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A salvianolic acid B-rich fraction of *Salvia miltiorrhiza* induces neointimal cell apoptosis in rabbit angioplasty model

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Summary. Apoptosis has been suggested to participate in stabilizing cell number in restenosis. *Salvia miltiorrhiza* (SM) Bunge which is a Chinese herb widely used for the treatment of cardiovascular disorders contains a potent antioxidant, Salvianolic acid B. To determine whether the antioxidant affects vascular apoptosis, the present study examined the frequency of apoptotic cell death in atherosclerotic plaques and in restenotic lesions of cholesterol-fed rabbits. New Zealand White rabbits were treated with a normal diet (normal), a 2% cholesterol diet (HC), a 2% cholesterol diet and endothelial denudation (HC-ED), a 2% cholesterol diet with 5% water-soluble extract of SM (4.8 g/Kg B.W./day) and endothelial denudation (HC-ED-SM), or with a 2% cholesterol diet containing probucol (0.6 g/kg B.W./day) and endothelial denudation (HC-ED-probucol). Apoptosis and associated cell types were examined in serial paraffin sections by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and immunohistochemistry. The expression of p53, an apoptosis-related protein, was also examined. Apoptosis was mainly detected in the neointima of the three groups with endothelial denudation. The percentage of apoptotic cells in SM-treated group (68.5±5.9%) was significantly higher than that of normal (0%), HC (1.9±1.2%), HC-ED (46.1±5.4%), and probucol-treated (32.8±3.9%) groups. The SM treatment markedly reduced the thickness of the neointima which was mainly composed of smooth muscle cells with few macrophages. In accordance with the apoptotic cell counts, positive immunoreactivity for p53 was observed in restenotic lesions from HC-ED, SM-treated and probucol-treated groups but not in the intima of the other two groups. These results suggest that the treatment with salvianolic acid B-rich fraction of SM induces apoptosis in neointima which in turn may help prevent the neointimal thickening.

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Introduction

Atherosclerosis and restenosis are characterized by excessive accumulation and proliferation of the intimal tissue alternating with tissue death. A great deal of progress has been made in clarifying the causes and effects of intimal proliferation in atherosclerosis and restenosis (Andres, 1998; Newby and Zaltsman, 1999), but specific information characterizing the events leading to cell death in the lesions remains incomplete. Apoptosis, a programmed cell death, is involved in the pathogenesis and progression of a variety of diseases including cancer, acquired immune deficiency syndrome, heart disease, and neurological disorders (Badley et al., 1997; Davies, 1997; Rudin and Thompson, 1997; Wyllie et al., 1999). Apoptosis is considered a physiologic event that regulates cell number and eliminates damaged cells. Recent reports provide information on apoptosis *in vitro* for several component cell types of the vascular wall. Apoptosis has been found to be rare in cultures of human arterial smooth muscle cells, human fibroblasts, and a human macrophage cell line but could be initiated in a large proportion of the cells if they were exposed to strongly oxidized low density lipoprotein (oxLDL) (Bjorkerud and Bjorkerud, 1996). Apoptosis has also been reported for rat smooth muscle cells (Leszczynski et al., 1994; Bennett and Boyle, 1998) and it can be promoted by oxLDL in a murine macrophage-like cell line (Reid et al., 1993). Recent studies have provided evidence for the occurrence of apoptosis in atherosclerotic and restenotic lesions (Geng and Libby, 1995; Isner et al., 1995; Björkerud and Björkerud, 1996; Kockx et al., 1998a) and in restenotic lesions of humans,

rats with arterial wall injury (Han et al., 1995; Holm et al., 2000), in cholesterol-fed rabbits (Kockx et al., 1996), and in hyperlipidemic knockout mice (Harada et al., 1997). These observations encouraged us to examine the occurrence of apoptosis in a well established cholesterol-fed rabbit model with or without vascular injury.

Restenosis appears to be the result of two processes: an accelerated formation of atherosclerosis induced by arterial injury and wound-healing response to severe intimal and medial damage (Libby et al., 1992; Faxon et al., 1997). Antioxidants may attenuate the process of restenosis after angioplasty through their ability to both reduce the progression of atherosclerotic plaque formation and to favorably influence wound healing. *Salvia miltiorrhiza* (SM) Bunge (Labitata), a traditional Chinese herb often used in the treatment of cardiovascular disorder (called blood stasis in traditional Chinese medicine), is a popular folk medicine used in China, Japan, and Taiwan (Lei and Chiou, 1986). Salvianolic acid B (Sal B), a water-soluble polyphenolic antioxidant isolated from the roots of this plant, has been found to scavenge 1,1-diphenyl-2-picryhydrazyl radicals and to inhibit LDL oxidation more effectively than probucol (Wu et al., 1998). Probucol is a well-known antioxidant which has been shown to inhibit atherosclerosis (Daugherty et al., 1991; Sasahara et al., 1994) and postangioplasty restenosis (Ferns et al., 1992; Schneider et al., 1993). Subsequent studies have shown that a Sal B-rich fraction of SM inhibits LDL oxidation and reduces the severity of atherosclerosis in hypercholesterolemic rabbits (Wu et al., 1998). In addition, SM reduced the neointimal thickness of abdominal aortas after balloon injury in cholesterol-fed rabbits (unpublished data). In this context, apoptosis-inducing ability could prove to be a key factor for the efficacy of the antioxidant treatment.

The purpose of the current study was to asses the occurrence rate of apoptosis and to characterize the cell types undergoing apoptosis in the normal, cholesterol-fed rabbits, and cholesterol-fed rabbits with arterial wall injury. Furthermore, the effects of two antioxidants-a Sal B-rich fraction of SM and probucol therapy on putative apoptosis in cholesterol-fed rabbits with arterial wall injury were examined. Our results showed that neointimal hyperplasia induced by endothelial denudation is accompanied by a marked increase in apoptotic death. SM treatment further induces neointimal apoptosis which correlates with a pronounced reduction in neointimal thickening.

Materials and methods

Animal preparation

A total of 30 New Zealand white rabbits (2.5 to 3.0 Kg) were used for this study. The experimental procedures, animal care and handling were carried out according to the "Guide for the care and use of the laboratory animals" published by the US National Institute of Health. After one week on a commercial rabbit chow diet (Purina Mills, USA), 24 rabbits were fed a 2% cholesteol diet (Purina Mills, USA) and randomly allocated to one of four groups: (1) a cholesterol-fed (HC) group, (2) a cholesterol-fed endothelium-denuded (HC-ED) group, (3) a cholesterol-fed, endothelium-denuded and SM-treated (HC-ED-SM) group: plus the oral ingestion of SM (4.8 g/Kg B.W./day), and (4) a cholesterol-fed, endothelium-denuded and probucol-treated (HC-ED-probucol) group: plus the oral ingestion of probucol (0.6 g/Kg B.W./day). The selection of SM dose was based on its antioxidant activity comparable to that of probucol in Cu^{2+}-induced LDL oxidation (Wu et al., 1998). Water was allowed *ad libitum*. Animals were bled periodically for the assessment of plasma cholesterol, liver function, and renal function. Three weeks after starting the above diets in the second, third, and the fourth groups, animals were anesthetized with intramuscular xylazine (5 mg/kg) and ketamine hydrochloride (35 mg/kg). The surgery was performed under sterile conditions. The right femoral artery was exposed through an incision line 1.5-2.0 cm below the inguinal ligament and an arteriotomy was performed. A 3 Fr. Fogarty arterial embolectomy catheter (Baxter Healthcare, Buckinghamshire, UK) was introduced retrogradely into the lower abdominal aorta for 16 cm, measured from the tip of catheter. Denudation was then performed by inflating the balloon with normal saline and slowly pulling it back with the feeling of resistance. This procedure was repeated three times. The surgical wound was closed and the animals were continued on a high cholesterol diet with different antioxidant treatments.

The remaining 6 rabbits with a control chow (a normal diet) for six weeks were used as the normal control group. At the end of the 6^{th} week of the experiment, the rabbits were euthanized with the intravenous injection of 35-40 mg/kg sodium pentobarbital and were sacrificed. Abdominal aortas were removed, gently dissected free of adhering tissues, and then rinsed with ice-cold phosphate buffer saline (PBS). A segment of each aorta was immersion-fixed with 4% paraformaldehyde for light microscopy and transmission electron microscopy; the remaining larger portion was immediately frozen in liquid nitrogen for DNA extraction.

DNA gel electrophoresis

Abdominal aortas were homogenized in liquid nitrogen and lysed in genomic DNA isolation reagent (GibcoBRL, Life Technologies). Then total DNA was precipitated by absolute ethanol. After centrifugation, the DNA pellets were dissolved in 8 mM NaOH solution, and electrophoresed in 2% agarose pre-impregnated with ethidium bromide. Gel was visualized and photographed under transmitted UV light with a Polaroid camera.

Transmission electron microscopy

Examination of transmission electron micrographs from the five groups confirmed and extended the observations made by light microscopy. Following fixation, each arterial segment was washed thoroughly in 0.1M phosphate buffer and postfixed in 0.1% buffered OsO_4 for 2 hours at 4 °C. The samples were dehydrated through a graded ethanol series followed by propylene oxide. Each sample was embedded in Epon 812. Ultrathin sections were placed on formvar grids, stained with uranyl acetate and lead citrate and viewed with JEOL JEM-2000EXII transmission electron microscope.

DNA nick-end labeling of tissue sections

Arterial segments were fixed in 4% buffered formaldehyde for 3 h at 4 °C, subsequently dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Transverse sections (4-5 μm thick) of paraffin-embedded tissues were mounted on slides coated with poly-L-lysine (Sigma). To examine the cellular localization of apoptosis, DNA *in situ* end labeling was performed in combination with immunocytochemistry in three serial sections. The first tissue section was used for TUNEL method. The second and the last sections were used to identify smooth muscle cells and macrophages, respectively. Apoptosis was detected with *in situ* labeling by terminal deoxynucleotidyl tranferase (TdT) of biotin-dUTP to 3'-OH DNA ends generated by the internucleosomal degradation of DNA (denoted TUNEL technique). The TUNEL method used the *in situ* Cell Death Detection Kit (TdT-FragEL™, DNA fragmentation detection kit, CALBIOCHEM, MA). Briefly, tissue sections were predigested with 20 μg/ml proteinase K in l0 mM Tris-HCl buffer (pH 8.0) for 20 minutes at room temperature (Geng and Libby, 1995; Han et al., 1995). Endogenous peroxidase was inactivated by incubation with 3% H_2O_2 in methanol for 5 min at room temperature. The samples were incubated with terminal deoxynucleotidyl transferase (TdT) and biotin labeled dUTP containing nucleotide mixture (TUNEL reaction mixture) in a humid atmosphere at 37 °C for 90 minutes. After rinsing in TBS, bound biotin was detected using streptavidin-HRP. Later slides were incubated with DAB and H_2O_2 solution for 10 min to yield a brown colored precipitate. The slides were mounted with glass coverslips and viewed by light microscopy. A negative control was included in each experiment by omitting the terminal deoxynucleotidyl transferase from the TUNEL reaction mixture.

Quantification

Quantitative measurements of the percentage of apoptotic cells in each group were made using the Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, MD, USA). Eight separate regions from each cross section were quantitatively analyzed. The percentage of apoptotic cells was calculated for each area (length: 0.3 mm; width: variable), and the overall percentage of apoptotic cells for each section was expressed as a mean of these eight values.

Immunohistochemistry

To identify the cell type undergoing apoptosis, two of the serial sections were examined by immunohistochemistry for macrophages and smooth muscle cells, respectively. The tissue sections were deparaffinised, rehydrated, washed with PBS, and treated with 5 mg/ml serum albumin in PBS for 1 hour to block non-specific binding. The second section was incubated with mouse anti-α-smooth muscle actin (1:400 dilution, 1A4, Sigma) and the last one was incubated with mouse anti-rabbit macrophage (1:50 dilution, Ram 11, Dako Corp.) to detect smooth muscle cells and macrophages, respectively. These two sections were then incubated with FITC-conjugated goat anti-mouse secondary antibody (1:400 dilution, Sigma) and observed by fluorescence microscopy.

For tissue sections undertaken for p53 immunostaining, the slides were boiled in 0.01M citrate buffer, pH 6.0, for 17 minutes at 100 °C to unmask the p53 antigen, followed by cooling at room temperature for 15 minutes. Sections were then incubated for 1 hour at room temperature with mouse anti-human p53 antibody (1:100 dilution, NeoMarkers). Bound primary antibody was detected with peroxidase-conjugated goat anti-mouse secondary antibody (1:100 dilution, Transduction). Antigen-antibody complexes were localized by employing 0.5 mg/ml 3,3'-dioaminobenzidine/0.01% hydrogen peroxide in 0.1M Tris-HCl buffer, pH 7.2 as a chromogen (Vector Lab, USA). Negative control was performed by omitting the incubation of the primary antibodies in the tissue sections.

Statistical analysis

Data are expressed as mean±SEM. All the data were stored in the computer and analyzed by Statistics Package for the Social Sciences (SPSS) for Windows. The statistical comparison was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni test. A value of $P<0.05$ was considered significantly different.

Results

During the experimental period, weight gain and final weight did not differ significantly among 5 study groups. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine also exhibited no significant difference among all groups. Plasma cholesterol and triglyceride levels showed rapid increase after feeding with 2% high-cholesterol diet in HC and HC-ED groups.

Neither probucol nor SM significantly prevented the elevation of plasma triglyceride; however, both SM and probucol treatment attenuated the increase in plasma cholesterol.

Evidence of apoptosis by gel electrophoresis

After experimental periods, the tissues from each group were collected, cellular DNA was extracted, and agarose gel electrophoresis was performed to analyze DNA fragmentation. The results are demonstrated in Fig. 1. In the normal and cholesterol-fed groups, there was no evidence of DNA fragmentation. On the other hand, the HC-ED group, the HC-ED-SM group, and the HC-ED-probucol groups on apoptosis were confirmed by DNA ladder formation.

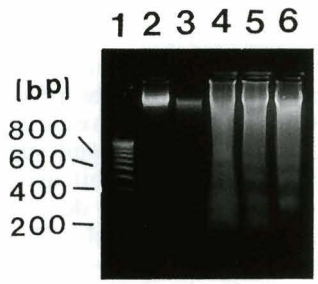


Fig. 1. Agarose gel electrophoresis analyses for DNA ladder formation. Fifteen micrograms of DNA extracted from aortas of each group was applied to each lane. Lane 1: DNA marker; lane 2: the normal group; lane 3: the HC group; lane 4: the HC-ED group; lane 5: the HC-ED-SM group; lane 6: the HC-ED-probucol group. DNA from the normal and the HC groups show no apparent DNA laddering. DNA extracted from the HC-ED group, the HC-ED-SM group and the HC-ED-probucol group indicate the DNA ladder (n=3, representative experiment).

Evidence of apoptosis by transmission electron microscopy

We also examined the ultrastructures of these tissues by transmission electron microscopy. For the normal group and the HC group, cells in the intima did not show the characteristics of apoptotic cells (data are not shown). In the arterial sections of the HC-ED, HC-ED-SM, and HC-ED-probucol groups, many neointimal cells were apoptotic, exhibiting condensation of chromatin, which are ultrastructural characteristics of apoptosis (Fig. 2A). A few neointimal cells, however, did not show the characteristics of apoptosis (Fig. 2B).

Evidence of apoptosis by TUNEL

In the normal group the intima was very thin (Fig. 3) and apoptotic cell death was not detected (Fig. 3A). In general, smooth muscle cells were found only in the media (Fig. 3B), whereas macrophages were not detected in the vascular wall immunohistochemically

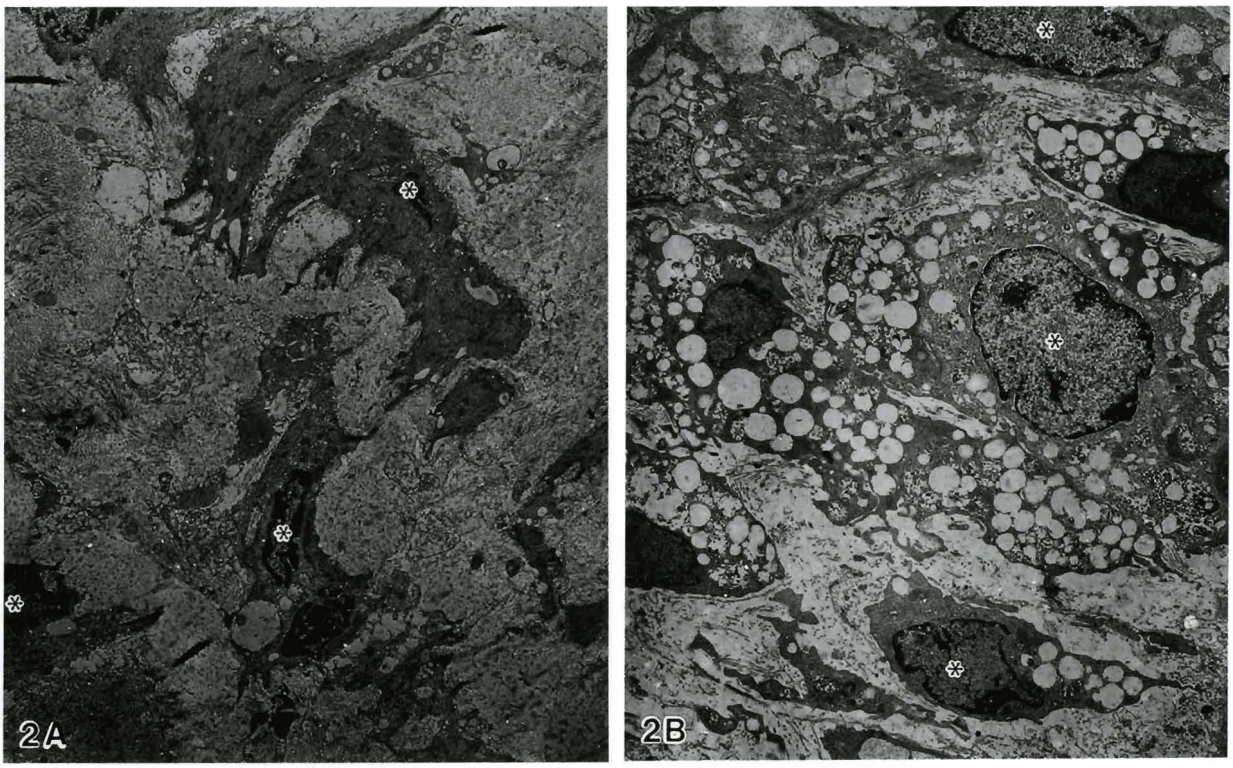
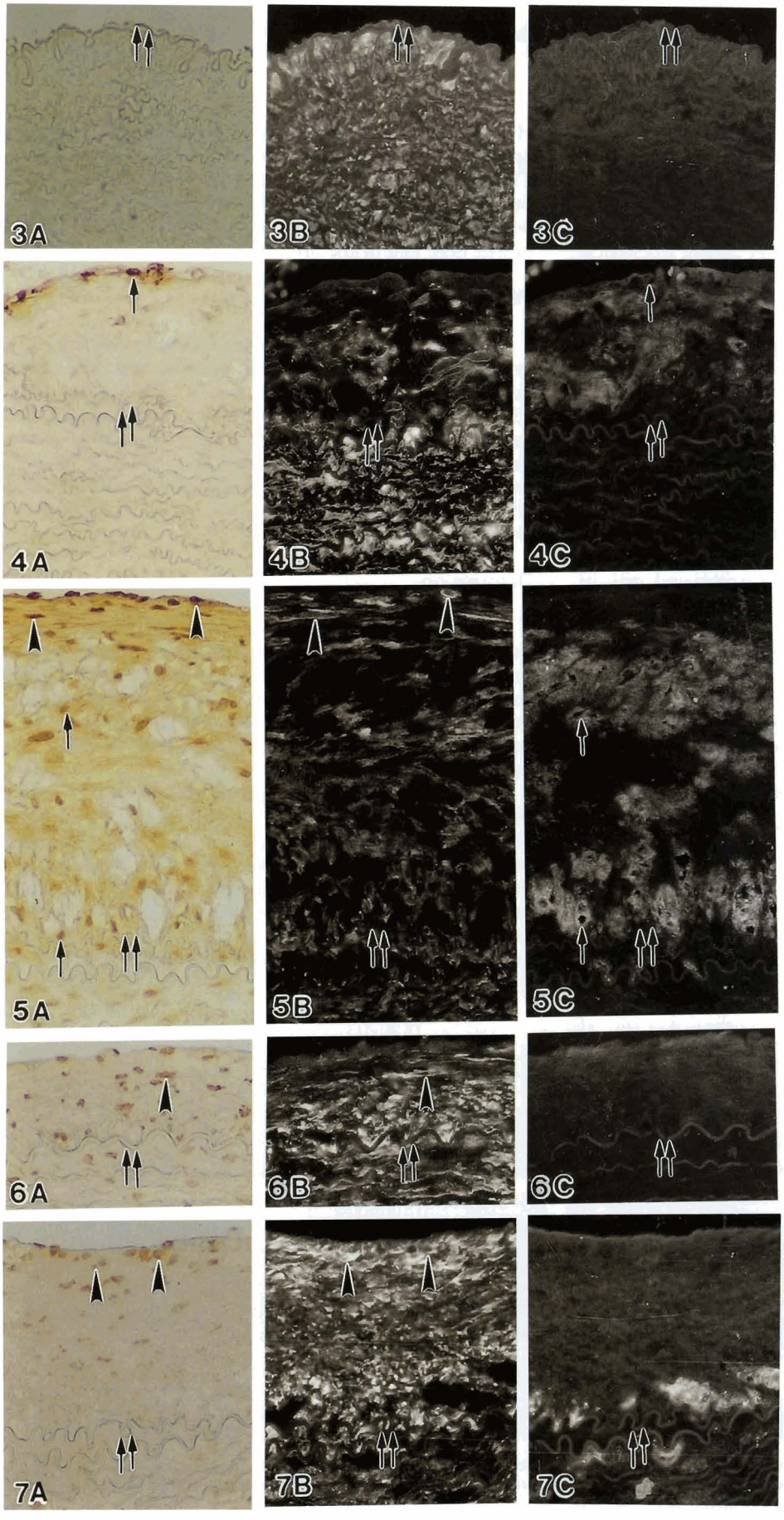


Fig. 2. Transmission electron micrographs of apoptotic **(A)** and nonapoptotic **(B)** cells in the neointima from the HC-ED-SM group. **A.** The nucleus (*) demonstrates a condensation of the chromatin. **B.** The nucleus (*) shows a normal structure. A, x 5,000; B, x 4,000



Figs. 3-7. Detection of apoptosis in serial sections of aorta from the normal **(Fig. 3)**, the HC **(Fig. 4)**, the HC-ED **(Fig. 5)**, the HC-ED-SM **(Fig. 6)**, and the HC-ED-probucol **(Fig. 7)** groups. The lumen is uppermost in all sections. The internal elastic membrane is indicated by double arrows. Single arrow and arrowhead indicate TUNEL-positive overlapping with smooth muscle cell or macrophage-specific staining. **A.** TUNEL for apoptosis. **B.** Staining for smooth muscle-specific α-actin. **C.** Staining for macrophage-specific antibody. x 260

(Fig. 3C). The HC group showed fatty streaks in the vascular wall (Fig. 4). Apoptotic cell death was rarely observed and was detected only in the superficial region (Fig. 4A). A few smooth muscle cells were present in the thickened intima in addition to the media (Fig. 4B). Numerous macrophages were present in the thickened intima (Fig. 4C). The HC-ED group showed markedly thickened intima (Fig. 5). Many apoptotic cells were found in the thickened intima (Fig. 5A) and were associated with both smooth muscle cells (Fig. 5B) and macrophages (Fig. 5C). The SM-treated group exhibited greatly reduced thickening of the neointima compared to the HC-ED group (Fig. 6). Many apoptotic cells were detected in the thickened intima (Fig. 6A) and were associated mainly with smooth muscle cells (Fig. 6B). Macrophages were not found in the neointima (Fig. 6C). The probucol-treated group also showed decreased thickening of the neointima compared to the HC-ED group (Fig. 7). Apoptotic cells were abundant in the thickened intima (Fig. 7A) and were associated mainly with smooth muscle cells (Fig. 7B). Few macrophages were present (Fig. 7C).

The occurrence rate of apoptotic cells in the five groups was systemically scanned under the light microscope. Apoptotic cells scanned in each aortic specimen were estimated by TUNEL-positive brown cells before hematoxylin staining, as shown in Figs. 8A (the HC group) and 8C (the HC-ED group). In the same area, the total cells scanned were counted after hematoxylin staining, as indicated in Figs. 8B (the HC group) and 8D (the HC-ED group). Fig. 9 shows the percentages of apoptotic cells in the intima of thirty rabbits from five groups. In the normal group, apoptotic cells were not detected. A small fraction (1.9±1.2%) of apoptotic cells was found in the HC group. TUNEL-positive cells accounts for 46% of the total cells in the HC-ED group. A high percentage (68.5±5.9%) of apoptotic cells was observed in the SM-treated group. The percentage of apoptotic cells is 32.8±3.9% in the probucol-treated group. The frequency of TUNEL-positive cells in the SM-treated group was significantly higher than that of the other groups.

P53 expression in the arterial wall

The expression of p53 in the intimal areas from five groups was examined by immunohistochemistry. In the normal group (Fig. 10A) and the HC group (Fig. 10B), p53 was not detected in the vascular wall. In the HC-ED group a highly pronounced expression of p53 was detected in the neointimal regions (Fig. 10C). In the SM-treated group (Fig. 10D) and the probucol-treated group (Fig. 10E) marked p53 expression was also observed in the neointima.

Discussion

By comparing DNA laddering, TUNEL reaction, immunohistochemical staining and transmission electron microscopy, this study provides evidence for a higher frequency of apoptosis on aortic walls in cholesterol-fed, endothelium-denuded rabbits. SM treatment further induces apoptosis which correlates with reduction in neointimal thickening.

In the present study apoptotic cell death was absent in the vascular wall of the normal group. In the fatty streaks of the HC group, apoptotic cell death was found primarily in the superficial layer with macrophage infiltration. The overall number of TUNEL-positive nuclei in atherosclerotic lesions was very low. The low occurrence of apoptosis in atherosclerotic lesions confirms the results of previous studies. Isner et al.

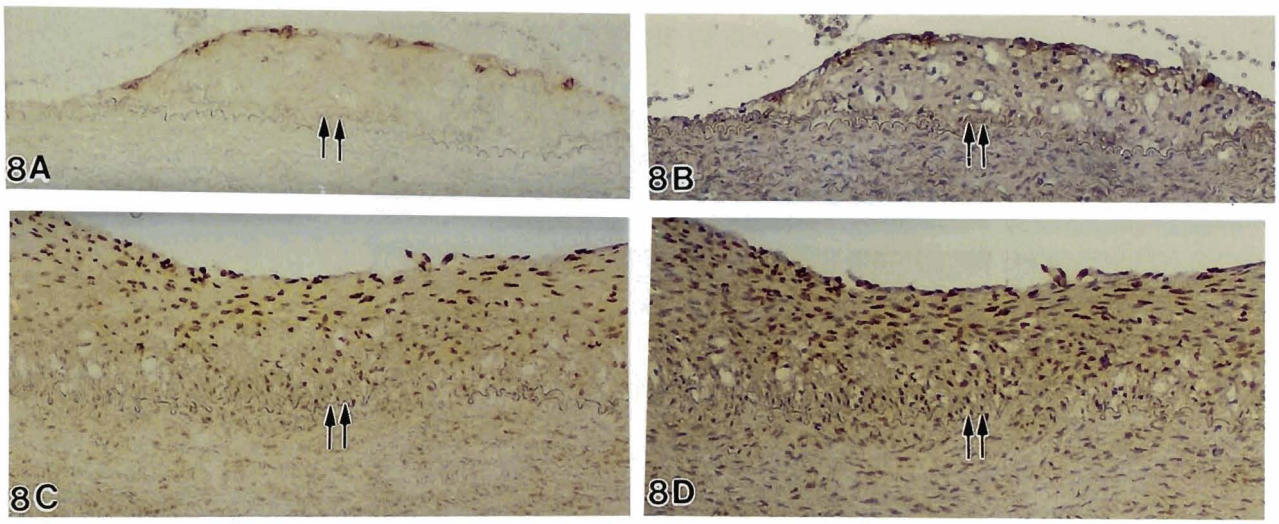


Fig. 8. Detection of apoptotic frequency in aortic sections. The lumen is uppermost in all sections. The internal elastic membrane is indicated by double arrows. **A.** TUNEL-positive cells in the HC group. **B.** An aortic section stained with both TUNEL and hematoxylin in the HC group. **C.** TUNEL-positive cells in the HC-ED group. **D.** An aortic section stained with both TUNEL and hematoxylin in the HC-ED group. x 130

(1995) reported that apoptotic cells represented <2% of cells in each atherectomized specimens. Kockx et al. (1996, 1998a) also reported that apoptotic cells accounted for approximately 1% of cells in atherosclerotic plaques of cholesterol-fed rabbits. Harada et al. (1997) reported that frequencies of apoptotic cells were very low in atherosclerotic plaques of apo-E and LDL receptor-deficient mice.

A better correlation is found between apoptosis and intimal hyperplasia of cholesterol-fed endothelium-denuded rabbits in the present study. This position further supported by the results of previous studies of human restenotic lesions obtained at necropsy or by directional atherectomy (Isner et al., 1995). TUNEL disclosed marked evidence of apoptosis at 1 day after balloon injury in the carotid artery of rats (Holm et al., 2000). In the aorta of normocholesterolemic rat apoptosis has also been described in the intimal thickening following endothelial denudation (Han et al., 1995). The number of apoptotic cells increased up to 40% by 9 days after injury, declined to 20% by 14 days and remained above basal level (10%) by 28 days after the initial injury. Geng and Libby (1995) reported that nearly 50% of the cells in advanced human atheroma were apoptotic, and these cells were located in intimal lesions near or in the lipid-rich core. In the present study approximately 46% of the cells in the neointima were undergoing apoptosis. The percentage of apoptosis remained high by 3 weeks after endothelial denudation. It may be triggered by both of cholesterol-feeding and endothelium-denudation. Kockx et al. (1998b) reported that TUNEL is not specific for apoptosis but also labelled DNA splicing and necrotic cells. In the present study, TUNEL method is combined with p53 immunohistochemistry and electron microscopy to observe apoptotic cells. Therefore data from percentages of apoptosis in each group are reliable.

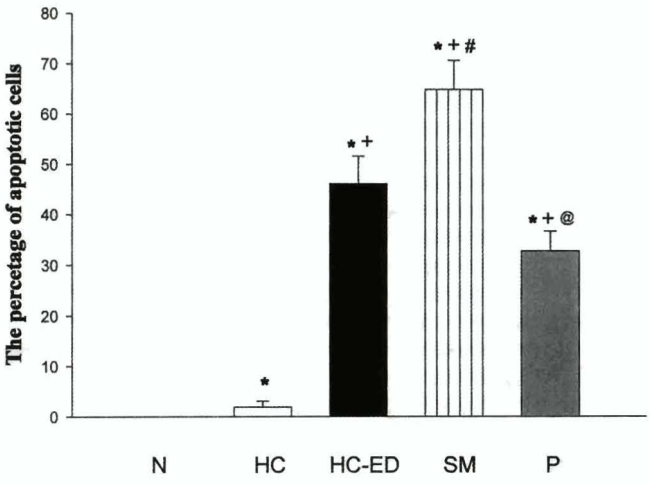


Fig. 9. Quantification of apoptotic rate in different groups of rabbit abdominal aortic specimens. *: denotes p<0.05, for data compared with the normal (N) group; +: denotes p<0.05, for data compared with the HC group; #: denotes p<0.05, for data compared with the HC-ED group; @: denotes p<0.05, for data compared with the SM-treated (SM) group.

We chose to test the effects of SM since it is routinely used as a blood-quickening stasis-dispelling agent in traditionally Chinese medicine. Its non-polar extracts contain tanshinones, which can inhibit platelet aggregation (Onitsuka et al., 1983) and protect myocardium against ischemia-induced derangement (Yagi et al., 1989). The aqueous extract of SM contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage (Liu et al., 1992). Among SM ingredients, Sal B is a potent hepatoprotective agent and water-soluble antioxidant. A Sal-B rich fraction of SM has been shown to scavenge DPPH radicals and inhibit Cu^{2+}-induced LDL oxidation (Wu et al., 1998). Its feeding reduced endothelial damage and the severity of atherosclerosis in cholesterol-fed rabbits (Wu et al., 1998). LDLs from SM-treated animals contained more Vitamin E and were more resistant to oxidation ex vivo (Wu et al., 1998). In the present study the percentage of apoptosis in the SM-treated group was significantly higher than that of the HC-ED group, suggesting that the reduction of intimal hyperplasia may result from apoptosis induced by Sal B-rich fraction of SM.

Apoptosis-promoting activity of Sal B-rich fraction of SM observed in the present study is shared by other antioxidants. Curcumin, a remarkable antioxidant and

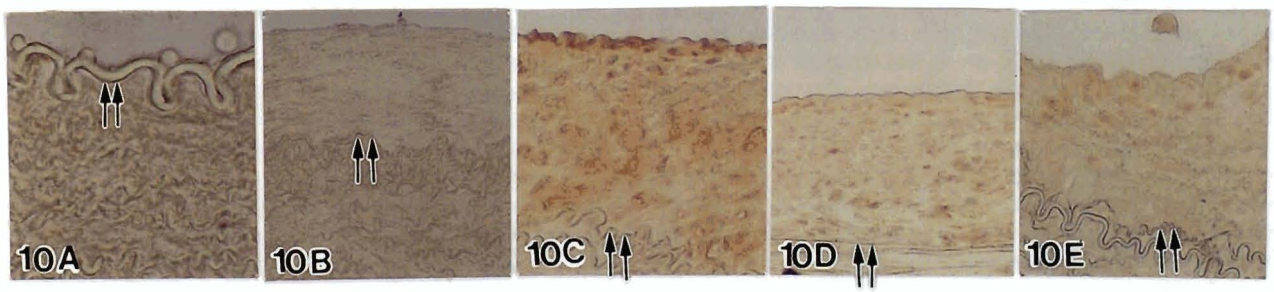


Fig. 10. Immunostaining of p53 in arterial sections. The lumen is uppermost in all sections. The internal elastic membrane is indicated by double arrows. Arterial sections of the normal **(A)**, HC **(B)**, HC-ED **(C)**, HC-ED-SM **(D)**, and HC-ED- probucol **(E)** groups were reacted with p53-specific antibody as described in Methods. **A.** The normal group: no p53 immunoreactivity was detected. **B.** The HC group: no staining was detected. **C.** The HC-ED group: intense brown stain was detected in neointima. **D.** The HC-ED-SM group: brown stain was frequent in nuclei of the neointima. **E.** The HC-ED-probucol group: brown stain was frequent in nuclei of the neointima. x 260

chemopreventive agent, has recently been found to be capable of inducing apoptosis in human hepatoma, leukemia, and basal cell carcinomal cells (Jee et al., 1998). Apoptosis in tumor growth regulates cell number and eliminate damaged cells. Tsai et al. (1996) has shown two antioxidants, pyrrolidinedithiocarbamate and N-acetylcysteine, induced apoptosis in rat and human smooth muscle cells. These results indicate that antioxidants induce apoptosis in vascular cells and hence retard the intimal hyperplasia in cholesterol-fed endothelium-denuded rabbits in a manner similar to curcumin. Interestingly, some antioxidants were recently reported to inhibit apoptosis. Pretreatment of smooth muscle cells with Vitamin C attenuated the cytotoxicity and apoptosis induced by moderately oxidized LDL (Siow et al., 1999). Antioxidants, N-acetylcystine and pyrroline dithiocarbamate, markedly attenuated the induction of apoptosis in response to rabbit common carotid artery with balloon distension injury (Pollman et al., 1999). In the present study probucol which is a lipophilic antioxidant did not induce apoptosis in cholesterol-fed endothelium-denuded rabbits. Probucol has been reported to reduce intimal hyperplasia in nonhuman primates and rabbits (Ferns et al., 1992; Schneider et al., 1993). Probucol attenuated oxLDL-induced apoptosis in cultured human coronary smooth muscle cells (Bachem et al., 1999). Based on these findings, SM and probucol are likely to reduce intimal hyperplasia through different mechanisms. Further investigation is required to clarify the induction mechanism of apoptosis by SM.

The TUNEL-positive cells in atherosclerotic lesions appeared to differ in both location and composition in different experimental groups. In the HC group without endothelial denudation, apoptotic cells are mainly present in the superficial layer, which contains numerous macrophage-originated foam cells. In the HC-ED group, the TUNEL-positive cells included smooth muscle cells and macrophages of the neointima. These findings are in contrast to those of Pollman et al. (1999), who reported that neointimal smooth muscle cells are more resistant than their medial counterparts to apoptosis at 30 minutes after balloon injury. The discrepancy between our results and the other study may simply reflect the differences in time points after balloon injury. The most dramatic changes in TUNEL-positive cells were detected in the SM-treated group. In addition to the marked increase in apoptotic cell counts, the neointima shows a strong reduction or even disappearance of the macrophages. Whether macrophages underwent apoptosis at a higher rate and thus disappeared before three weeks following endothelial denudation remains unknown. Another possibility is the potential inhibitory effect of SM on the adherence of circulating monocytes to the endothelium, hence decreasing macrophages in the vascular wall. Further studies examining tissues at earlier time points after the SM treatment should help to unveil the fate of the disappearing macrophages. Nonetheless, the extraordinarily high frequency of apoptotic cells within the neointima in the SM-treated group is likely to account for the marked neointimal regression.

There is an increasing evidence that protein encoded by the tumor-suppressor gene p53 may induce apoptosis (Lowe et al., 1993). In the present study, immunoreactivity for p53 was detected in restenotic lesions, including those with apoptosis. These findings are consistent with the recent observation that immunopositivity for p53 may be identified in atheroectomy specimens retrieved from restenotic but not primary lesions (Speir et al., 1994; Isner et al., 1995). Although the expression of p53 is inducible in the SM-treated or the probucol-treated group in the present study, our data showed that p53 is not highly expressed in the SM-treated group. It is possible that the expression of p53 peaked earlier and has decreased three weeks after endothelial denudation. These results may also indicate that apoptosis is regulated in mammalian cells by multiple prolife and prodeath factors (Bennett and Boyle, 1998; Haunstetter and Izumo, 1998). However, the expression of p53 in the neointimal cells indicates that these cells become susceptible for apoptosis.

In summary, we demonstrated that SM treatment is able to induce the highest frequency of apoptosis in cholesterol-fed balloon-injury rabbits, accompanied by the presence of p53. The decrease in intimal hyperplasia could result from the loss of smooth muscle cells and macrophages through apoptosis. These observations should provide a basis for further understanding of the mechanisms of SM treatment.

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