Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2009, Article ID 456208, 8 pages
doi:10.1155/2009/456208

Research Article

Effect of Oxysterol-Induced Apoptosis of Vascular Smooth Muscle Cells on Experimental Hypercholesterolemia

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Received 12 February 2009; Revised 24 May 2009; Accepted 7 July 2009

Recommended by Seamus J. Martin

Smooth muscle cells (SMCs) undergo changes related to proliferation and apoptosis in the physiological remodeling of vessels and in diseases such as atherosclerosis and restenosis. Recent studies also have demonstrated the vascular cell proliferation and programmed cell death contribute to changes in vascular architecture in normal development and in disease. The present study was designed to investigate the apoptotic pathways induced by 25-hydroxycholesterol in SMCs cultures, using an in vivo/in vitro cell model in which SMCs were isolated and culture from chicken exposed to an atherogenic cholesterol-rich diet (SMC-Ch) and/or an antiatherogenic fish oil-rich diet (SMC-Ch-FO). Cells were exposed in vitro to 25-hydroxycholesterol to study levels of apoptosis and apoptotic proteins Bcl-2, Bcl-X_L and Bax and the expression of bcl-2 and bcl-x_L, genes. The quantitative real-time reverse transcriptase-polymerase chain reaction and the Immunoblotting western blot analysis showed that 25-hydroxycholesterol produces apoptosis in SMCs, mediated by a high increase in Bax protein and Bax gene expression. These changes were more marked in SMC-Ch than in SMC-Ch-FO, indicating that dietary cholesterol produces changes in SMCs that make them more susceptible to 25-hydroxycholesterol-mediated apoptosis. Our results suggest that the replacement of a cholesterol-rich diet with a fish oil-rich diet produces some reversal of cholesterol-induced changes in the apoptotic pathways induced by 25-hydroxycholesterol in SMCs cultures, making SMCs more resistant to apoptosis.

1. Introduction

The dedifferentiation and proliferation/apoptosis of smooth muscle cells (SMCs) in the arterial intima represent one of the changes found in early atherosclerotic lesions, when the disease is still reversible [1–3]. Cultured SMCs from an atherosclerotic plaque showed a greater susceptibility to apoptosis that did not vary with subculture [4]. This finding suggests that stable and cell-intrinsic changes in the expression of proapoptotic or antiapoptotic genes exert a greater control over apoptosis in SMCs than do cell-cell interactions or the microenvironment in the plaque, indicating that this susceptibility develops in initial stages of the disease.

The decision phase of apoptosis entails the expression of specific pro- and antiapoptotic genes. If the sum of signals gives apoptosis as a result, the whole protein machinery for destruction is unleashed. The Bcl-2 protein family also regulates the apoptosis pathways to influence cell survival; this family forms heterodimers between apoptosis-inhibiting proteins such as Bcl-2, Bcl-XL, and A1, and inducing proteins such as Bax, Bad, Bid, regulating cell survival [5]. The ratio of anti- to proapoptotic proteins determines susceptibility to apoptosis, especially the Bcl-2/Bax ratio [6]. SMCs express low levels of Bcl-2 in vitro and in vivo [4, 7]. Although SMCs in an atherosclerotic process show no major changes in bcl-2 expression, the balance between antiapoptotic and proapoptotic proteins can change in favor of the latter and trigger apoptosis, mainly mediated by Bax [8, 9].

Cholesterol and its oxides are involved in the initiation of atherosclerosis [10–12]. The most numerous cholesterol oxides found in human atheromatous plaque are 7-hydroxycholesterol and 7-ketocholesterol, while 7-ketocholesterol, and 25-hydroxycholesterol are the most

abundant in the aortic wall of experimental animals after a cholesterol-rich diet and are the most effective to induce cell death, especially 25-hydroxycholesterol [13]. Dietary cholesterol is a risk factor for the development of these atherosclerotic changes [3]. Thus, chickens have been reported to readily develop hypercholesterolemia with a cholesterol-rich diet, producing atherosclerotic lesions in some cases [14–16]. Preliminary studies demonstrated that a 20-day diet enriched with 5% cholesterol is sufficient to cause hypercholesterolemia and gives rise to lipid deposits in the main arteries that are similar to the fatty streaks observed in the first stages of atherosclerosis [17].

Because the presence of apoptosis in atherosclerotic lesions can have a major impact on the progression of the disease, characterization of oxysteroid-induced cell death is important to understand the development of atherosclerosis, since it is not clear how cholesterol and its oxides induce apoptosis in SMCs. Therefore, the objective of this study was to investigate the apoptotic pathways induced by oxysterols, using a cell model in which SMCs were exposed to atherogenic factors (cholesterol-rich diet) or antiatherogenic factors (fish oil-rich diet) [18]. These cells were then exposed in vitro to 25-hydroxycholesterol, studying levels of apoptosis and apoptotic proteins Bcl-2, Bcl-X_L, and Bax and the expression of genes that encode the proteins involved in apoptosis, bcl-2, bcl-x_L.

2. Methods and Materials

2.1. Animals. The protocol of this study was approved by the Animal Laboratory Service of the University of Granada (Spain) and chickens received humane treatment according to the regulations for Animal Research of the European Union. Newborn White Leghorn male chicks (*Gallus domesticus*), supplied by the Animal Laboratory Service of the University of Granada, were kept in a chamber with a light cycle from 09.00 to 21.00 hours and controlled temperature of 29–31°C and allowed free access to food and water.

2.2. Diet and Treatment. The diet was started at hatching and kept on until the chicks were killed (20 days after). Water was available at all time. None of the chicks died a natural death during the treatment nor developed any illness. Three groups of chicks twenty days old were used, the control diet group (C-group) was kept on a standard diet (Sanders A-00) while treated group (Ch-group) was fed on the same diet supplemented with 5% w/w powdered cholesterol mixed homogeneously (Panreac reagent Barcelona, pure grade). The third group (Ch-FO-group) was fed for 10 days with a diet of 5% cholesterol and then cholesterol diet was withdrawn and the standard diet was supplemented with 10% of fish (menhaden) oil for 10 days more. Experimental diet was prepared daily to minimize the oxidation deterioration. Standard diet free of cholesterol contained (w/w) 42% carbohydrate (mainly starch), 3.5% fat, and 20.5% protein. Experimental diets were obtained by supplementation to de standard diet with 5% of cholesterol (Ch diet) and 10% fish (menhaden) oil (FO diet). Fatty acid composition of analysed diets is given in Table 1 [19]. No significant differences were observed in fatty acid composition of each diet during the experiments. After each treatment, animals were decapitated and the aortic arch was removed. Animals did not suffer at all at any stage of the experiment because our laboratory usually uses the anesthetic ketamine (60 mg/kg of body weight) and sodium pentobarbital (50 mg/kg of body weight) according to the regulations of the Animal Research of the European Union. The work has a positive inform from the Ethics Committee of the Granada University. Animals received therapy and euthanasia methods. The research staff had the preparation and the Certification to do experimental work with animals. All the stages of the experiment are adapted to the established protocol.

Table 1: Fatty acid composition of control and experimental diet.

Fatty acid	Control	+ 10% fish oil
14	0.8	6.6
16	22.3	22.3
18	8.6	5.8
Total sat.	31.7	34.8
16 : 1 n-7	3.3	9.2
18 : 1 n-9	32.4	19.5
20 : 1 n-9	0.0	0.4
Total MUFA	35.7	29.1
18 : 2 n-6	24.6	10.7
20 : 2 n-6	2.5	2.4
20 : 3 n-6	1.1	0.6
20 : 4 n-6	1.6	1.4
Total n-6	29.8	15.1
18 : 3 n-3	0.8	0.6
20 : 5 n-3	0.0	12.0
22 : 5 n-3	1.7	1.6
22 : 6 n-3	0.0	6.8
Total n-3	2.5	21.0
Total PUFA	32.3	36.1
Total unsat.	68.0	65.2
Sat./unsat.	0.47	0.53
Sat./PUFA	0.98	0.96
20 : 5/22 : 6	0.00	1.76
n-3/n-6	0.08	1.39

Results (% of fatty acid) are expressed as means of 3 determinations

2.3. Smooth Muscle Cell Culture. SMCs were isolated from the aortic arch of the chicks as described elsewhere [17] with slight modifications and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with D-glucose (4.5 g/L), L-glutamate (0.584 g/mL), antibiotic cocktail composed of penicillin (100 μg/mL) and amphotericin (0.25 μg/mL) (Sigma-Aldrich, Inc.), as well as 10% (v/v) fetal bovine serum (FBS). Medium was buffered with bicarbonate and cultures were kept at 37°C in humidified atmosphere of 95% air and 5% CO_2. Medium was renewed three times a week. Secondary cultures were initiated after either low or

high passages using 0.05%/0.02% Trypsin-EDTA solution. All experiments were conducted using 3 or 5 passages. Cells were determined to be vascular SMCs by their hill-and-valley configuration at confluence and positive fluorescence staining for smooth muscle actin and myosin [17].

2.4. 25-Hydroxycholesterol Treatment and Cytotoxicity Assay. Cytotoxicity was analyzed by using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, bromide. Sigma). SMCs were plated in 96-well plates at a density of 25000 cells/well. After adhering during overnight culture, cells were treated for 24 and 48 hours with 25-hydroxycholesterol (5–40 μg/mL) dissolved in absolute ethanol. The final concentration of ethanol in the culture medium never exceeded 0.8% and no effect on culture was observed at or below this concentration.

MTT was dissolved in DMEM at a concentration of 5 mg/mL. An amount of this solution equal to 10% of the culture medium volume was added to cell cultures. After 2 hours, cultures were removed from the incubator and washed with PBS. The formazan crystals were solubilized by adding 200 l μL of solubilization solution (0.05 N HCl in isopropanol). Metabolic activity was quantified by subtracting light absorbance at 630 nm from absorbance at 570 nm.

2.5. Real-Time PCR Analysis. Total RNA was isolated with Tri-Reagent/Trizol (Invitrogen, Ltd, UK). Single-stranded cDNA was synthesized from 4 μg total RNA using an Oligo$(dT)_{12–18}$ as primer and PowerScript reverse transcriptase (Clontech Laboratories, Inc., Calif, USA). Real-time PCR was performed with the Fast Start DNA Master SYBR Green I Kit (Roche) and Light Cycler system (Roche). For the Light Cycler reaction, a master mix of the following reaction components was prepared to the indicated final concentration: 12.6 l μL H_2O, 2.4 l μL $MgCl_2$ (4 mM), 1 l μL forward primer (0.5 μM), 1 l μL reverse primer (0.5 μM), and 2.0 l μL of the Fast Start DNA Master SYBR Green I mix (Roche). The primer sequences used in this study are given in Table 2 and optimized at an annealing temperature of 55°C. The cDNA of the genes studied in the different samples (treated in vivo and in vitro) were diluted 1 : 100 and amplified to obtain the Cp value for each sample. Light Cycler products of the different gene expressions were analyzed by agarose gel electrophoresis and a Light Cycler melting curve was constructed to test for a single product at the end of each PCR reaction. A mathematical model developed by Pfaffl [20] was used for the relative quantification of bcl-2, bcl-x_L, c-myc, and p53 mRNA expression in real-time PCR with respect to the reference β-actin gene transcript. The efficiency of the assay for each studied gene was β-actin: 1.86; bcl-2: 1.89; bcl-x: 1.9; c-myc: 1.91; p53: 1.81.

2.6. Immunoblotting. Cell monolayer (1×10^7 cells) was disrupted by incubation with RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/mL PMSF, 2 μg/mL aprotinin, 0.18 mg/mL sodium orthovanadate). Protein concentration was determined by using the micro-BCA procedure (Bio-Rad Laboratories, Inc., Calif, USA). Proteins were denatured in 1 × sample buffer with 5% 2-mercaptoethanol at 95° for 10 minutes. Bcl-2, Bcl-X_L, and Bax protein expression was detected by immunoblotting. Total cell lysate was separated under reducing conditions by 12% SDS-polyacrylamide gel electrophoresis (Mini Protean II, Bio-Rad). Gel-resolved proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF) using a Mini Trans-Blot Cell apparatus (Bio-Rad). Membranes were blocked for 1 hour at room temperature in blocking reagent (5% milk power, 0,05% Tween-20 in TBS or PBS, pH 7.4) and probed at 4°C overnight with the primary antibody: anti-Bcl-X_L (Cell signaling Technology, Inc., Mass, USA) 1 : 1000, anti-Bax 1 : 100 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif, USA), and anti-Bcl-2 1 : 500 (BD Biosciences, Calis, USA). Primary antibodies were detected using a horseradish peroxidase- (HRP-) conjugated secondary antibody. The immunocomplex was detected with the ECL-Plus kit (Amersham, Buckinghamshire, UK), and the band density was analyzed using QuantiScan software (Biosoft, Cambridge, UK).

2.7. Statistical Analysis. Results are expressed as mean ± SEM. Data were analyzed by Student's *t*-test when the variability was the same in each group, because the *t*-test assumes that standard deviations of two datasets are equal. A difference between groups was considered significant when the *P*-value was $\leq .05$.

3. Results

3.1. 25-Hydroxycholesterol Treatment and Cytotoxicity Assay in the In Vivo/In Vitro Cell Culture Model. To evaluate the cytotoxicity of 25-hidroxycholesterol on SMC-C, SMC-Ch, and SMC-Ch-FO cultures, cells in third passage were incubated for 24 hours in 25-hidroxycholesterol at concentrations of 0, 5, 10, 20, 40, and 80 μg/mL, were used. The 25-hidroxycholesterol was previously dissolved in the ethanol and was diluted in cultures in order to obtain the concentrations of previous experiments. The concentration of ethanol in cultures never exceeded 0.8%, wich previously verified that it was not toxic for the cells. As Figure 1 shows, the citotoxicity of 25-hidroxycholesterol during 24 hours is the same in the three types of cultures. Even then, a slightly greater viability was observed in the SMC-Ch-FO cultures with respect to the control. With a concentration of 20 μg/mL, the cellular viability is approximately of 50%.

3.2. Bax, Bcl-2, and Bcl-X_L Protein Levels in the In Vivo/In Vitro Cell Culture Model. Expression of proteins of the Bcl-2 family of oncogenes (bax, bcl-x_L, and bcl-2) was studied (Figure 2). Because of the homodimerization and heterodimerization capacity of Bax, Bcl-X_L, and Bcl-2, the relationship between expression levels of Bcl-2/Bax and Bcl-X_L/Bax determines whether the cells will undergo

Table 2: Primer sequences and PCR product lengths for β-actin, bcl-2, and bcl-xl.

Gen	forward primer	Reverse primer	length
β-actin	GCTCCGGCAATGTGCAA	AGGTTCATGAGGTAGT	515
bcl-2	TACCTGCTTACACTTAGGAA	ATGACTATGATGCGATGGCA	307
bcl-xl	GGAGGAAGAGGATGAGAA	ATCTCCTTGTCCACG	383

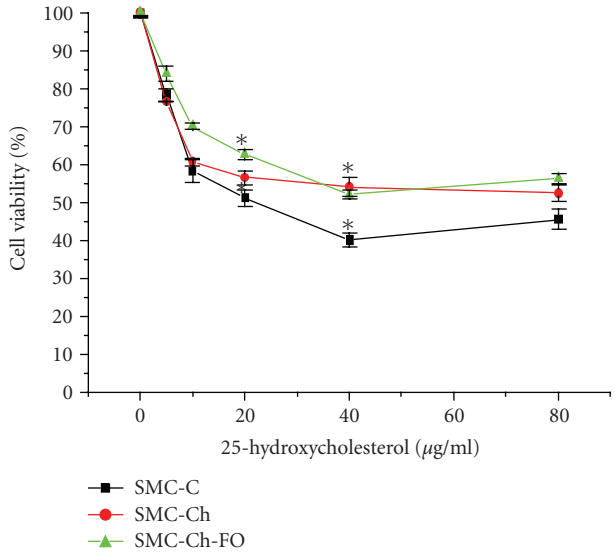

Figure 1: Toxicity of 25-hydroxycholesterol to SMC as measured by MTT assay. SMCs were treated with 25-hydroxycholesterol for 24 hours. Lowest values indicate highest toxicity. Results represent mean values ± SEM ($n = 3$). ($^*P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$).

apoptosis or not after an apoptotic stimulus. Table 3 shows the antiapoptotic/proapoptotic ratios at baseline (after in vivo treatments) and after 25-hydroxycholesterol in vitro treatment. At baseline, expression of Bcl-2 and Bax (Figure 2) was similar among the three culture types, but expression of Bcl-X_L was higher in SMC-Ch and SMC-Ch-FO than in SMC-C. Hence, the Bcl-2/Bax ratios were very similar among cultures, whereas the Bcl-X_L/Bax (Table 3) was significantly higher in SMC-Ch-FO. The SMC-Ch-FO (Table 3) showed a highly significant increase in the Bcl-X_L/Bax ratio but a similar Bcl-2/Bax ratio to that of the control cells, indicating some resistance to apoptosis. Therefore, the replacement of a cholesterol-rich diet with a dish oil-rich diet not only reverses the effects of cholesterol but also protects the SMCs from apoptotic stimuli.

3.3. Effect of 25-Hydroxycholesterol on Bax, Bcl-2, and Bcl-X_L Protein Levels. The addition of 25-hydroxycholesterol produced a highly significant increase in Bax levels in all three culture types (Figure 2) but no change in Bcl-X_L, except for a nonsignificant increase in the SMC-C. Hence, there was a major reduction in the Bcl-2/Bax ratio. Moreover, a large reduction in the Bcl-X_L/Bax ratio was observed for the SMC-C and an intermediate reduction for the SMC-Ch-FO (Table 3).

Table 3: Bcl-2/Bax and Bcl-xl/Bax expression ratios in SMC cultures: Bcl-2/Bax and Bcl-xl/Bax expression ratios in SMC cultures at baseline (SMC-C, SMC-Ch, and SMC-Ch-FO) and Bcl-2/Bax and Bcl-xl/Bax Expression ratios for SMC cultures treated with 20 μg/ml 25-hydroxycholesterol for 24 hours. Ratios were calculated by using O.D protein and reading and normalized against a control (SMC-C) for each protein. Each datum indicated mean ± SEM of three independent experiments. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus SMC-C.

	Bcl-X_L/Bax	Bcl-2/Bax
SMC-C	1	1
SMC-Ch	1.3*	0.97
SMC-Ch-FO	1.7**	1.13
25-Hydroxycholesterol 20 μg/ml	Bcl-X_L/Bax	Bcl-2/Bax
SMC-C	0.77	0.56***
SMC-Ch	0.99	0.61**
SMC-Ch-FO	0.82**	0.61**

3.4. Expression of bcl-2 and bcl-X_L Genes in the In Vivo/In Vitro Cell Culture Model. We also studied the expression of genes that encode proteins implicated in apoptosis (bcl-2, bcl-x_L). At baseline (after in vivo experiments), the amount of mRNA of bcl-2 gene remained unchanged in the SMC-C and SMC-Ch (Figure 3). However, the SMC-Ch-FO showed a slight increase in bcl-2 ($P < .05$). No major differences were observed in baseline expression of the bcl-x_L gene (Figure 4) in the SMC-C, SMC-Ch, and SMC-Ch-FO cells, as also found for bcl-2 and protein levels.

3.5. Effect of 25-Hydroxycholesterol on the Expression of bcl-2 and bcl-x_L Genes. Incubation of the cultures with 25-hydroxycholesterol increased the mRNA of bcl-2 ($P < .001$) to a greater degree in SMC-Ch than in SMC-C ($P < .05$), see Figure 3. Moreover, the expression of bcl-x_L markedly increased in the three culture types after the addition of 25-hydroxycholesterol ($P < .001$), see Figure 4.

4. Discussion

Hyperlipemia and similar conditions, in particular, high cholesterol, change the expression of genes in endothelial cells and SMCs, inducing atherosclerotic lesions in which proliferation and apoptosis are both present [1, 8, 21]. The dedifferentiation and proliferation/apoptosis of SMCs in the arterial intima represent one of the changes found in early atherosclerotic lesions, when the disease is still reversible [2, 3]. In light of this, it is predictable that SMC culture

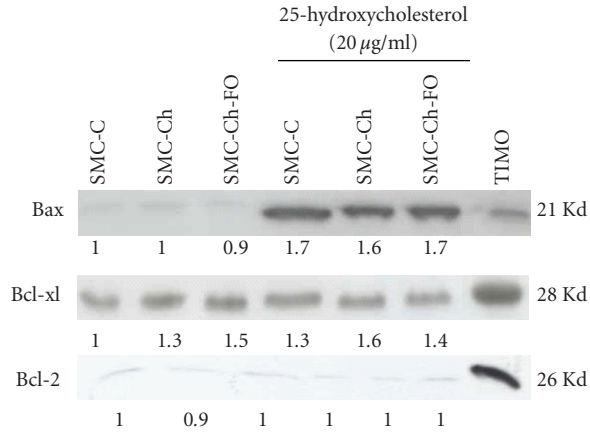

Figure 2: Representative Western blots showing expression levels of Bax, Bcl-xl, and Bcl-2 on SMC cultures at baseline (SMC-C, SMC-Ch, and SMC-Ch-FO) and changes in protein expression after treatment of SMC cultures with 20 μg/mL 25-hydroxycholesterol for 24 hours. Equal amounts of proteins were separated by electrophoresis and processed for immunoblotting. Similar results were obtained in three separate experiments. The ratios were calculated by using O.D protein band reading and normalized against a control (SMC-C) for each protein.

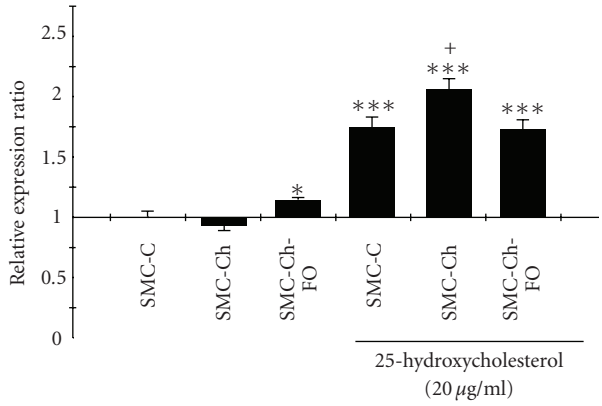

Figure 3: bcl-2 mRNA quantification in SMC cultures (SMC-C, SMC-Ch, SMC-Ch-FO) and SMC cultures treated with 20 μg/mL 25-hydroxycholesterol for 24 hours. mRNA levels were quantified by real-time semiquantitative reverse-transcription PCR. Results are shown as relative expression ratio of bcl-2 in SMC cultures with respect to control culture and expressed in comparison to reference gene β-actin. Bars represent the means ± SEM for 3 different experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus SMC-C, $^{+}P < .05$, $^{++}P < .01$, $^{+++}P < .001$ versus SMC-C treated with 20 μg/mL 25-hydroxycholesterol.

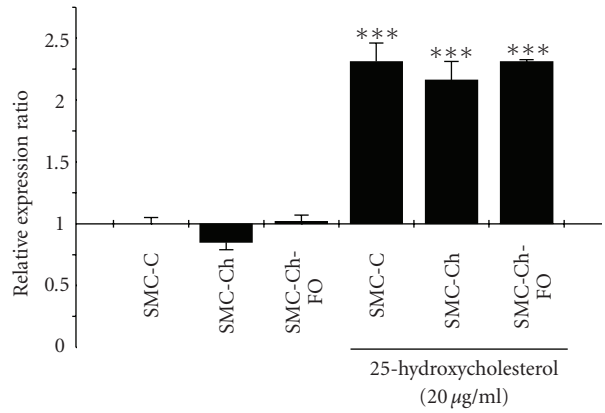

Figure 4: bcl-xl mRNA quantification in SMC cultures (SMC-C, SMC-Ch, SMC-Ch-FO) and SMC cultures treated with 20 μg/mL 25-hydroxycholesterol for 24 hours. mRNA levels were quantified by real-time semiquantitative reverse-transcription PCR. Results are shown as relative expression ratio of bcl-2xl in SMC cultures with respect to control culture and expressed in comparison to reference gene β-actin. Bars represent the means ± SEM for 3 different experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus SMC-C, $^{+}P < .05$, $^{++}P < .01$, $^{+++}P < .001$ versus SMC-C treated with 20 μg/mL 25-hydroxycholesterol.

models from animals fed with fish oil diet could produce some reversal of the changes induced by cholesterol in apoptotic proteins Bcl-2, Bcl-X_L, and Bax and the expression of bcl-2 and bcl-x_L genes [22, 23]. The SMC culture model generated in our laboratory by isolating the cells from control and cholesterol-fed chicks [17] produces changes in SMCs that make them more susceptible to 25-hydroxycholesterol-mediated apoptosis. Replacement of a cholesterol-rich diet with a fish oil-rich diet produces some reversal of the cholesterol-induced changes, increasing the resistance of SMCs to apoptosis. The very low level of apoptosis showed in SMCs is similar to the 4% death rate usually observed in cultured cells. These data are in agreement with previous reports that cultured SMC from the arterial media, even those from an atherosclerotic plaque, showing no apoptosis in culture, unlike observations in SMC from the intima [4]. In early atherosclerosis, the cells in the arterial media that will proliferate and migrate, modulating their phenotype, are in some way protected against apoptosis [24].

In our experimental cell model, Bcl-2 expression was very low in SMCs from chicks after a 20-day calorie-rich diet, whereas Bax expression showed virtually no change. These results are consistent with previous reports that Bcl-2 is undetectable in most SMCs from atherosclerotic plaque or healthy arteries [25, 26], explaining its poorly defined role, and that the expression of Bax does not differ between SMCs with synthetic and contractile phenotypes. However, other studies suggested that Bcl-2 is expressed in SMCs with contractile phenotype but not in those with synthetic phenotype. It has, therefore, been postulated that Bcl-2 may be repressed throughout the modulation from contractile to synthetic phenotype [27]. This modulation in SMCs of the arterial intima is observed in early atherosclerotic lesions, when the disease is still reversible. Cells in fatty streaks have lipid deposits in small vacuoles in the cytoplasm [27–29]. They subsequently become so-called foam cells [30, 31], and both cell types show a high expression of Bax. Therefore, although the increase in Bax expression in our cells did not reach significance, it suggests that the lesion may still be in an early and reversible state, as are initial adaptive streaks in which Bax is not detectable [30]. In contrast, the Bcl-X_L/Bax ratio increased in the SMC-Ch. The expression of Bcl-X_L has

been reported in SMCs from atherosclerotic plaques, and is believed to play the main role in preventing apoptosis in atherosclerosis [32]. Hence, it is thought to act largely in initial stages of atherosclerosis, in which proliferation must predominate. In fact, an increase in Bcl-X_L was observed in SMC from the intima in early atherosclerotic lesions [33]. Other members of the Bcl-2 family, for example, Bak, are abundantly expressed in SMCs from atherosclerotic plaques and tend to heterodimerize with Bcl-X_L [33].

Oxysterols, especially 25-hydroxycholesterol, can reduce the expression of bcl-2 y bcl-x_L [34–36], activate Bad, and increase the expression of genes that encode BH3-only proapoptotics, for example, Bim, which in turn activates Bax/Bak. These actions appear to be mediated by the inhibition of AKT survival kinases [37]. Beside the protein levels, we also studied the expression of genes of bcl-2 family that encode proteins implicated in apoptosis (bcl-2, bcl-x_L), the amount of mRNA of bcl-2 gene (prototype of antiapoptotic genes) remained unchanged in the SMC-C and SMC-Ch, consistent with previous findings of no modifications in the expression of bcl-2 during atherosclerotic alterations [9, 25, 26]. However, the SMC-Ch-FO showed a slight increase in bcl-2, which may imply some resistance to apoptotic stimuli. In fact, many genes involved in the response to oxidative stress, for example, NF-κB survival factor genes, are activated by a fish oil-rich diet and are also involved in the regulation of bcl-2 [38–40]. No major differences observed in baseline expression of the bcl-x_L gene in the SMC-C, SMC-Ch and SMC-Ch-FO cells, as also found for bcl-2 and protein levels. The bcl-x_L gene is thought to play a critical role in the prevention of cell death in atherosclerotic plaques, and a reduction in bcl-x_L expression levels may be a prerequisite for apoptotic induction [9]. It has also been reported that the inhibition of NF-κB (activator of cell survival genes reduces the expression of bcl-x_L, which makes the cells more susceptible to apoptosis [41]) .

The increase in the mRNA of bcl-2 to a greater degree in SMC-Ch than in SMC-C may appear contradictory, since Bcl-2 protein levels are known to be reduced by oxysterol treatment of the cells [36]. However, this reduction is largely produced by dimerization of this protein with Bax, which increases in the presence of oxysterols, as demonstrated by the results of our protein studies. The marked increase of the expression of bcl-x_L in the three culture types after the addition of 25-hydroxycholesterol again appears contradictory because Bcl-X_L protein levels are reduced, which may be explained by the dimerization of Bcl-X_L with Bax and especially with Bak [36].

The aortic arches of male chick were chosen for this study for two reasons: first, for the ability of chicks to absorb high amount of dietary cholesterol [14, 42]; and second, because intimal thickening in the aortas from these hypercholesterolemic chicks can be detected after only 20 days [43, 44]. This short diet period is very convenient and contrasts with those reported for other experimental animal models, mainly non-human primates [45] and rabbits [46], which must be a cholesterol-containing diet for between 1 and 18 months to obtain an atherosclerotic lesion. The short time of the chicken model is comparable with that necessary to induce mitotic activity in aortas of white carneau pigeons or swine [47]. Serum cholesterol levels observed in 10-day-old control chicks are normal because newly hatchet chicken reabsorb the yolk during the first week of live [48], thus developing hypercholesterolemia, which becomes almost three times higher in cholesterol-fed chicks. The significantly elevated cholesterol and triglyceride plasma levels are in accordance with previously reported plasma lipids levels [42].

Avian models were used in trials of atherosclerosis, and, in fact, cholesterol feeding has been described elsewhere as producing atherosclerotic lesions in fowl species [16]. We have also used chicks previously to study lipids metabolism after cholesterol administration [49] and to develop a cell-culture avian model to look into, in vitro, the effect of a cholesterol diet, in vivo, on the transformation of SMC [17]. Thus, in our laboratory, we isolated SMCs from cholesterol-fed chicks (SMC-Ch cultures) which are very proliferative in culture compared with SMC isolated from control-fed chicks (SMC-C cultures), and with identical maintenance in culture of the two lines of SMC [17]. In this way, DNA synthesis in the S phase was 4-fold higher and after 20 days of culture, SMC-Ch increased their cholesterol content to double that of SMC-C, giving SMC-Ch cultures under conditions mimicking such a cholesterol diet and a very early atherosclerosis in vivo/in vitro model at the SMC level [17]. We have studied the cholesterol synthesis and HMG-CoA reductase gene expression in these cultures, showing great differences between SMC-C and SMC-Ch [50, 51]. Also we demonstrated the existence of cyclic fluctuations of HMG-CoA reductase activity in nonsynchronized SMC cultures not correlated to the cultured feeding and not with the increase of mRNA, suggesting the posttranscriptional modulation of the HMG-CoA reductase and the relationships between HMG-CoA reductase activity and cell division [52]. Finally, we have examined the morphological, molecular, and proliferation change in arterial SMC mimicking such a cholesterol diet. Consequentially, this transformed SMC is a good model to study the alterations of the differentiated state of SMC, caused not only by cholesterol-rich diet but also by fish oil-rich diet as well as the apoptotic pathway induced by oxysteroid in SMCs.

5. Conclusion

In conclusion, we investigated the apoptotic pathway induced by oxysteroid in SMCs isolated from male chicks exposed to a control diet (SMC-C) and an atherogenic cholesterol-rich diet (SMC-Ch) or an antiatherogenic fish oil-rich diet (SMC-Ch-FO). We found that 25-hydroxycholesterol-induced apoptosis in SMCs, mediated by a high increase in Bax protein and bax gene expression. These changes were more evident in SMC isolated from chicks exposed to an atherogenic than to an antiatherogenic one. Thus, we deducted that the replacement of a cholesterol-rich diet with a fish-oil rich diet might produce some reversal of cholesterol-induced changes in oxysterol-activated apoptotic pathway, making SMC more resistant to apoptosis. Moreover, high cholesterol diet and fish-oil rich diet in

the animals, before obtaining the SMCs cultures, induced changes in the apoptotic gene expression studied. Thus, the mRNA concentration was downregulated in SMC-Ch-FO versus SMC-Ch. Since we reported previously that, the nutritional culture conditions, the intracellular cholesterol concentration, and the ultrastructural morphology were the same during the first days of cultures; the change in gene expression must have been induced by the cholesterol or fish oil diet in the aortic SMC in vivo. In this way, this in vivo/in vitro model is relevant to study the nutritional control and gene regulation of the differentiation and apoptosis in SMC. New studies would be necessary to demonstrate the implication of the cell signalling pathways, as well as to show if new protein synthesis is required for the Bax increase or if some peaks of gene expression correlate with other regulatory events.

Acknowledgments

We would like to thank Maria Romero for her kindly help in the mattering cell cultures and also to the Centro de Instrumentación Cientifica, Flow Citometry Service and Animal Laboratory Service of the University of Granada (Spain). This work was supported partly by Junta de Andalucía (Group code CTS 168) and also by the research grants from Instituto de Salud Carlos III, ISCIII (Grant no. PI030829).

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