Regulatory effect of epigallocatechin gallate on expression of Acyl-CoA:cholesterol acyltransferase and caspase-3 genes in an experimental model of atherosclerosis

1. INTRODUCTION

Acyl-CoA:cholesterol acyltransferase (ACAT), a membrane-associated enzyme, is primarily localized in the endoplasmic reticulum. The localization of ACAT in the endoplasmic reticulum is consistent with its demonstrated role in cholesterol transport within the liver and intestinal mucosa. ACAT is primarily responsible for the esterification of cholesterol in all mammalian cells; it has also been implicated in intestinal mucosal absorption of cholesterol, and in the synthesis of the cholesterol esters which are incorporated into VLDL or stored in fatty cells (Krause et al., 1993; 1995; Katsuren et al., 2001). Accumulation of ACAT reaction-products, such as foamy lipid droplets in the cytoplasm of macrophages and SMCs, is a characteristic feature of early lesions of atherosclerotic plaque (Brown and Goldstein, 1983). In atherosclerosis, modified LDLs such as Ox-LDL are taken up into macrophages, which form foam cells via the accumulation of cholesterol esterified by ACAT (Brown et al., 1980; Holvoet and Collen, 1998; Miyazaki et al., 1998). Over-expression of hepatic ACAT also possibly contributes to the pathogenesis of hypercholesterolemia (Nosratola and Liang, 2002).

Apoptosis is a physiologic process that is important in the normal development and homeostasis of multicellular organisms (Raff, 1992). However, derangement of apoptosis can have deleterious consequences as exemplified by several human diseases, including acquired immunodeficiency syndrome, neurodegenerative disorders, cancer and atherosclerosis. Apoptosis is reported to occur in several experimental models (Bennett et al., 1995; Bochanton Piallat et al., 1995; Isner et al., 1995; Rembold, 1996). Apoptosis of vascular cells may also be involved in the progression of atherosclerosis.
Cell death, via apoptosis or necrosis of macrophages, plays a pathogenetic role in the development of atherosclerotic lesions and destabilization of plaques (Libby et al., 1996; Mitchinson et al., 1996; Hegyi et al., 2001).

Caspases, a family of cysteine proteases, play a pivotal role in the process of apoptosis. Apical caspases may be activated by two independent pathways that involve activation of receptor-intermediate caspase-8 and mitochondria-cytochrome-c-intermediate caspase-9. Both caspase-8 and caspase-9 activate the executioner, caspase-3, which, in turn, is responsible for the terminal phase of apoptosis (Nunez et al., 1998). Induction of caspase-3 proteolytic activity appears to be one of the most important events in apoptosis (Harvey et al., 1998; Sakahira et al., 1998). Caspase-3-mediated apoptosis may be modulated by cellular oxidative stress.

The direct involvement of oxidative stress in apoptosis has been demonstrated in a variety of cell types, such as aortic endothelial and smooth muscle cells (Hockenbery et al., 1993). Increased formation of oxygen radicals facilitates oxidation of LDL and influences oxidation-sensitive mechanisms (Napoli et al., 2001). It is likely that the apoptotic effects of Ox-LDL occur due to oxygen radicals interfering with oxidation-sensitive signaling pathways. Indeed, various antioxidant enzymes and scavengers of free radicals inhibit activation of caspases and other apoptotic effects of Ox-LDL, such as generation of ROS (de Nigris et al., 2000; Napoli et al., 2000). Antioxidants of various types, including vitamin E, have been shown to inhibit both apoptotic and necrotic cell death (Coffey et al., 1995; Harada-Shiba et al., 1998; Li et al., 1999; Maziere et al. 2000; Hsieh et al., 2001).

Epidemiological studies have shown that consumption of tea reduces the risk of high cholesterol levels (Maron et al., 2003; Vinson et al., 2004), diabetes mellitus (Vinson and Zhang, 2005), arthritis (Haqqi et al., 1999; Roy et al., 2008), osteoporosis (Hegarty et al., 2000) and dental caries (Otake et al., 1991). Green tea and its bioactive
ingredients, mainly catechins, have shown protective effects against various diseases including CVD (Khan and Mukhtar, 2007; Babu and Liu, 2008). EGCG, the principal constituent of green tea (Giakoustidis et al., 2006), is a well-characterized antioxidant (Xu et al., 2004) and is known to inhibit a vast array of biomedically relevant molecular targets and disease-related cellular processes (Doss et al., 2005; Khan et al., 2006). EGCG has been shown to exhibit anti-cancer, anti-mutagenic, anti-neurodegenerative and anti-colitic effects (Koh et al., 2003; Mochizuki and Hasegawa, 2005). The present chapter focuses on an evaluation of the effect of EGCG on the expression of the ACAT and caspase-3 genes in Wistar rats fed an atherogenic-diet.

2. MATERIALS AND METHODS

2.1. Isolation and purification of EGCG from Camellia sinensis

EGCG was isolated from Camellia sinensis and purified by methods already described in Chapter I.

2.2. Experimental design

In this phase of the study, the experimental design was similar to that described in chapter V. At the end of the experimental period, all the animals were sacrificed by cervical decapitation. From each animal, blood samples were collected, and the hepatic tissue was excised.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA transcripts of ACAT and caspase-3

Total RNA was extracted from hepatic tissue using Trizol (Sigma-Aldrich, St. Louis, USA) reagent (1 ml/100 mg tissue) according to the manufacturer's instructions. The concentration and purity of total RNA were determined by absorbance at 260/280 nm in a UV-spectrophotometer (Sambrook et al., 1989). The purity of RNA obtained was > 1.8.
RT-PCR was performed to quantify the mRNA transcripts of ACAT and caspase-3 against the internal control, GAPDH. The primer sequences for ACAT, caspase-3 and GAPDH are gene-specific; the following primers were used for the generation of cDNAs: ACAT (Son et al., 2003) (amplicon size-370 bp), sense primer: 5'-CCTCCCGGTTTCATTCTGATA-3', anti-sense primer: 5'-ACACCTGAGATGGAGTT-3'; caspase-3 (Tamada et al., 2000) (amplicon size-463 bp), sense primer: 5'-GGACCTGTGGAGCCTGGAATAA-3', anti-sense primer: 5'-TACCCACTCCAGCTATTAC-3'; rat GAPDH (Nakajima et al., 2002) (amplicon size-207 bp), sense primer: 5'-TCAAAGAAGTGCTGTGAAGCCAGG-3', anti-sense primer: 5'-GGATCCACCACCTCGGTGCTGT-3'.

Two micrograms of total RNA were reverse transcribed by Qiagen one-step RT-PCR kit (Qiagen, Germany) according to the manufacturer's instructions and further amplified by PCR. The reverse transcriptase (RT) reaction was performed at 50°C for 30 min followed by initial PCR activation at 95°C for 15 min. The three-step PCR cycle included (i) denaturation at 94°C for 1.5 min, (ii) annealing at 60°C for 1.5 min and (iii) extension at 72°C for 3 min. PCR amplification was carried out through 30 cycles and, to ensure that the products were extended completely, a final extension at 72°C for 10 min was performed.

Ten microliters of each PCR product were analyzed by gel electrophoresis on a 2% agarose gel. The molecular size of the amplified products (ACAT, caspase-3 and GAPDH) was determined by comparison with molecular weight markers (100 bp DNA ladder, Genei, Bangalore, India) run in parallel with the RT-PCR products. Gels were subjected to densitometric scanning and the band intensities of the cDNA fragment of the ACAT and caspase-3 genes were normalized against the band intensity of the cDNA fragment of the reference gene, GAPDH, using Quantity One Software (Bio-Rad, USA).
2.4. **Statistical analysis**

The values are expressed as mean ± SD for nine animals in each group. Differences between groups were assessed by one-way ANOVA using SPSS software package for Windows (Version 11.5; SPSS Inc., Chicago, IL, USA). Post-hoc testing was performed for intergroup comparisons using the least significance difference test. Values of $P<0.001$ have been denoted by distinct symbols in the figures.

3. **RESULTS**

**Transcript levels of ACAT and caspase-3 mRNA**

ACAT and caspase-3 mRNA was analyzed by RT-PCR and the transcript levels were compared between the experimental groups (Fig. 20). In group III (atherogenic diet-fed, EGCG-treated) rats, the levels of the transcripts of both ACAT and caspase-3 genes were found to be significantly ($P<0.001$) lower than those in group II (atherogenic diet-fed, saline-treated) rats. The levels of both ACAT and caspase-3 gene transcripts were found to be significantly ($P<0.001$) higher in group II rats than those in normal rats (group I).

4. **DISCUSSION**

Intracellular cholesterol esterification, catalyzed by the microsomal enzyme ACAT, is believed to play an important role in development of atherosclerosis (Suckling and Stange, 1985). ACAT catalyzes the synthesis of cholesterol esters that accumulate in macrophages, thereby promoting macrophage foam-cell formation in the arterial wall, a hallmark of early atherosclerotic lesions (Brown and Goldstein, 1983). Furthermore, ACAT synthesizes cholesterol esters that are incorporated into lipoproteins and secreted from the intestine and liver, and thus ACAT activity may modulate plasma cholesterol levels, an important risk factor for atherosclerosis (Chang *et al.*, 1994; 1997). It has been postulated that the inhibition of ACAT is a rational and efficient approach to the design of novel hypocholesterolemic agents by interfering with the intestinal absorption of cholesterol (Sliskovic and White, 1991; O' Brien and Sliskovic, 1992). In the present investigation, the expression of the ACAT gene in the
Fig. 20. (a). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of acyl-CoA:cholesterol acyltransferase (ACAT) and caspase-3 mRNA transcript levels in hepatic tissue visualized on an ethidium bromide-stained agarose gel. M-100 bp DNA ladder; L1- Group I; L2- Group II; L3- Group III

(b) and (c). The results depicted are normalized to levels of the reference GAPDH gene. Data are expressed as mean ± SD Value of ratios of intensity for ACAT and caspase-3 genes divided by that for GAPDH (tested in triplicate)

*Significance at P<0.001; a-Group I vs. group II values; b-Group II vs. group III values
Group I- Normal; Group II -ATH for 45 days, saline treatment for 15 days;
Group III-ATH for 45 days, EGCG treatment for 15 days.
hepatic tissue of the experimental rats was analysed by RT-PCR. The level of the ACAT gene transcript was higher in hepatic tissue of atherogenic diet-fed, saline treated (group II) rats than in normal (group I) rats. This result corroborates earlier observations of increased ACAT mRNA expression in hypercholesterolemic rats (Son et al., 2003). Studies have shown that the herbal and antioxidant compounds that down-regulate the expression of ACAT enzyme are able to lower plasma cholesterol and triglyceride levels by inhibiting absorption and storage of metabolic fatty acid, therein reducing VLDL production in the liver and possibly directly blocking the formation of atherosclerotic lesions (Son et al., 2003; Miike et al., 2008). In the present study, administration of EGCG for 15 days to rats that had been fed an atherogenic diet appeared to lead to a decreased level of mRNA transcript of ACAT in hepatic tissue, when compared to rats that had been fed an atherogenic diet and treated with saline. Similarly, Sudhahar et al. (2006b) reported that the administration of lupeol and lupeol linoleate to hypercholesterolemic rats resulted in decreased mRNA expression of the ACAT gene in hepatic tissue. Thus, the results of the present study suggest that the administration of EGCG alleviates or prevents abnormalities in rats fed an atherogenic diet by inhibiting the expression of the ACAT gene.

A key event in plaque disruption is death of macrophages, leading to generation of the necrotic or lipid core (Schaefer, 1981; Ball et al., 1995). Apoptosis of macrophages has been proposed as a mechanism by which lipids accumulate within the coronary vasculature and thereby contribute to formation and progression of plaques (Ball et al., 1995; Colles et al., 2001). It has also been reported that the apoptosis of endothelial cells contributes to endothelial dysfunction and destabilization of atherosclerotic plaques and thrombosis (Asai et al., 2000; Chen et al., 2005), suggesting that apoptosis of endothelial cells plays an important role in initiation and progression of atherosclerosis. However, in vivo observations suggest that atherosclerotic plaques contain both necrotic and apoptotic cells (Mitchinson et al., 1996; Crisby et al., 1997; Kockx, 1998). In various EC, SMC and monocytic/macrophage-like cells, apoptosis induced by Ox-LDL has been found to
simulate apoptotic systems, including up-regulation of, and increased sensitivity to, activation of caspase-3 (Dimmeler et al., 1997; Harada-Shiba et al., 1998; Wintergerst et al., 2000; Hsieh et al., 2001). In the present investigation, the expression of caspase-3 gene in the hepatic tissue of the experimental rats was analysed by RT-PCR (Fig. 20). The level of the caspase-3 gene transcript was higher in hepatic tissue of atherogenic diet-fed, saline treated (group II) rats than that in atherogenic diet-fed, EGCG-treated (group III) rats and that in normal (group I) rats. Thus, the administration of EGCG appeared to down-regulate the expression of effector caspase-3. Yu et al. (2005) reported that the administration of the phenolic compound, ellagic acid, suppressed the expression of caspase-8 and caspase-9 in the aorta of rabbits fed an atherogenic diet. These findings suggest that EGCG treatment is able to modulate caspase-3 expression level, therein reducing the risk of atherosclerosis. Thus, EGCG may inhibit atherosclerosis by the additional mechanism of suppressing the activation of caspase-dependent apoptosis.

5. CONCLUSION

The results of the present investigation appear to suggest that EGCG prevents atherogenic diet-induced atherosclerosis by protecting against abnormal expression of genes involved in esterification of cholesterol and in apoptosis.