallocatechin on various cell types

	n	IC_{50} (10^{-6} M)	*Max. suppresion at* 10^{-4} M (%)
A7r5 cells	5	3.8±0.6	80.0±4.0
Rabbit VSMC	5	3.3±1.0	92.3±1.5
Human CASMC	5	2.7±0.4	84.3±5.7
Human CEM lymphocytes	5	0.4±0.1	81.9±2.4

The inhibitory effect of test compound was determined by [^{3}H]-thymidine incorporation stimulated by serum in various cells. The inhibitory activities are expressed as percentages of control values (% of control). The serum stimulated control values in the absence of epigallocatechin were 7266±755 c.p.m./well for rat vascular smooth muscle cells (A7r5 cells), 11,283±879 c.p.m./well for rabbit cultured vascular smooth muscle cells (rabbit VSMC), 3250±837 c.p.m./well for human coronary artery smooth muscle cells (human CASMC), and 7031±332 c.p.m./well for human CEM lymphocytes (CEM cells). Values are means±s.e.mean for five triplicate experiments.

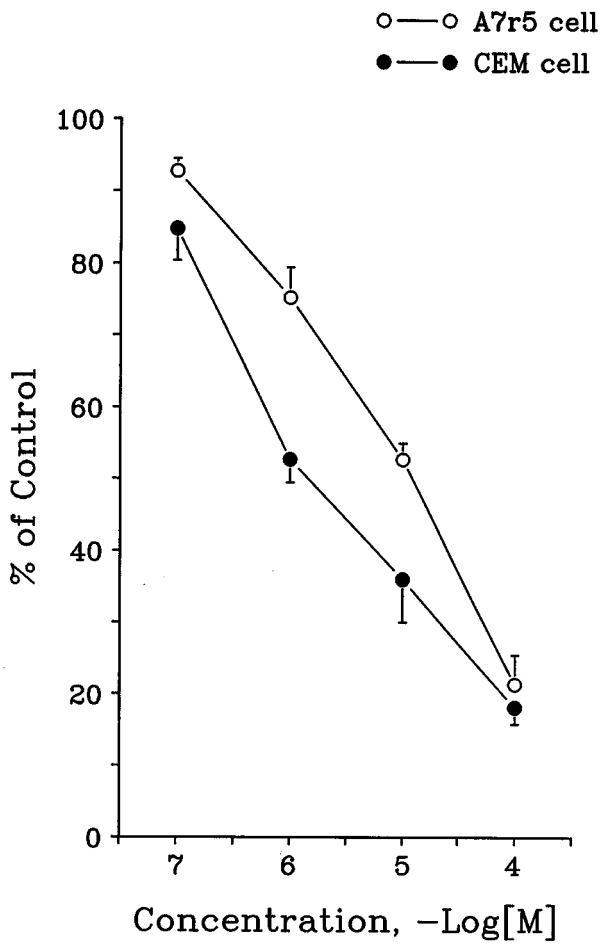

Figure 2 Effects of epigallocatechin on the proliferative responses of A7r5 and CEM cells. The proliferative response was determined from the uptake of tritiated thymidine. The control values for [^{3}H]-thymidine incorporation induced by serum without test compound were 7266±755 and 7031±332 c.p.m./well for A7r5 and CEM cells, respectively. The inhibitory activities of the test compound are expressed as percentage of the control values (% of control). Each point with vertical line represents the mean and s.e.mean ($n=5$).

Effect on protein tyrosine kinase activity

The membranous protein tyrosine kinase activity in serum-stimulated A7r5 cells was 3.2±0.2 pmol mg^{-1} protein min^{-1} over the basal activity ($n=20$) (the basal activity of the membranous protein tyrosine kinase without serum-stimulation was 1.15±0.06 pmol mg^{-1} protein min^{-1}). The serum-stimulated protein tyrosine kinase activity was significantly reduced by 10^{-5} M EGC (19.3±2.1% inhibition, $n=5$) (Figure 4). However, EGC at 10^{-4} M did not significantly alter the enzyme activity. At a higher concentration, EGC may exert other effects to counteract the inhibitory activity. In our assay system, 10^{-5} M genistein, a protein tyrosine kinase inhibitor, inhibited protein tyrosine kinase activity by 31.3±4.6% ($n=5$).

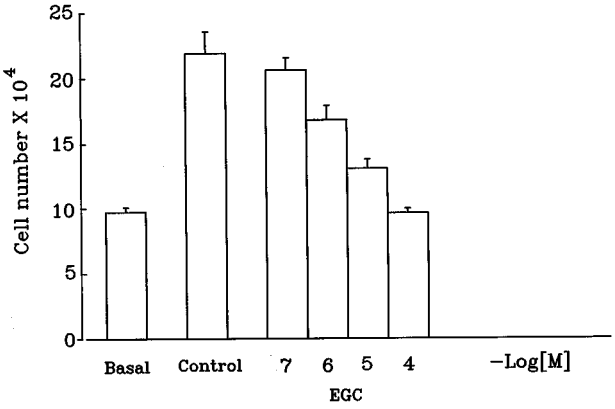

Figure 3 Effect of epigallocatechin on A7r5 cells growth. Quiescent A7r5 cells (basal level, 10^5 cells) were stimulated with serum. After addition of serum of 20 h in the absence (control level) or presence of test compound, cells were harvested and the viability examined by trypan blue dye exclusion test. The number of viable cells was estimated using a haemocytometer. Each column represents the mean±s.e.mean ($n=5$).

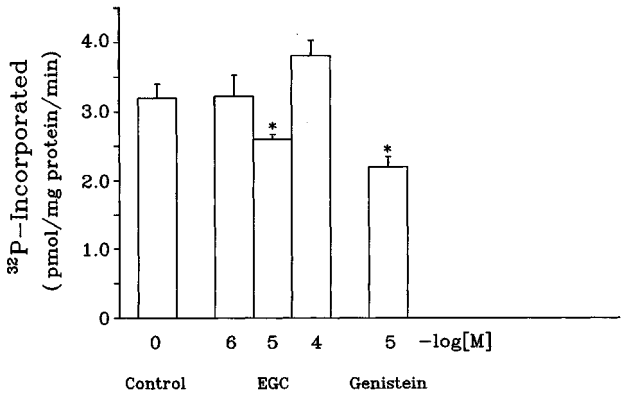

Figure 4 Effect of epigallocatechin on membranous protein tyrosine kinase activity in A7r5 cells. Subcellular fractions were prepared as indicated in Methods. The membranous protein tyrosine kinase was stimulated by serum in the absence (control level) or presence of test compound. The effect of test compound was compared to that of 10^{-5} M genistein (a protein tyrosine kinase inhibitor). Protein tyrosine kinase activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean±s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without test compound.

The levels of tyrosine phosphorylated proteins were also analysed by Western blot assay using the anti-phosphotyrosine monoclonal antibody PY-20. Quiescent A7r5 cells were stimulated by 5% v/v FCS for different time periods (0.5 and 1 h) to activate the protein tyrosine kinase. After immunoblotting with PY20, the phosphorylation of several proteins with different molecular weights was observed at both 0.5 and 1 h (Figure 5, lanes 2 and 4). EGC (10^{-5} M) reduced the levels of tyrosine phosphorylated proteins of at least two proteins of molecular weight 220 and 97 kDa (Figure 5, lanes 3 and 5). Genistein (10^{-5} M) also inhibited the tyrosine phosphorylation of proteins (Figure 5, lanes 6 and 7). Similar observations were obtained in five independent experiments.

Effect on protein kinase C activity

The cytosolic protein kinase C activity of A7r5 cells was 22.0 ± 1.4 pmol mg^{-1} protein min^{-1} ($n=16$) in the presence of 39 μM phorbol-12-myristate-13-acetate. The protein kinase C activity was unaffected by directly incubating with EGC ($10^{-6}-10^{-4}$ M) (Figure 6) ($n=5$). In our assay system, 10^{-5} M H-7, a protein kinase C inhibitor, inhibited protein kinase C activity by $25.2 \pm 1.5\%$ ($n=5$).

Effects on c-fos, c-jun *and* c-myc *mRNA expression*

The mRNA levels of three protooncogenes, *c-fos*, *c-jun* and *c-myc* in serum-stimulated A7r5 cells untreated and treated with EGC were measured using the RT-PCR technique. The quiescent A7r5 cells responded to serum-stimulation by markedly increasing *c-fos*, *c-jun* and *c-myc* mRNA levels over their respective basal levels within 0.5 h. The signal intensities of protooncogenes and GAPDH were quantified by an image analyser, and changes in the signal intensities of test genes relative to GAPDH are shown (Figure 7 and bottom of Figure 8a,b,c). In Figure 7, a semi-quantitative analysis of *c-fos*, *c-jun*, and *c-myc* mRNA at different time points (0.5, 1 and 2 h) after serum-stimulation is shown. The mean expressions of all three mRNAs at 1 h 2 h were less than those at 0.5 h (Figure 7) ($n=7$). The effects of EGC (10^{-5} M) on the levels of *c-fos*, *c-jun* and *c-myc* mRNA are shown in Figure 8. The levels of *c-fos* and *c-myc* mRNA were not significantly affected by EGC (Figure 8a,c) ($n=6$). However, EGC significantly reduced the serum-stimulated expression of *c-jun* mRNA by 39.5 ± 7.8 and $29.5 \pm 11.9\%$ at 1 and 2 h, respectively (Figure 8b) ($n=6$).

Effects on expression and phosphorylation of JNK and ERK

For Western blot analysis of expression and phosphorylation of JNK (c-Jun N-terminal kinase) or ERK (extracellular signal-regulated kinase), antibodies against JNK1 (46 kDa), ERK1 (44 kDa) and ERK2 (42 kDa) were used. Concomitant with the phosphorylation, a shift of the protein band occurred. As shown in Figure 9a and Figure 10, the unphosphorylated enzyme is apparent as the faster migrating band. Phosphorylation induces a mobility shift resulting in a slower migrating band. In a preliminary experiment, tumour necrosis factor-α

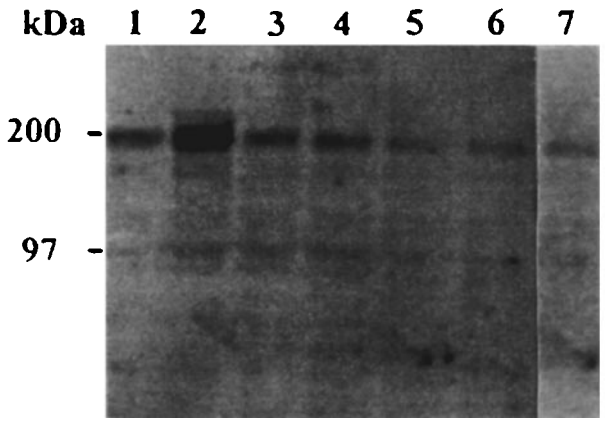

Figure 5 Effect of epigallocatechin on the levels of tyrosine phosphorylated proteins using Western blot analysis. Quiescent A7r5 cells were stimulated by serum in the absence and presence of test compound for the indicated time periods. The levels of tyrosine phosphorylated proteins were analysed by Western blot assay using the anti-phosphotyrosine monoclonal antibody PY-20 as indicated in Methods. Tyrosine-phosphorylated proteins of 200 and 97 kDa are indicated. Lane 1, cells in the basal condition; lane 2, serum-stimulated control at 0.5 h; lane 3, 10^{-5} M EGC-treated and serum-stimulated at 0.5 h; lane 4, serum-stimulated control at 1 h; lane 5, 10^{-5} M EGC-treated and serum-stimulated at 1 h; lane 6, 10^{-5} M genistein-treated and serum-stimulated at 0.5 h; lane 7, 10^{-5} M genistein-treated and serum-stimulated at 1 h. Similar observations were obtained in five independent experiments.

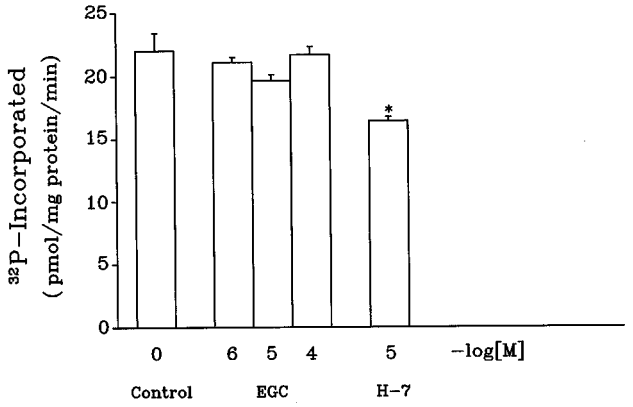

Figure 6 Effect of epigallocatechin on protein kinase C activity in A7r5 cells. Subcellular fractions were prepared as indicated in Methods. They cytosolic protein kinase C was stimulated by phorbol ester in the absence (control level) or presence of test compound. The effect of test compound was compared to that 10^{-5} M H-7 (a protein kinase C inhibitor). Protein kinase C activities are expressed as pmol ^{32}P incorporated mg^{-1} protein min^{-1}. Each column represents the mean $\pm$ s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without test compound.

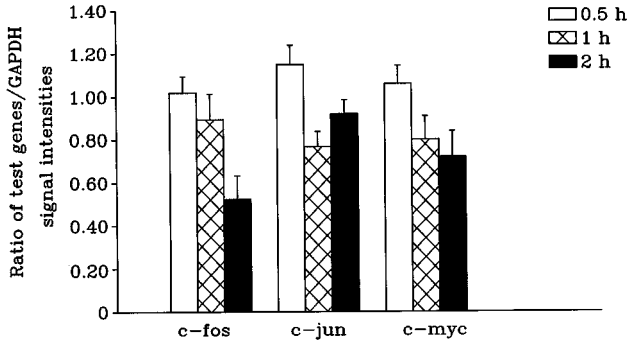

Figure 7 Time courses of *c-fos, c-jun* and *c-myc* mRNA accumulation in A7r5 cells. The serum-stimulated protooncogene mRNA levels at 0.5 h, 1 h and 2 h were analysed by RT-PCR amplification as indicated in Methods. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The signal intensities of protooncogenes and GAPDH were quantified by an image analyser, and changes in signal intensities of test genes relative to GAPDH are shown ($n=7$).

(TNF-α, 10^{-9} M) induced more expression and phosphorylation of JNK1 than did FCS (5% v/v) in A7r5 cells. As shown in Figure 9, the increases in levels of both unphosphorylated and phosphorylated JNK1 were measurable 0.5 and 1 h after stimulation by 10^{-9} M TNF-α (Figure 9a, lanes 2 and 4). EGC (10^{-5} M) treatment significantly reduced the phosphorylated JNK1 levels 0.5 and 1 h after TNF-α-stimulation (Figure 9a, lanes 3 and 5) ($n=5$). The changes in unphosphorylated JNK1 did not achieve significance ($n=5$).

On the other hand, the ERK1 and ERK2 were basally expressed and the expression and phosphorylation of both ERK1 and ERK2 were increased 0.5 and 1 h after serum-stimulation (Figure 10, lanes 2 and 4). However, the levels of the serum-stimulated ERK phosphorylation were not sig-

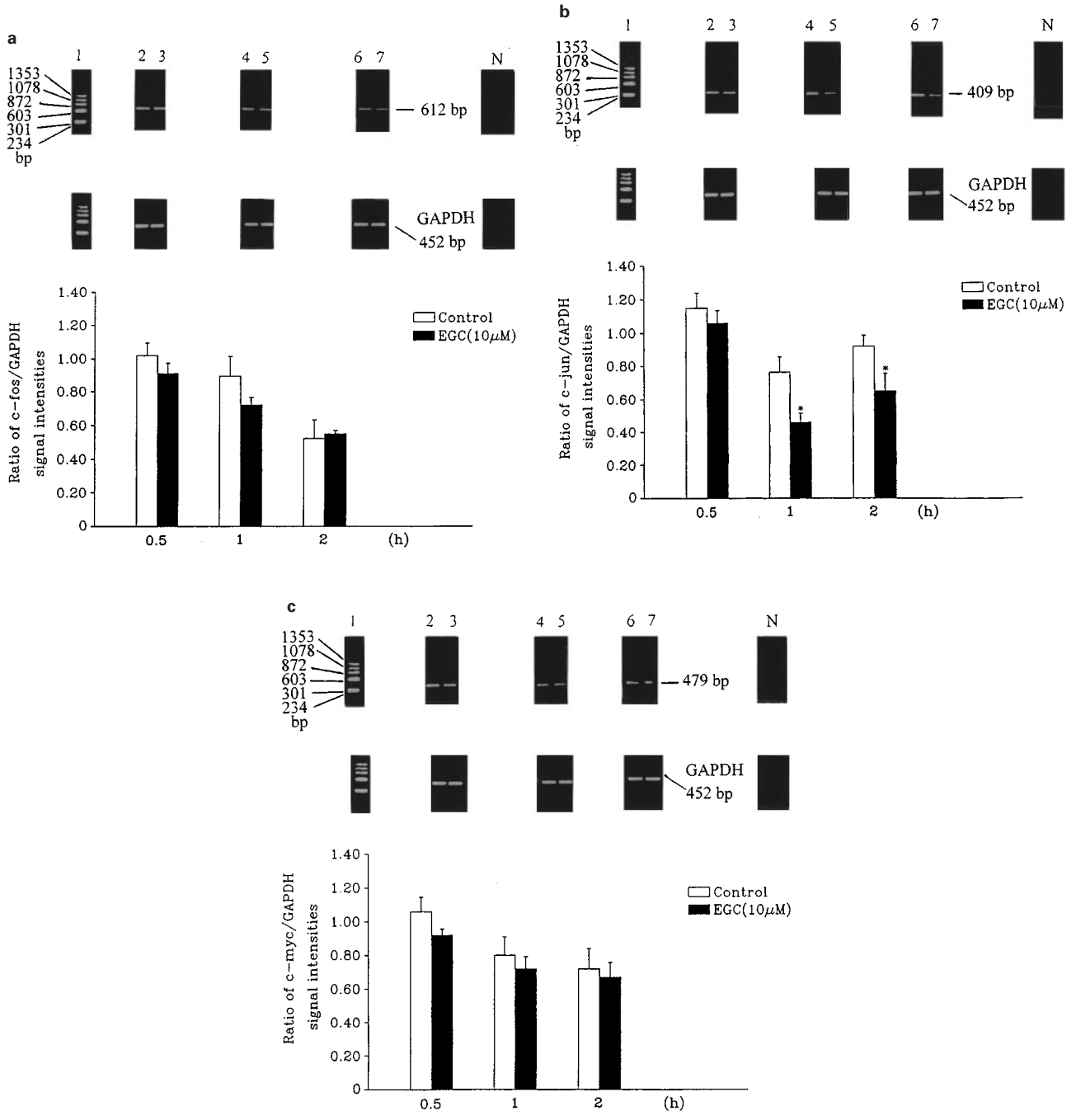

Figure 8 Effects of epigallocatechin on (a) *c-fos* (b) *c-jun* and (c) *c-myc* mRNA levels in A7r5 cells. Quiescent A7r5 cells were stimulated by serum in the absence and presence of test compound for the indicated time periods. The serum-stimulated protooncogene mRNA levels untreated and treated with EGC were analysed by RT-PCR amplification as indicated in Methods. Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The signal intensities of protooncogenes and GAPDH were quantified by an image analyser, and changes in the signal intensities of test genes relative to GAPDH are shown (bottom) ($n=6$). On the top of each panel, corresponding electrophoretic patterns of PCR products are shown. Lane 1, φ X174/*Hae*III DNA size marker; lane 2, serum-stimulated control at 0.5 h; lane 3, 10^{-5} M EGC-treated and serum-stimulated at 0.5 h; lane 4, serum-stimulated control at 1 h; lane 5, 10^{-5} M EGC-treated and serum-stimulated at 1 h; lane 6, serum-stimulated control at 2 h; lane 7, 10^{-5} M EGC-treated and serum-stimulated at 2 h lane N, negative control, no RT. Each column represents the mean ± s.e.mean ($n=6$). $^*P<0.05$, when compared to the control value without test compound.

nificantly affected by 10^{-5} M EGC (Figure 10, lanes 3 and 5) ($n=5$). These findings suggest that EGC preferentially suppresses signalling events in A7r5 cells leading to phosphorylation of JNK, rather than the ERK system.

Kinase activity assay of JNK1

Anti-JNK1 antibody was used for kinase immunoprecipitation from the soluble fraction of cell lysates. Kinase activity was then assessed by immune complex kinase assay using GST-c-Jun as a substrate. The JNK1 activity of A7r5 cells was markedly increased 0.5 h after TNT-α stimulation (Figure 9b, lane 2). EGC (10^{-5} M) treatment significantly inhibited TNF-α stimulated JNK activation (Figure 9b, lane 3) ($n=5$).

Discussion

The present results demonstrate for the first time that tea catechins concentration-dependently inhibited the proliferation of CEM lymphocytes and various vascular smooth muscle cells, including rat aortic smooth muscle cells (A7r5), rabbit cultured aortic smooth muscle cells, and human coronary artery smooth muscle cells. Among the catechin derivatives tested, catechin and epicatechin were less effective in inhibiting the serum-stimulated cell proliferation than epigallocatechin, epicatechin gallate and epigallocatechin gallate, indicating that the galloyl group seems to be important for full inhibitory activity. Tea catechins have been shown to exhibit hypolipidaemic and antioxidant effects. Matsuda *et al.* (1986) demonstrated that tea catechins were effective in reducing the serum cholesterol level. Ikeda *et al.* (1992) further showed that tea catechins reduced cholesterol absorption from intestine. On the other hand, catechins have been shown to inhibit low density lipoprotein (LDL) oxidation and the subsequent degradation of the oxidized-LDL by human monocyte-derived macrophages (Mangiapane *et al.*, 1992). In the present study, tea catechins were effective inhibitors of serum-stimulated mitogenesis in vascular smooth muscle cells and CEM lymphocytes. Atherosclerosis and post-angioplasty restenosis are characterized by the abnormal accumulation of vascular smooth muscle cells, inflammatory cells, and extracellular matrix proteins (Lundergan *et al.*, 1991; Ross, 1993). Thus, tea catechins may be useful in the study and treatment of atherosclerosis and post-angioplasty restenosis. Although there are limited studies on the bioavailability of the polyphenols, the absorption of catechins in man has been shown (Rice-Evans, 1995). Epidemiological studies, though inconclusive, suggest a protective effect of tea consumption on human cancer and coronary heart disease (Stoner & Mukhtar,

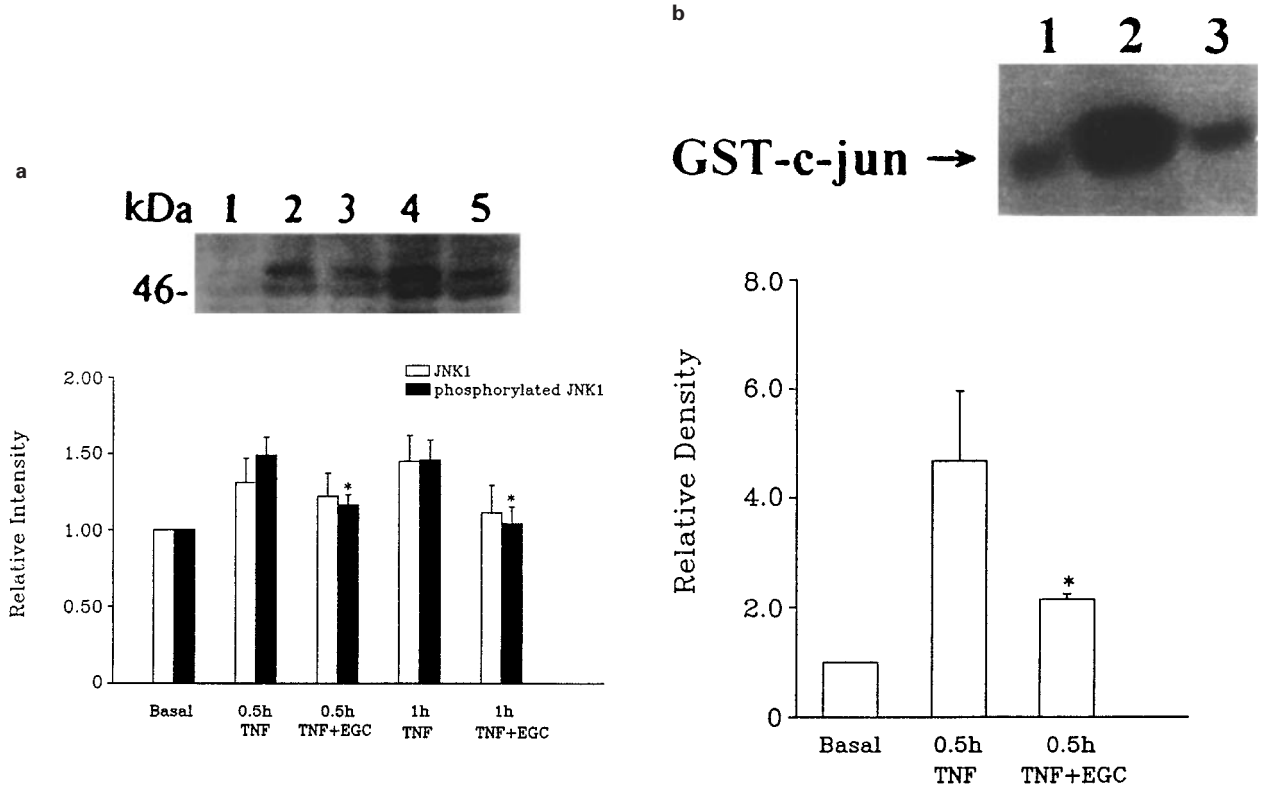

Figure 9 (a) Effect of epigallocatechin on JNK1 expression and phosphorylation in A7r5 cells. Quiescent A7r5 cells were stimulated by TNF-α in the absence and presence of test compound for the indicated time periods. The levels of unphosphorylated and phosphorylated kinase were analysed by Western blotting using anti-JNK1 antibody as indicated in Methods. The signal intensities were quantified and changes in protein levels relative to untreated basal levels are shown (bottom) ($n=5$). Lane 1, cells in the basal condition; lane 2, TNF-stimulated control at 0.5 h; lane 3, 10^{-5} M EGC-treated and TNF-α-stimulated at 0.5 h; lane 4, TNF-α-stimulated control at 1 h; lane 5, 10^{-5} M EGC-treated and TNF-α-simulated at 1 h. Each column represents the mean$\pm$s.e.mean ($n=5$). *$P<0.05$, when compared to the control value without EGC. (b) Effect of epigallocatechin on JNK1 activity in A7r5 cells. Quiescent A7r5 cells were stimulated by TNF-α in the absence and presence of test compound for 0.5 h. Anti-JNK1 antibody was used for kinase immunoprecipitation from the soluble fraction of cell lysates. Kinase activity was then assessed by immune complex kinase assay using GST-c-Jun as a substrate. The signal intensities were quantified by densitometer, and changes in protein levels relative to untreated basal levels are shown (bottom) ($n=3$). Lane 1, cells in the basal condition; lane 2, TNF-α-stimulated control at 0.5 h; lane 3, 10^{-5} M EGC-treated and TNF-d-stimulated at 0.5 h. Each column represents the mean$\pm$s.e. mean ($n=5$). *$P<0.05$, when compared to the control value without EGC.

1995; Thelle, 1995). The present results suggest that green tea available in everyday life may be a practical chemopreventive agent for proliferative diseases, such as cancer, atherosclerosis and restenosis. Tea catechins, which possess hypolipidaemic and antiproliferative activities, may be useful as a template for the development of drugs to prevent the pathological changes of atherosclerosis and restenosis. Cell viability assay verified that the effects of EGC were not due to general cellular toxicity. Our results show that EGC may inhibit the proliferative responses through inhibition of transduction signals leading to DNA synthesis. There are some signalling pathways for growth factors and mitogens (Powis & Kozikowski, 1991). The receptors for growth factors such as platelet-derived growth factor and epidermal growth factor are protein tyrosine kinases. Tyrosine kinase activity has also been found to be associated with other growth factors and oncoproteins (Bishop, 1983). On the other hand, peptides such as vasopressin and bombesin partly act through cell surface receptors, coupled to a guanine nucleotide binding protein to activate a specific phospholipase C that hydrolyzes phosphatidylinositol-4, 5-bisphosphate to inositol-1,4,5-trisphosphate and diacylglycerol. Diacylglycerol then activates protein kinase C (Powis & Kozikowski, 1991). In the present study, EGC reduced protein tyrosine kinase activity. The inhibitory effect of EGC on tyrosine kinase activity was demonstrated by biochemical method. We also performed Western blot analysis using the anti-phosphotyrosine monoclonal antibody PY-20. EGC reduced the levels of tyrosine phosphorylated proteins, indicating that EGC may inhibit the protein tyrosine kinase activity or stimulate the protein phosphatase activity.

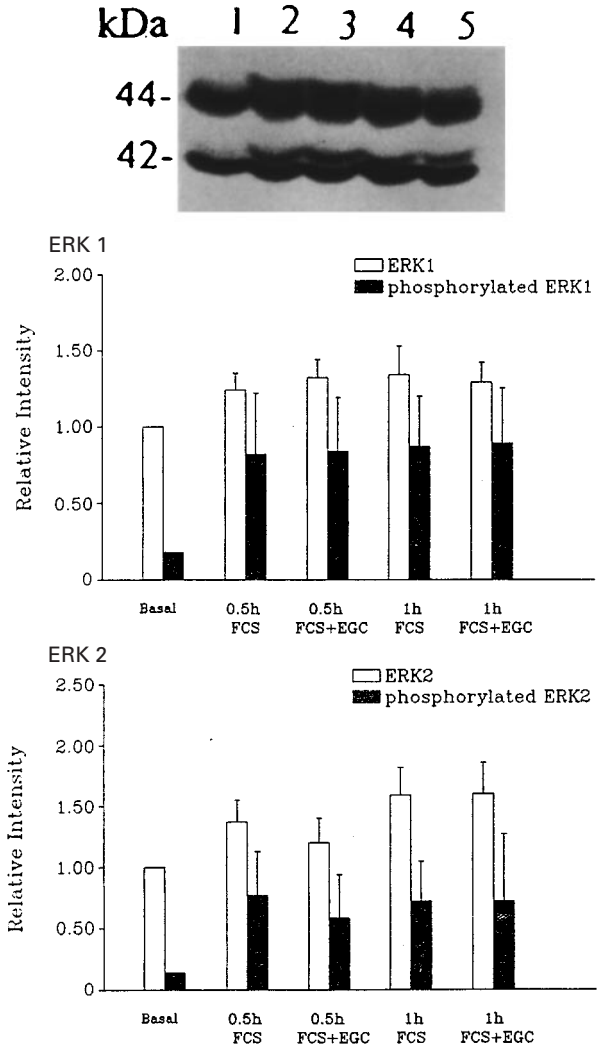

Figure 10 Effect of epigallocatechin on ERK expression and phosphorylation in A7r5 cells. Quiescent. A7r5 cells were stimulated by serum in the absence and presence of test compound for the indicated time periods. The levels of unphosphorylated and phosphorylated kinase were analysed by Western blotting using anti-ERK antibody as indicated in Methods. The signal intensities were quantified and changes in protein levels relative to untreated basal levels as shown (bottom) ($n=5$). Lane 1, cells in the basal condition; lane 2, serum-stimulated control at 0.5 h; lane 3, 10^{-5} M EGC-treated and serum-stimulated at 0.5 h; lane 4, serum-stimulated control at 1 h; lane 5, 10^{-5} M EGC-treated and serum-stimulated at 1 h.

We also examined the effects of EGC on the expression of proto-oncogenes mRNA. The induction of *c-fos*, *c-jun* and *c-myc* mRNA is one of the earliest transcriptional events associated with growth factor stimulation. The products of these oncogenes appear to function as transcriptional regulatory proteins which are involved in the G_1 phase of the cell cycle (Reed *et al*., 1986; Pardee, 1989). Our results demonstrate that the reduction of DNA synthesis in vascular smooth muscle cells by EGC is associated with alteration of *c-jun* mRNA expression. Reduction in serum-stimulated proto-oncogene expression may partly be involved in the antiproliferative effect of EGC. EGC preferentially reduced *c-jun*, but had less affect on *c-fos* or *c-myc*, mRNA levels in the present results, indicating that EGC may preferentially reduce the activation of the JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) signal transduction pathway. SAPKs define a stress-activated signalling pathway which regulates the activity of c-Jun (Kyriakis *et al*., 1994). The SAPKs are activated in response to cellular stresses, including ischaemia, u.v. light, heat shock, and to inflammatory cytokines (Kyriakis *et al*., 1994; Knight & Buxton, 1996). The c-Jun protein can be activated by JNK/SAPK or by an increase in transcription of the *c-jun* gene (Angel *et al*., 1988; Kyriakis *et al*., 1994). Mammalian MAP kinase (mitogen-activated protein kinases) singalling pathways leading to activation of the MAP kinases, ERK, JNK, and p38 have been identified (Davis, 1994; Waskiewicz & Cooper, 1995; Whitmarsh & Davis, 1996). These are the MAP kinase itself, which is activated by phosphorylation on Thr and Tyr by a dual-specificity MAP kinase kinase (MAPKK), which in turn is activated by Ser/Thr phosphorylation by a MAP kinase kinase kinase (MAPKKK) (Davis, 1994; Waskiewicz & Cooper, 1995; Whitmarsh & Davis, 1996). And, MAP kinase can be inactivated by a unique family of dual specificity phosphatases, MAP kinase phosphatases (MKPs) (Hirsch & Stork, 1997). By using Western blot analysis of phosphorylated kinase and immune complex kinase activity assay, we further demonstrated that EGC reduced the phosphorylation and kinase activity of JNK1. The JNK have been shown to participate in cellular proliferation via transcriptional activation (Derijard *et al*., 1994; Kallunki *et al*., 1994). The activation of JNK, which phosphorylates the c-Jun activation domain, results in enhanced transcription of AP-1-dependent genes. Therefore, a reduction in JNK activity may partly contribute to the antiproliferative effect of EGC. On the other hand, it has been found that the ERK pathway prolongs survival, whereas activation of the JNK pathway promotes cell death (Xia *et al*., 1995). And, sightings of apoptosis have been obtained from every corner of cardiovascular medicine, ranging from restenosis to conduction system defects in congestive heart failure (Yeh, 1997). EGC could also be

developed to form a novel therapy for apoptosis. It has been shown that green tea catechins bind to various hepatic cytochrome P450 and inhibit P450-dependent functions (Wang *et al*., 1988). An examination of the structure-activity relationship of catechins demonstrated that the inhibitory effect on the microsomal enzyme system may be due to the galloyl groups or hydroxyl groups on the molecule. Tea catechins has also been reported to block the interaction of tumour promoters, hormones and growth factors with their receptors (Komori *et al*., 1993). Studies to elucidate further the structure-activity relationship of the inhibitory effect of EGC on JNK activity are in progress in our laboratory.

In summary, we found that tea catechins exhibited an antiproliferative effect in vascular smooth muscle cells and CEM lymphocytes. The antiproliferative effect of epigallocatechin in vascular smooth muscle cells may partly be mediated through inhibition of protein tyrosine kinase activity, reduction in *c-jun* mRNA expression, and inhibition of JNK1 activation. Tea catechins which possess antiproliferative as well as hypolipidaemic activities, may be useful as a template for the development of drugs to prevent the pathological changes of atherosclerosis and post-angioplasty restenosis. For treatment of atherosclerosis and restenosis, the dose range of antiproliferative agent which can slow down the overdrive response of cell division without deleterious effect on the stability of atherosclerosis plaques should be used.

This investigation was supported by grants from the National Science Council and Department of Health, Taiwan.

References

ANGEL, P., HATTORI, K., SMEAL, T. & KARIN, M. (1988). The *jun* protooncogene is positively autoregulated by its product, Jun/AP-1. *Cell,* **55,** 875–885.

BISHOP, J.M. (1983). Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.,* **52,** 301–354.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

DAVIS, R.J. (1994). MAPKs: new JNK expands the group. *Trends Biochem. Sci.,* **19,** 470–473.

DERIJARD, B., HIBI, M., WU, I.-H., BARRETT, T., SU, B., DENG, T., KARIN, M. & DAVIS, R.J. (1994). JNK-1: a protein-kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell,* **76,** 1025–1037.

GRAHAM, H.N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.,* **21,** 334–350.

HANNUN, Y.A., LOOMIS, C.R. & ROBERT, M.B. (1985). Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.,* **260,** 10039–10043.

HIRSCH, D.D. & STORK, P.J.S., (1997). Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways *in vivo*. *J. Biol. Chem.,* **272,** 4568–4575.

HUANG, H.C., HSIEH, L.M., CHEN, H.W., LIN, Y.S. & CHEN, J.S. (1994a). Effects of baicalein and esculetin on transduction signals and growth factors expression in T-lymphoid leukemia cells. *Eur. J. Pharmacol. Molec. Pharmacol.* **268,** 73–78.

HUANG, H.C., HUANG, Y.L., CHANG, J.H., CHEN, C.C. & LEE, Y.T. (1992a). Possible mechanism of immunosuppressive effect of scoparone (6,7-dimethoxycoumarin). *Eur. J. Pharmacol.,* **217,** 143–148.

HUANG, H.C., JAN, T.R. & YEH, S.F. (1992b). Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. *Eur. J. Pharmacol.,* **221,** 381–384.

HUANG, H.C., LAI, M.W., WANG, H.R., CHUNG, Y.L., HSIEH, L.M. & CHEN, C.C. (1993). Antiproliferative effect of esculetin on vascular smooth muscle cell: Possible roles of signal transduction pathways. *Eur. J. Pharmacol.,* **237,** 39–44.

HUANG, H.C., WANG, H.R. & HSIEH, L.M. (1994b). Antiproliferative effect of baicalein, a flavonoid from Chinese herb, on vascular smooth muscle cells. *Eur. J. Pharmacol.,* **251,** 91–93.

IKEDA, I., IMASATO, Y., SASAKI, E., NAKAYAMA, M., NAGAO, H., TAKEO, T., YAYABE, F. & SUGANO, M. (1992). Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochim. Biophys. Acta.,* **1127,** 141–146.

KALLUNKI, T., SU, B., TSIGELNY, I., SLUSS, H.K., DERIJARD, B., MOORE, G., DAVIS, R. & KARIN, M. (1994). JNK2 contains a specificity determining region responsible for efficient c-Jun binding and phosphorylation. *Gene. Develop.,* **8,** 2996–3007.

KNIGHT, R.J. & BUXTON, D.B. (1996). Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. *Biochem. Biophys. Res. Commun.,* **218,** 83–88.

KOMORI, A., YATSUNAMI, J., OKABE, S., ABE, S., HARA, K., SUGANUMA, M., KIM, S.J. & FUJIKI, H. (1993). Anticarcinogenic activity of green tea polyphenols. *Jpn. J. Clin. Oncol.,* **23,** 186–190.

KYRIAKIS, J.M., BANERJEE, P., NIKOLAKAKI, E., DAI, T., RUBIE, E.A., AHMAD, M.F., AVRUCH, J. & WOODGETT, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature,* **369,** 156–160.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

LUNDERGAN, C.F., FOEGH, M.L. & RAMWELL, P.W. (1991). Peptide inhibition of myointimal proliferation by angiopeptin, a somatostain analogue. *J. Am. Coll. Cardiol.,* **17,** 132B–136B.

MANGIAPANE, H., THOMSON, J., SALTER, A., BROWN, S., BELL, G.D. & WHITE, D.A. (1992). The inhibition of the oxidation of low density lipoprotein by (+)-catechin, a naturally occurring flavonoid. *Biochem. Pharmacol.,* **43,** 445–450.

MATSUDA, H., CHISAKA, T., KUBOMURA, Y., YAMAHARA, J., SAWADA, T., FUJIMURA, H. & KIMURA, H. (1986). Effects of crude drugs on experimental hypercholesterolemia. I. Tea and its active principles. *J. Ethnopharmacol.,* **17,** 213–224.

MIANO, J.M., TOTA, R.R., VLASIC, N., DANISHESKY, K.J. & STEMERMAN, M.B. (1990). Early protooncogene expression in rat aortic smooth muscle cells following endothelial removal. *Am. J. Pathol.,* **137,** 761–765.

PARDEE, A.B. (1989). G_1 events and regulation of cell proliferation. *Science,* **246,** 603–608.

POWIS, G. & KOZIKOWSKI, A. (1991). Growth factor and oncogene signaling pathways as targets for rational anticancer drug development. *Clin. Biochem.,* **24,** 385–397.

REED, J.C., ALPEVS, J.D., NOWELL, P.C. & HOOVER, R.G. (1986). Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **83,** 3982–3986.

RICE-EVANS, C. (1995). Plant polyphenols: free radical scavengers or chain-breaking antioxidants? *Biochem. Soc. Symp.,* **61,** 103–116.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801–809.

SAMBROOK, J., FRITSCH, E.F., & MANIATIS, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Habor, NY; Cold Spring Habor Laboratory.

STONER, G.D. & MUKHTAR, H. (1995). Polyphenols as cancer chemopreventive agents. *J. Cell. Biochem., Suppl.* **22,** 169–180.

THELLE, D.S. (1995). Coffee, tea and coronary heart disease. *Curr. Opin. Lipidol.,* **6,** 25–27.

WANG, Z.Y., DAS, M., BICKERS, D.R. & MUKHTAR, H. (1988). Interaction of epicatechins derived from green tea with rat hepatic cytochrome P-450. *Drug Metab. Dispos.,* **16,** 98–103.

WANG, A.M., DOYLE, M.V. & MARK, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 9717–9721.

WASKIEWICZ, A.J. & COOPER J.A. (1995). MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.,* **7,** 798–805.

WATABE, S., SENDO, F. KIMURA, S. & ARAI, S. (1984). Activation of cytotoxic polymorphonuclear leukocytes by in vivo administration of a streptococcal preparation, OK432. *J. Natl. Cancer Inst.,* **72,** 1365–1370.

WHITMARSH A.J. & DAVIS R.J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.,* **74,** 589–607.

XIA, Z., DICKENS, M., RAINGEAUD, J., DAVIS, R.J. & GREENBERG, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science,* **270,** 1326–1331.

YANG, C.S. & WANG, Z.Y. (1993). Tea and cancer. *J. Natl. Cancer Inst.,* **85,** 1038–1049.

YEH, E.T.H. (1997). Life and death in the cardiovascular system. *Circulation,* **95,** 782–786.

(Received October 21, 1997
Revised March 5, 1998
Accepted March 30, 1998)