Fish Oil Supplementation Prevents Neointima Formation in Nonhypercholesterolemic Balloon-Injured Rabbit Carotid Artery by Reducing Medial and Adventitial Cell Activation

Elisabetta Faggin, Massimo Puato, Angela Chiavegato, Rafaealla Franch, Paolo Pauletto, Saverio Sartore

Abstract—We asked whether balloon-injured neointima formation in the presence of high/low serum cholesterol (CT) levels might be affected by dietary supplementation with fish oil (FO). To test this hypothesis, we examined the differentiation, proliferation, or apoptosis profile of smooth muscle cell (SMC) and adventitial cell response to a mild injury induced via a Fogarty catheter in the carotid artery of adult rabbits that had been fed a standard chow or 0.5% CT-enriched diet starting 4 weeks before the lesion. One week before surgery, animals received FO supplementation. This regimen was continued for the following 3 weeks. The effect of FO on the early proliferative/migratory response of carotid SMCs was also examined in 2- and 7-day–injured normocholesterolemic rabbits. As controls, animals subjected to 3-week endothelial injury and animals kept on a 7-week CT diet were used. Carotid cryosections from the various animal groups were evaluated for morphometry (image analysis), differentiation (immunofluorescence with monoclonal antibodies specific for smooth muscle markers, ie, myosin isoforms, SM22, and fibronectin), proliferation (bromodeoxyuridine incorporation), and apoptosis (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling). FO treatment significantly reduced the development of intimal thickening in normocholesterolemic rabbits but had no efficacy in the presence of relatively higher serum CT levels. At day 2 (adventitia) and day 7 (neointima, media, and adventitia), the proliferation index of SMCs in FO-treated injured rabbits was markedly lower than in untreated injured controls. Concomitantly with the antiproliferative effect, FO was able to decrease the size of 2 cell types involved in the cell growth response to endothelial injury, namely, the “fetal-type” medial SMC subpopulation and the fibroblast-derived adventitial myofibroblasts. Thus, in our experimental conditions, a low CT level is a permissive condition for FO to prevent neointima formation to a considerable extent. This event is attributable to the early postinjury effect of FO on SMC/adventitial cell proliferation/differentiation patterns. (Arterioscler Thromb Vasc Biol. 2000;20:152-163.)

Key Words: smooth muscle cells ■ adventitia ■ fish oil ■ endothelial injury ■ atherogenesis

The low occurrence rate of coronary heart disease in fish-consuming populations has raised the possibility that an increased consumption of the n (-3) polyunsaturated fatty acids (PUFAs) might be able to decrease the development of atherosclerosis in the vascular system.1–5 Despite the expectancy raised by epidemiological studies,5 contrasting results have been reported on the efficacy of FO as an antiatherogenic agent (in experimental animals6 –17) and anti-restenotic drug (in clinical trials18 –26). The experimental studies published so far focused almost exclusively on the wide array of effects consequent to PUFA/FO supplementation are all pertinent to the initial phase of atherogenesis, we examined the effect of FO dietary supplementation on the early response of vessel wall to endothelial injury. In this investigation, particular emphasis is given to the parietal cell types to which previous and recent studies28 –30 assign a key role in neointima formation and whose activation occurs soon after lesion: SMCs28 –30 and adventitial cells.31–33 Because medial SMCs are a structurally and functionally heterogeneous population,34 –36 it might be that FO action is carried out selectively on distinct SMC subtypes, possibly influencing the specific differentiation profile of these cells. It is also known that medial SMCs are phenotypically unstable34 –36 and are able to modify their structural and functional commitment soon after lesion, in association with the alteration in the proliferation and apoptosis profile and before the migration “wave” to the subendothelial space occurs.29,35 If FO
may potentially be able to influence these processes in the media/neointima, it is reasonable to assume that the marine oils may also affect the phenotypic stability of adventitial fibroblasts (ie, the inherent property of these cells of becoming myofibroblasts) once the wall is damaged. These cells are considered to be a hybrid fibroblast-SMC phenotype or an intermediate cell phenotype between the 2 cell types.

We have also sought to compare the potential efficacy of FO treatment on injured carotid arteries in the presence of high or low serum CT levels. Because lipid accumulation in the vascular wall is preceded by intimal SMC proliferation and LDL-CT can alter the in vitro phenotypic and the proliferative level of SMCs, the possibility exists that this atherogenic risk factor may interfere with the putative antineointima action of FO.

Using a combined morphometric and immunocytochemical approach, we determined that the efficacy of FO in preventing neointima formation is restricted to the normcholesterolemic rabbit. A possible mechanism through which FO may exert its effect is also delineated in this study, ie, by reducing the 2 arterial wall cell populations that are markedly increased in cell growth response to endothelial injury, the “fetal”-type medial SMCs and the adventitial myofibroblasts.

**Methods**

**Animals**

Sixty-two male 3- to 4-month-old New Zealand White rabbits were divided into 6 animals groups (see Figure 1). A different feeding protocol (duration, 7 weeks) was applied for the various groups: the standard chow diet, standard chow diet containing 0.5% CT (CT), and fish-oil–treated (FO) rabbits. Animals were anesthetized with sodium pentobarbital (30 mg/kg; sodium pentothal; Abbott Laboratories) and urethane (70 mg/kg) administered via a marginal ear vein. A 2F Fogarty embolectomy catheter (Baxter Healthcare Co) was introduced through an aseptic ear vein and positioned approximately at the origin of the common carotid artery. An acute balloon injury was performed by inflating the balloon with 0.2 mL saline solution and then gently pulling it back along the entire length of the common carotid artery with constant rotation. The procedure was repeated 3 times. The catheter was then removed, the artery branch ligated, and the surgical wound closed. The animals were allowed to recover under observation before being placed in their cages. Injection of Evans blue dye 3 hours after surgery confirmed the homogenous removal of endothelium from injured carotid artery. Histological examination of this area revealed a marked structural alteration of SMCs in the presence of a substantially intact wall organization (not shown). Because the lesion apparently did not involve disruption of the medial integrity and a direct contact of the blood with the adventitia, we define the impact of the lesion on the carotid wall as “mild” injury.

At the end of the study period (days 2, 7, and 21), animals were killed under pentobarbital anesthesia. The left common carotid artery and the contralateral vessel were excised with great care to avoid any damage to the adventitial layer. The vessel segments were then perfused with OCT mounting medium (Tissue Tek, Miles Inc) under constant pressure and then inserted into the lumen of the thoracic aorta. The mounted block was immersed in liquid nitrogen and stored at −80°C. The contralateral carotid artery was used as control.

**Determination of Serum Lipoproteins**

Total serum CT and triglycerides were determined by the CHOD-PAP method and analyzed with an automatic Hitachi 717 analyzer (Hitachi/Boehringer Mannheim).

**Bromodeoxyuridine Labeling**

Bromodeoxyuridine (BrdU; 30 mg/kg IP; Boehringer Mannheim) dissolved in PBS, pH 7.2, was injected 12 and 24 hours before euthanasia (at 2 and 7 days and 3 weeks).

**Morphometric Measurements and Image Analysis**

Calculation of tissue areas as well as counting of BrdU-incorporating cells, fetal-type medial SMCs, adventitial myofibroblasts, and total cell number were carried out by computerized image analysis systems. For tissue area calculations, transverse cryosections 10 μm thick prepared from each carotid segment were fixed in 1.5% formaldehyde in PBS and then stained with hematoxylin-eosin. Morphometric analysis of the medial and intimal areas was assessed in carotid segments from each animal with a computerized planimetry system (VIDS V). Measurements were carried out blinded on 6 sections per animal. The ratio of intimal area/medial area was taken as index of carotid neointima formation. Data expressed as mean±SD were compared by 2-way ANOVA. Then statistically significant data were corrected with the Bonferroni unpaired t test for direct post hoc comparisons. A probability of P<0.001 was considered statistically significant.

A detailed blinded quantification of fetal-type medial SMCs and adventitial myofibroblasts from carotid arteries injured on days 2 and 7 (treated or not treated with FO) (6 sections per animal, 3 microscopic fields for each cryosection) was performed by counting immunoperoxidase-positive cells for nonmuscle (NM)-F6 anti-NM myosin and SM-type α-actin antibody, respectively, in comparison

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Animal groups used in this study. Balloon-injured (B), cholesterol-fed (CT), and fish-oil–treated (FO) rabbits.

**Endothelial Lesion**

Animals were anesthetized with sodium pentobarbital (30 mg/kg; sodium pentothal; Abbott Laboratories) and urethane (70 mg/kg) administered via a marginal ear vein. A 2F Fogarty embolectomy catheter (Baxter Healthcare Co) was introduced through an aseptic neck incision produced in the external left carotid artery and positioned approximately at the origin of the common carotid artery. An acute balloon injury was performed by inflating the balloon with 0.2 mL saline solution and then gently pulling it back along the entire length of the common carotid artery with constant rotation. The procedure was repeated 3 times. The catheter was then removed, the artery branch ligated, and the surgical wound closed. The animals were allowed to recover under observation before being placed in their cages. Injection of Evans blue dye 3 hours after surgery confirmed the homogenous removal of endothelium from injured carotid artery. Histological examination of this area revealed a marked structural alteration of SMCs in the presence of a substantially intact wall organization (not shown). Because the lesion apparently did not involve disruption of the medial integrity and a direct contact of the blood with the adventitia, we define the impact of the lesion on the carotid wall as “mild” injury.

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with the total hematoxylin-positive cell nuclei. Cryosections were viewed with a high-resolution, charged-coupled device (CCD), TV video camera (Sony DXC-102P) attached to a Zeiss microscope (UMSP80) fitted with an ×25 objective. The resulting images were contrast-enhanced, digitalized, and measured with an AT-IBAS Image Analysis System (Kontron). According to these settings, the size of the image analyzed was 26 000 μm². After image acquisition and contrast enhancement, the thresholding procedure was applied to measure in each field the total area covered by negative and positive cytoplasm, respectively. The threshold cytoplasm antigen level for positive nuclei was also estimated by measuring for each sample 15 randomly chosen nuclear profiles. The mean number of nuclei was then counted for each sample by calculation of the ratio between the mean total area covered by positive or negative nuclei and the corresponding mean nuclear area. The mean number of cells was determined by the same procedure using the mean cellular area value.

The BrdU-incorporation profile in the various types of carotid arteries was studied by use of an immunoperoxidase protocol and the procedure described above for the AT-IBAS Image Analysis System. Twenty-four separate microscopic fields were measured for each sample. Counting of red (BrdU-incorporating) and of blue-violet + red (total number) nuclei was expressed as mean ± SD and normalized to the area unit (mm²). Labeling index (per mm²) expresses the ratio between BrdU-positive nuclei and the total number of nuclei for each animal group.

Statistical analysis of data was carried out by 2-way ANOVA followed, when statistically significant, by Bonferroni’s correction performed with the unpaired t test for direct post hoc comparisons. A probability of P < 0.05 was considered statistically significant.

The semiquantitative evaluation of apoptotic cells in the carotid arteries from normocholesterolemic, hypercholesterolemic, and injured animals was determined by counting the yellow terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)-positive versus the TUNEL-negative nuclei as they appeared after fluorescein labeling. Cryosections were observed with a Zeiss Axiosplan microscope equipped with a Hamamatsu CCD camera coupled with a Power Macintosh computer. Images were captured by a Hamamatsu frame grabber and analyzed with Optilab 2.3.1 software.

Antibodies

The following monoclonal antibodies were used in this study: SM-E7 anti–SM-type myosin heavy chain (MyHC) and NM-F6 and NGM-G2 anti-NM MyHC. As previously reported, the SM-E7 reacts with both SM1 and SM2 SM-type MyHC, whereas NM-G2 and NM-F6 are able to differently recognize an antigenic epitope localized in the platelet-type MyHC isoforms MyHC-A_plt and MyHC-A_plt', respectively. IST-9 anti-fibronectin EIIIA isoform (EIIIAFn; a generous gift of Dr L. Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), RAM anti–monocyte-macrophage (Dako, Dakopatts), and anti–SM-specific E-11 anti-SM22α,4 anti–SM-type α-actin was purchased from Sigma Chemical Co, and anti-vimentin was from Boehringer Mannheim.

Immunocytochemistry

Primary antibodies (except for the E-11) were applied to freshly cut unfixed cryosections as previously described. For anti-SM22α antibodies, cryosections were first fixed in 1.5% formaldehyde in PBS, pH 7.2,33,44 In these procedures, the secondary antibody was the anti-mouse IgG coupled with rhodamine isothiocyanate or horseradish peroxidase. In the latter circumstance, bound IgGs were revealed by incubation in aminoethylcarbazole solution. Counterstaining was performed with Mayer’s hematoxylin. Controls were made by omitting the primary antibody and using nonimmune IgG followed by the secondary antibody. Nuclei were stained with bis-benzimide (Hoechst 33258).

BrdU-incorporating nuclei in normal and injured vascular tissue from end-labeling experiments were detected with a specific anti-BrdU antibody (Dako) according to the procedure reported in Reference 45. Negative and positive BrdU-incorporating tissues (myocardium and small intestine, respectively) from the same animals as used for BrdU labeling of injured vessels were used as controls.

TUNEL Analysis

Freshly cut cryosections from normal and injured carotid wall were processed for apoptosis by the TUNEL procedure with the Boehringer Mannheim kit. The experiments and the negative control were performed according to the manufacturer’s instructions, including an incubation with 3% citric acid solution and omitting the proteinase K pretreatment as recommended by some authors. In addition, the cardiac muscle tissue and a leiomyosarcoma was used as alternative negative and positive controls, respectively.

Results

Effect of FO on Serum Lipids

Rabbits belonging to the various groups examined remained healthy throughout the study period. At the end of the experiments, there was no significant difference among the groups with respect to body weight (average, 3 kg/animal).

The total serum CT and triglyceride levels present in injured rabbits subjected to the various treatments/feedings is shown in the Table. There was no difference between the injured and injured + FO groups with regard to the CT level, whereas there was a slight, not significant, decrease in the triglyceride content after FO treatment. Total CT/triglyceride values in CT-fed animals and injured animals fed a CT-enriched diet were slightly lower than the corresponding values obtained after FO supplementation (B + CT + FO...
group). As expected, the values shown by the injured animals kept on standard chow diet are very similar to those of uninjured normocholesterolemic rabbits.

**Effect of FO on Carotid Lesion Area**

Figure 2 shows the results obtained with the morphometry measurements on cryosections from the various carotid arteries obtained from the 6 scrutinized animal groups. Importantly, the segment of carotid artery used in this study did not show, under normal cholesterol diet regimens, morphometrically detectable tunica intima (Figures 2 and 3); it was also selected because it is hardly susceptible to developing histologically visible atherosclerotic lesions at the CT feeding condition used here and despite a relatively high serum CT

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**Figure 3.** Micrographs showing the immunofluorescence pattern of serial cryosections from uninjured carotid artery of normocholesterolemic rabbit with SM-E7 (A), NM-G2 (B), NM-F6 (C), E-11 (D), and IST-9 (E) antibodies. The antigenic target of each antibody is shown at the top right of the respective panel. Note the numerous medial (m) SMCs stained with NM-G2 that are also positive with SM-E7 but negative with NM-F6, corresponding to postnatal-type SMCs. Medial SMCs are heterogeneous with E-11, some of them being particularly reactive with this antibody (black asterisks in D); a few SM22-positive cells may occasionally be visible in the adventitia (a; white asterisks in D). A thin layer of IST-9-positive SMCs can be identified just below the internal elastic lamina (iel; small arrowheads). Bar = 100 μm.
level (Figure 2 and Reference 49). The intima/media ratio is significantly lower in injured + FO-treated rabbits than in the injured animals (0.229 ± 0.032 versus 0.402 ± 0.076). If injured rabbits were fed a CT diet, the values obtained were very close to those of injured animals (0.382 ± 0.090 versus 0.402 ± 0.076). In this case, FO treatment was not able to reverse the higher intima/media ratio (0.400 ± 0.142) to that obtained with standard diet in the injured animal group. Thus,

**Figure 4.** Micrographs showing the immunofluorescence pattern of serial carotid cryosections from 3-week injured (A through E) and the corresponding injured + FO-treated (insets in A through E) rabbits with SM-E7 (A), NM-G2 (B), NM-F6 (C), E-11 (D), and IST-9 (E) antibodies. The antigenic target of each antibody is shown at the top left of the respective panel. Note that the neointimas (it) from injured carotid artery cells are homogeneously labeled with SM-E7 and NM-G2 and rather weakly with NM-F6. E-11 is almost unreactive with the medial (m) SMCs, except for small clusters of cells (asterisk in D). The neointimal cells are weakly stained, and some small regions near the internal elastic lamina (iel) appear negative (star in D). On FO treatment, the SM-E7 is still homogeneously reactive, whereas for the other antibodies, the reactivity is particularly evident in the subendothelial region. e indicates endothelium. Bar=100 μm.
the efficacy of FO supplementation on intimal thickening development is restricted to the animal group kept on a standard diet.

Effect of FO on SMC Composition 3 Weeks After Lesion

The potential effect of FO on the differentiation pattern of carotid SMCs was studied by immunophenotyping the expression of SM- and NM-type MyHC isoforms in the injured vessel wall from normcholesterolemic rabbits. The combined use of SM-E7, NM-G2, and NM-F6 anti-myosin antibodies in developing and adult rabbit arterial SMCs has allowed us to identify 3 categories of SMCs, namely, “fetal” (labeled with SM-E7+NM-F6, ie, containing SM+MyHC-\(A_{\text{pla}1}\) myosin), “postnatal” (labeled with SM-E7+NM-G2, ie, containing SM+MyHC-\(A_{\text{pla}2}\) myosin), and “adult” (labeled...
with SM-E7 only, ie, containing SM myosin exclusively). In this investigation, we also included 2 other antibodies: IST-9, whose recognized antigenic target (EIIIA fibronectin), in our model, permits the identification of “dedifferentiated” SMCs; and E-11, specific for a SMC lineage–specific marker (the SM22 isoform complex).

Figures 3 and 4 show the results obtained with the immunophenotyping study performed on cryosections from the normocholesterolemic intact (N group; Figure 3) and 3 weeks–injured/injured + FO treatment (B/B+FO group) (Figure 4) rabbits. In uninjured carotid artery (see Figure 3), the majority of cells are adult (Figure 3A), some are postnatal (Figure 3B), and none are fetal (Figure 3C). SM22 labels all medial SMCs, although with different intensities (Figure 3D). IST-9 is localized exclusively to the subendothelial cells (Figure 3E), where the majority of postnatal-type SMCs are accumulated (Figure 3B).

In injured carotid arteries, monocyte/macrophage contaminants are absent from both the neointima and the underlying media. Neointimal SMCs in the injured vessels are represented by postnatal SMCs (Figure 4A and 4B), being almost negative or weakly positive with NM-F6 (Figure 4C) or E-11 and E-11, specific for a SMC lineage–specific marker (the SM22 isoform complex).
Effect of FO on Adventitial Cells

Considering the role that adventitial cells might play in our model, we studied whether the locally activated cells, ie, the myofibroblasts31–33,41 (identified on the basis of SM-type α-actin and vimentin) are distributed differently in injured FO-treated versus untreated animals. Figure 7 shows that the FO supplementation induces a marked decrease in the actin-positive myofibroblasts in the adventitia. Vimentin immunostaining resembles the actin pattern very closely (not shown). Cell counting of VD-myofibroblasts54 performed on carotid cryosections from rabbits injured on days 2 and 7 and injured+FO rabbits confirmed that a considerable decrease of SM-type α-actin–containing cells occurs after FO treatment in the carotid adventitia (Figure 8).

Effect of FO on BrdU Incorporation Level

To establish whether the early (2/7 days) phenotypic changes and the late (3 weeks) morphometric gain occurring with FO treatment in endothelium-injured carotid wall is related to a proliferation profile of cells in the carotid media and adventitia, we performed a BrdU-incorporation study. Figure 9 shows that FO treatment is able to significantly reduce the number of BrdU-positive cells in adventitia at both day 2 and day 7 postinjury (from 73.2±38.2 to 27.7±24.5 at day 2; from 9.7±11.4 to 0.0±0.0 at day 7). FO can also decrease the number of BrdU-reactive cells in the media (from 18.7±13.1 to 17.5±8.8) at day 2 and day 7 after the induction of lesion. The BrdU incorporation levels attained by neointima and media at 3 weeks after injury is similar to the control rabbit (N group) reported in Reference 55. Figure 10 shows the tissue-specific distribution of BrdU-positive cells at days 2, 7, and 21 after injury. The majority of BrdU-incorporating cells are localized to the adventitia soon after lesion, whereas at day 7, adventitia is almost deprived of positive cells in favor of the innermost layer of the media and the developing neointima. FO treatment markedly reduces the presence of BrdU-positive cells in all the wall compartments. By 3 weeks after injury, no positive cells can be detected, irrespective of the experimental condition.

Effect of FO on Apoptosis Distribution Pattern

Apoptosis distribution in carotid cryosections from uninjured, injured, and injured+FO-treated animals is shown in Figure

Figure 8. Effect of FO treatment on SM-type α-actin–containing adventitial cells expressed as percentage (±SD) of the total cell number in days 2– and 7–injured carotid artery. B indicates injured and B+FO, injured+FO-treated rabbits. Two-way ANOVA and post hoc Bonferroni’s test. n=4 (B, days 2 and 7); n=5 (B+FO, day 2 and 7).

Figure 9. Effect of FO treatment on BrdU incorporation in days 2– (top) and 7– (bottom) injured carotid artery expressed as percentage of BrdU-positive nuclei with respect to the total cell number. B indicates injured and B+FO, injured+FO-treated rabbits. Two-way ANOVA and post hoc Bonferroni’s test. P<0.001, n=17.

Figure 10. Tissue-specific distribution of BrdU-positive cells at days 2, 7, and 21 after injury.
In the intact vessel, TUNEL-positive cells are present almost exclusively in the adventitia (Figure 11A), whereas at day 2, in both FO-treated and untreated animals, numerous positive cells are found in the endothelial layer but not in the medial or adventitial layer (Figure 11B and 11C). At days 7 (Figure 11D and 11E) and 21 (Figure 11F and 11G) after surgery, <1% of SMCs are apoptotic, irrespective of the tissue layer or the treatment.
Discussion

The results of this study indicate that lesion area development is significantly reduced after 3 weeks of FO only in the animal group fed a low-cholesterol diet. In fact, in the injured hypercholesterolemic animal group, the efficacy of FO treatment as lesion antagonist is dramatically lost. We are in concordance with Chen et al, who reported that a more severe effect of FO on the myointimal growth in the injured rabbit abdominal aorta is achieved in the presence of low CT levels. Our data are also in keeping with the results obtained by Sassen et al and Fincham et al, who found that in porcine injured arteries, the retardation in the progression or the lack of regression (ie, stabilization) of atherosclerotic lesions is related to the plasma CT content.

The serum CT-dependent efficacy of FO treatment in endothelium-injured arterial wall might be related to the differential cellular assortment of the lesion at low or high CT level, ie, the number of monocytes-macrophages/lymphocytes versus SMCs and/or the amounts of native versus oxidized LDL. Although in our experimental conditions, FO treatment in injured rabbits subjected to short-term CT feeding and FO supplementation was unable to substantially alter the total serum CT level (Table), it might be that LDL particles accumulated at the site of the lesion were present in an abnormal quantity or were qualitatively prone to be chemically modified. Oxidized LDL can stimulate arterial SMC growth indirectly, via induction of growth factors (eg, platelet-derived growth factor) from the same or other cells (macrophages and endothelial cells), or

Figure 11. Apoptotic cell distribution in cryosections from uninjured carotid artery (A); days 2– (B), 7– (D), and 21– (F) injured carotid artery from animals without FO treatment (–FO); and days 2– (C), 7– (E), and 21– (G) injured carotid artery from animals treated with FO (+FO). Note that no apoptotic (yellow) cells are seen in the media (m) from uninjured vessel, although rare positive cells are detectable in the adventitia (a, arrowheads). At day 2 (B), some apoptotic cells can be seen in the endothelium (e; arrowheads), in the muscle (double arrowheads) immediately beneath the internal elastic lamina (iel), and in the adventitia. At later stages after injury, the apoptosis level is scarce in both the treated and untreated carotids (media, neointima [it], or adventitia). Bar = 130 μm.
directly, via activation of the pathway of mitogen-activated protein kinases.\textsuperscript{37} Triglyceride levels did not show appreciable changes in the animal group treated with FO, in keeping with other reports.\textsuperscript{27,56}

The study of SMC differentiation has furnished some indications about the mechanism through which FO acts at low CT concentrations. We purposely selected a segment of the rabbit common carotid artery that is histologically unable to develop an atherosclerotic plaque when subjected to the diet regimen used in this study.\textsuperscript{49} In addition, this vessel is relatively enriched with postnatal-type SMCs\textsuperscript{35,37} and thus is potentially at risk for developing SMC growth and migration.\textsuperscript{35,37} Twenty-one days after injury, in the FO-treated group, there was a slight increase in the differentiation pattern of both neo-intimal and medial SMCs, although a complete maturation was not achieved (high signal for fetal fibronectin\textsuperscript{19} and scarce presence of SM22\textsuperscript{10}; see Figure 4). In the early phase of response to injury (days 2 and 7), the effect of FO treatment became more evident. Here, both the general organization of arterial wall and the degree of SMC differentiation were markedly improved. In particular, the media contained fewer fetal-type SMCs (Figure 5), in which the injured carotid artery is particularly high and are directly implicated in neo-intima formation\textsuperscript{35} (Figure 5A, 5C, and 5E). Similarly, the adventitia from injured–FO-treated artery contained fewer vimentin- and SM α-actin–containing myofibroblasts (the VA myofibroblasts\textsuperscript{54}), which are known to participate in the medial cell incorporation/neointima formation.\textsuperscript{31–33}

Taken together, these findings are fairly consonant with our previous data about the correlation existing between the reduction of plaque formation induced by calcium channel blockers and the shift toward more differentiated SMCs\textsuperscript{51–63} (both decrease in the fetal-type and increase in the adult-type SMCs). The change in the phenotypic pattern of medial SMCs induced by FO treatment soon after injury is strictly linked to the modification of proliferation indices in the adventitia and media, but not the apoptotic cell distribution, which remains substantially unaffected compared with the injured carotid artery. We cannot rule out completely the possibility that FO may influence apoptosis, inasmuch as the burst of this event seems to occur within hours after injury.\textsuperscript{47} Interestingly, a decrease of the adventitial myofibroblast content in FO-treated day 2–injured carotids is mirrored by a marked decrease in the proliferation ability of these cells, whereas the BrdU-incorporation level of the medial SMCs remains unaffected. It is at day 7 postinjury that adventitia, media, and neo-intima all display a much lower propensity to proliferate on FO treatment. Recent experiments indicate that neo-intimal SMCs may derive from the dedifferentiated or phenotypically modulated medial SMCs and by recruitment and incorporation of adventitial myofibroblasts (porcine coronary arteries and rabbit carotid artery).\textsuperscript{28–33} Thus, the antiproliferative effect of FO treatment on the injured carotid seems to be dual, affecting both medial and adventitial cells. In particular, the reduction of proliferative index exerted by FO on adventitial cells suggests that the local cell conversion pathway from fibroblast to myofibroblast,\textsuperscript{31–33,41} as part of the response-to-injury process, may be controlled by FO.

Although the results of this study might have important implications for the still-debated use of FO as an antirestenotic agent, some caution must be taken. First, the dose used is certainly not in the range of that used up to now in human studies. Second, the different lipid metabolism in rabbit versus human does not allow any firm conclusions about the real efficacy of FO as an antiatherogenic or antistenotic agent. Third, the relatively short period of FO pretreatment that precedes the cellular wall response triggered by the endothelial lesion might have led to an underestimation of FO efficacy. However, on the basis of the data presented here and those of Chen et al.,\textsuperscript{27} we are in a position to suggest that relatively high doses of FO might have a short-term beneficial effect on restenosis soon after angioplasty. Certainly, this hypothesis needs to be preliminarily tested in experimental settings in which FO treatment is applied to animals with a well-developed atherosclerotic plaque at the time of angioplastic intervention.

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