A comparison of oxidized LDL-induced collagen secretion by graft and aortic SMCs: role of PDGF

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Absood, Aaf, Akira Furutani, Tsutomu Kawamura, and Linda M. Graham. A comparison of oxidized LDL-induced collagen secretion by graft and aortic SMCs: role of PDGF. Am J Physiol Heart Circ Physiol 287: H1200–H1206, 2004. First published May 13, 2004; 10.1152/ajpheart.00228.2004.—Smooth muscle cells (SMCs) from prosthetic vascular grafts constitutively secrete higher levels of collagen than aortic SMCs. Lipid oxidation products accumulate in grafts, and we postulated that they stimulate SMC production of collagen. The effect of oxidized low-density lipoprotein (oxLDL) on type I collagen secretion by aortic and graft SMCs was compared. SMCs isolated from the canine thoracic aorta or Dacron thoracoabdominal grafts (n = 10) were incubated with native LDL or oxLDL (0–400 μg cholesterol/ml) for 72 h. Type I collagen in the conditioned medium was measured by ELISA. OxLDL increased collagen production from SMCs from 4.1 ± 0.3 to 11.0 ± 0.4 ng/μg DNA and by aortic SMCs from 2.3 ± 0.1 to 3.5 ± 0.2 ng/μg DNA. Native LDL had little effect. LY-83583, a superoxide generator, stimulated a dramatic increase in collagen secretion by graft SMCs and a smaller but significant elevation by aortic SMCs. OxLDL has been shown to increase PDGF production by graft SMCs, and PDGF can stimulate collagen production. Anti-PDGF antibody inhibited the increase in collagen production by graft SMCs that was stimulated by oxLDL, implicating PDGF as one mechanism of oxLDL-induced collagen production. Lipid oxidation products that accumulate in prosthetic vascular grafts can cause an oxidative stress that stimulates PDGF production by graft SMCs that in turn stimulates collagen production, contributing to the progression of intimal hyperplasia.

SMCs in intimal hyperplastic lesions of grafts are in a synthetic phenotype, and these SMCs produce high levels of PDGF (6, 23, 29, 37) and collagen (30). Although collagen deposition accounts for the increase in mass in the later stages of lesion development after balloon injury or graft placement in experimental animals (10, 21), factors controlling collagen production are unknown. The elevated rate of collagen synthesis in the aorta of animals on a hypercholesterolemic diet compared with controls (34), and the positive correlation between the amount of collagen and cholesterol in aortic specimens (32, 44), suggests a role for lipids in the excessive collagen deposition that occurs in intimal hyperplasia. The role of oxidized low-density lipoprotein (oxLDL) in stimulating collagen synthesis was suggested by Jimi et al. (20) when they reported that collagen production by aortic SMCs in vitro increased when they were incubated with LDL under oxidizing conditions and that antioxidants abrogated the effect. The causal relationship between the accumulation of lipid oxidation products and collagen deposition in the development of anastomotic intimal hyperplasia, however, is not proven.

Previous studies have shown that SMCs cultured from prosthetic grafts produce higher levels of PDGF and collagen compared with aortic SMCs (30, 37). These phenotypic differences between graft and aortic SMCs are maintained for several passages in culture. Because oxLDL stimulates PDGF production by graft SMCs (1), and PDGF stimulates collagen production under some circumstances, we studied the ability of oxLDL to stimulate collagen production by graft and aortic SMCs and the role of PDGF in this stimulation. Graft SMCs not only produced more collagen than aortic SMCs under basal conditions but also the graft SMCs responded to oxLDL and a superoxide generator, LY-83583, with a larger increase in collagen production than aortic SMCs. The oxLDL-induced increase in collagen production was blunted by the addition of an anti-PDGF blocking antibody, suggesting that PDGF mediates the stimulation of collagen production by graft SMCs. The oxLDL-induced collagen production may contribute to the development of anastomotic intimal hyperplasia that eventually can cause synthetic vascular graft occlusion.

MATERIALS AND METHODS

Graft implantation and removal. Adult female mongrel dogs underwent placement of thoracoabdominal grafts using Cooley doublevelour knitted Dacron grafts (Meadox Medicals), 8-mm internal diameter and 22 cm long, as described previously (13). The protocol for preparing the grafts and the surgical procedure have been described previously (31). Bilateral thoracoabdominal grafts were placed using the Cooley double-velour technique by the author (M.D.T.) as described previously (31). The dog in Figure 1A demonstrated normal thoracoabdominal graft flow. Vascular grafts can cause an oxidative stress that stimulates PDGF production by graft SMCs that in turn stimulates collagen production, contributing to the progression of intimal hyperplasia.

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for animal studies was approved by the institutional committee on animal use. All procedures and care complied with the standards stated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC: National Academy Press, 1996). After 16–26 wk, the graft and native vessels were carefully isolated from surrounding tissue and flushed with medium 199 (M199) tissue culture medium (Sigma). Tissue on the outer surface of the graft was removed before cell harvest.

**SMC harvest and culture.** Cells were harvested from the proximal aorta (ascending aorta and aortic arch), the distal abdominal aorta below the graft anastomosis, and sections of the midgraft as previously described (1). Briefly, the luminal surface was incubated in collagenase A (630 U/ml, Boehringer Mannheim Biochemicals) for 10 min at 37°C, and endothelial cells were removed mechanically. After this, the medial layer of the aorta and the graft were minced into 2 × 2-mm² pieces and incubated in M199 that contained 15 U/ml elastase type III (Sigma), 170 U/ml collagenase A, 2 mg/ml crystalline bovine serum albumin (Boehringer Mannheim), and 0.375 mg/ml soy bean trypsin inhibitor (Boehringer Mannheim). SMCs were collected, plated in serum-coated tissue culture plates, and grown to confluence in M199 with 20% FBS (HyClone Laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml). For all experiments, passage 2 or 3 SMCs were grown to confluence in M199 containing 20% FBS at 37°C in a 5% CO₂ atmosphere and then changed to medium containing 5% FBS and the compound to be tested. The viability of the cells as determined by the trypan blue exclusion principle was 97–98%. The identity of the SMC population was confirmed immunohistochemically using an antibody to SMC α-actin (Sigma).

**ELISA for canine collagen type I.** A sandwich assay was developed for quantifying collagen type I production by canine SMCs. Briefly, Nunc-immuno 96-well ELISA plates (MaxiSorp; Naperville, IL) were coated with 50 µl/well affinity-purified polyclonal goat anti-human collagen type I capture antibody (Southern Biotech, S/B No. 1310-01) at a dilution of 2.0 µg/ml solution of borate buffer (0.6 M NaCl, 0.26 M H₃BO₃, and 0.08 N NaOH, pH 9.6) for 18 h at 4°C. After the removal of excess capture antibody and being blocked with 3% bovine serum albumin in phosphate-buffered saline for 120 min at 25°C, 100-µl aliquots of recombinant human collagen (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 ng/ml, S/B No. 1200-01s) or samples were added and incubated for 18 h at 4°C. Detection antibody, biotin-conjugated polyclonal goat anti-human collagen type I (100 µl/well, S/B No. 1310-08), and 0.5 µg/ml of blocking buffer were added for 2 h at room temperature in the dark. After samples were washed, streptavidin peroxidase (Sigma) was added for 45 min, and α-phenylendiamine chromogen substrate (0.1 µl/well, DAKO) was the added for 20 min. Reactions were terminated with the addition of 100 µl/well of 0.5 M H₂SO₄. Plates were read on an ELISA plate reader (ELX 808, Bio-TEK Instruments) at 492 nm. The detection limit for collagen was 0.3 ng/ml. The ELISA was validated for canine collagen type I by using standards prepared with stock calf thymus DNA (Sigma Chemical) and an equal volume of bisbenzimide solution (Sigma) was added. Samples (200 µl) and DNA standards prepared with stock calf thymus DNA (Sigma Chemical) were measured using cholesterol calibrators (Sigma). Purified LDL was filtered through a 0.45-µm filter. LDL was stored in the dark at 4°C under nitrogen and oxidized with 1.5 µM copper sulfate as previously described (1). The level of LDL oxidation was monitored by the formation of thiobarbituric acid-reacting substances (TBARS), measured by the method of Schuh and colleagues (40). TBARS were extracted from the LDL and native vessels with methanol, filtered through a 0.45-µm membrane and the concentration determined using a spectrophotometric assay. LDL oxidation was measured by TBARS of the oxLDL used in the experiments ranged from 5 to 8 nmol MDA/mg cholesterol, whereas TBARS for the native LDL ranged from 0.3 to 0.5 nmol MDA/mg cholesterol.

**RT-PCR.** RT-PCR was used to amplify the procollagen α1(I) and GAPDH mRNA isolated from the canine SMCs. For canine collagen and GAPDH, primers and probe were designed using a software program (Premier Biosoft International) and the cDNA sequence downloaded from the NCBI database (14). Oligonucleotides were synthesized commercially (Operon Technologies). The oligonucleotide sequences used for procollagen type I were as follows: sense primer (5'-TGG CAT GAT GTG ATT TGC G-3'), antisense primer (5'-CCA GGG ACC AAG AAA CCA CAG G-3'), and probe (5'-TGC GAC GAA ACC AAG AAC ACC TGC C-3'). The corresponding sequences used for canine GAPDH were as follows: sense primer (5'-CAT GTT GTG TAT GGG CCT GAA C-3'), antisense primer (5'-GTC GCA GAT ATG GCA TGG AC-3'), and probe (5'-TGG ATG ACT TTT CTA GAG G-3'). SMCs were lysed, and mRNA was extracted using FastTrack 2.0 kits (Invitrogen; San Diego, CA). cDNA was synthesized from the isolated mRNA using Malmey murine leukemia virus reverse transcriptase, random hexamers, and deoxynucleotides. Collagen and GAPDH cDNA were then selectively amplified in a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer) using GeneAmp RNA PCR kits (Perkin-Elmer) with specific primers, excess nucleotides, and heat-stable DNA Taq polymerase. Amplification was performed in the thermal cycler using 35 cycles with a denaturing temperature of 94°C for 60 s, annealing temperature of 60°C for 90 s, and an extension temperature of 72°C for 90 s. The number of cycles was selected to remain in the linear phase of PCR amplification. To confirm specificity of the amplified products, 10 µl of the final PCR reaction yield were applied on a 2% agarose gel and visualized by ethidium bromide staining. Products of predicted size were detected, and nonspecific bands were not seen in the region of interest. The PCR parameters were used for PCR-ELISA to assess gene expression.

**PCR-ELISA (digoxigenin detection).** After it was confirmed that primers and amplification parameters yielded the predicted products, PCR-ELISA was performed using a modification of the method described by Ruth et al. (39). Briefly, to quantitate procollagen α1(I) and GAPDH mRNA under the experimental conditions, 10 µl of 200 ng/µl of digoxigenin-labeled procollagen α1(I) or GAPDH cDNA were added to the PCR mixture, and reverse transcription and amplification were performed as described above. Products were detected by PCR-ELISA using a commercially available PCR ELISA digoxigenin.
The effect of oxLDL on type I collagen production by aortic and graft SMCs was explored. Constitutive secretion of type I collagen by graft SMCs over 72 h was statistically significantly higher than that by aortic SMCs, 4.1 ± 0.3 and 2.3 ± 0.1 ng collagen/µg DNA, respectively, \( P < 0.0001 \) (Fig. 1). OxLDL (TBARS range of 5–8 nmol MDA/mg cholesterol) induced a concentration-dependent increase in collagen production by graft SMCs, which was greater than aortic SMCs. Collagen production increased with increasing concentrations of oxLDL, being 11.0 ± 0.4 and 3.5 ± 0.2 ng collagen/µg DNA for graft and aortic SMCs, respectively, when they were incubated with 400 µg cholesterol/ml. Native LDL (TBARS range of 0.3–0.5 nmol MDA/mg cholesterol) had a significant effect on collagen production by graft but not aortic SMCs. The concentrations of oxLDL studied were not toxic in 72 h as shown by stable levels of DNA and protein synthesis. OxLDL induced a statistically significant increase in type I collagen production by graft SMCs \( (P < 0.001) \) and a smaller but significant increase in collagen production by aortic SMCs.

The time course of the increase in graft SMC production of collagen was investigated. Collagen production in response to oxLDL was assessed at 24, 48, and 72 h. OxLDL stimulated an increase in collagen production compared with control or LDL, and the increase was significant after 24 h (Fig. 2).

OxLDL is known to produce an oxidative stress, which might be responsible for the increase in collagen production. To determine the effect of other oxidative stresses, collagen type I production was measured after incubation of graft and aortic SMCs with \( \text{H}_2\text{O}_2 \) or LY-83583 (6-anilinoquinoline-5,8-quinone, Sigma), a substance known to induce superoxide production by SMCs \( (4) \). As with studies using oxLDL, graft SMCs were found to be much more responsive to the stimulatory effects of LY-83583 than were the aortic SMCs. Collagen production by graft SMCs increased from 3.5 ± 0.2 to 15.2 ± 0.5 ng collagen/µg DNA \( (P < 0.0001) \) as the LY-83583 concentration was increased from 0 to 1.2 µM (Fig. 3). Over the same concentration range, collagen production by aortic SMC production rose from 2.2 ± 0.2 to 6.7 ± 0.6 ng collagen/µg DNA \( (P < 0.001) \). \( \text{H}_2\text{O}_2 \) did not increase collagen in the medium over concentrations from 0 to 50 µM. Collagen in graft SMC medium was relatively constant, changing from 2.4 ± 0.3 to 3.4 ± 0.2 ng collagen/µg DNA, and collagen in aortic SMC medium changed from 2.4 ± 0.2 to 2.7 ± 0.3 ng collagen/µg DNA over the \( \text{H}_2\text{O}_2 \) concentration range from 0 to 50 µM (data not shown). At concentrations above 50 µM, decreases in DNA were noted, suggesting toxicity. These findings suggest that superoxide, but not \( \text{H}_2\text{O}_2 \), causes an increase SMC production of collagen and that graft SMCs have a larger response to the oxidative stress than aortic SMCs.

Protein synthesis by graft and aortic SMCs was measured to assess cell viability and determine the specificity of changes in collagen secretion after incubation with agonists. SMC protein synthesis was determined in conditions identical to those for the collagen assay. \(^{[3]}\text{H}\)leucine (0.5 mCi/ml) was added to confluent aortic and graft SMCs for the determination of protein synthesis. Secreted protein synthesis and cell-associated protein synthesis after incubation with agonists, oxLDL

**Fig. 1.** Effect of oxidized low-density lipoprotein (oxLDL) and native LDL on type I collagen production by graft and aortic smooth muscle cells (SMCs). Passage 2 graft and aortic SMCs \( (n = 10) \) were grown to near confluence in medium 199 (M199) with 20% FBS and then changed to M199 with 5% FBS and varying concentrations of LDL or oxLDL. After 72 h, collagen in the conditioned medium was measured by ELISA, and the cells harvested for DNA measurement. Collagen levels were standardized per microgram of DNA. Data are means ± SE. * \( P < 0.01 \) vs. graft SMCs with no oxLDL. ** \( P < 0.001 \) vs. aortic SMCs with no oxLDL.

**Fig. 2.** Time course of collagen production by graft SMCs. Graft SMCs \( (n = 4) \) were grown to near confluence, and oxLDL (300 µg/ml) was then added. Conditioned medium and cells were collected at 24, 48, or 72 h for measurement of collagen by ELISA and DNA by fluorometric assay. Collagen levels were standardized per microgram of DNA. Depicted is the type I collagen secretion in the presence of oxLDL minus the collagen produced in medium alone during the same time.
**Fig. 3.** Effect of LY-83583 on type I collagen production by graft and aortic SMCs. Graft and aortic SMCs (n = 10) were grown to near confluence in M199 with 20% FBS and then changed to M199 with 5% FBS and varying concentrations of LY-83583. After 72 h, collagen in the conditioned medium was measured by ELISA, and the cells were harvested for DNA measurement. Type I collagen levels were standardized per microgram DNA. Data are means ± SE. *P < 0.001 vs. graft SMCs with no LY-83583. **P < 0.001 vs. aortic SMCs with no LY-83583.

(300 μg/ml) or LY-83583 (1.2 μM), were the same as under control conditions (Fig. 4). This suggested that the stimulatory effect of oxLDL and LY-83583 was relatively specific for collagen and that the increase in collagen was not simply part of a global increase in cellular protein synthesis. In addition, agonists were not toxic in the concentrations used as demonstrated by protein synthesis data and visual morphological analysis of SMCs.

The studies with oxLDL and LY-83583 suggest that reactive oxygen species may alter collagen production. Therefore, the ability of antioxidants to prevent the increase in collagen production by graft SMCs was evaluated. SOD (500 U/ml), catalase (4,000 U/ml), and N-acetylcysteine (NAC; 1 mM) were tested for their effect on the oxLDL-mediated increase in collagen production by graft SMCs. SOD and catalase had no effect, even when linked to polyethylene glycol (data not shown). This may have been due to their relatively short duration of action. NAC also had no effect.

Previous studies in our laboratory showed that oxLDL significantly increased PDGF production by graft SMCs, with aortic SMCs being less responsive (1), in a pattern similar to that for type I collagen production presented in the present report. This suggested that either a common mechanism upregulated both simultaneously or that PDGF was responsible for the increase in collagen. Therefore, the ability of antibody to PDGF to block the increase in collagen production stimulated by oxLDL was assessed. Anti-PDGF antibody significantly reduced the oxLDL-induced collagen production (Fig. 5).

The mechanism by which collagen production was increased was explored by determining gene expression for the α1-chain of collagen [procollagen α1(I) mRNA]. Collagen in the conditioned medium of graft and aortic SMCs incubated with agonists for 72 h was measured by ELISA. mRNA was isolated from the SMCs, and procollagen gene expression was determined using RT-PCR and represented as the ratio of the procollagen α1(I) signal to the GAPDH signal (Fig. 6). Collagen production and mRNA expression were increased in parallel by oxLDL and collagen. OxLDL and LY-83583 induced greater increases in collagen mRNA expression in graft SMCs than in aortic SMCs.

**DISCUSSION**

Intimal hyperplasia is a common cause of late graft failure in the clinical setting (11, 38). It is most severe adjacent to the anastomoses of prosthetic grafts where arterial cells grow onto the graft and lipids accumulate (5, 31). Although lesion development initially involves the migration and proliferation of SMCs, deposition of the extracellular matrix accounts for the later increase in lesion size (10). Lipid oxidation products that accumulate in grafts may contribute to the development of intimal hyperplasia by stimulating SMC proliferation, migration, and production of extracellular matrix proteins. Hypercholesterolemia causes more severe intimal hyperplasia in vein
expressed as the ratio of optical density (OD) for collagen to OD for GAPDH.

LDL (300 μg/ml), oxLDL (300 μg/ml), and LY-83583 (1.2 μM) were added. After 72 h, SMCs were harvested, and mRNA was isolated. Gene expression for collagen and GAPDH was determined by RT-PCR-ELISA. Results were expressed as the ratio of optical density (OD) for collagen to OD for GAPDH. A representative of 3 separate experiments is shown. Data are means ± SE.

grafts and prosthetic grafts in experimental animals (5, 19, 22, 25, 45) and decreases graft patency in humans (2, 8). Cholesterol-lowering agents reduce the development of vein graft stenoses and restenosis after coronary angioplasty (7, 26). Hyperlipidemia may contribute to intimal hyperplasia by stimulating collagen production, because hypercholesterolemic animals have a 10-fold higher rate of aortic collagen synthesis than controls (34).

SMCs from normal arteries produce low levels of collagen, but modified SMCs from prosthetic grafts exhibit a synthetic phenotype and produce higher levels of collagen. Our earlier investigations document higher collagen synthesis and procollagen α(I) gene expression by graft SMCs compared with aortic SMCs (30), but the cause of this was not explored. The present study shows that the constitutive production of collagen, as well as agonist-induced collagen production, is higher in graft SMCs than aortic SMCs. Jimi et al. (20) reported that collagen production by porcine aortic SMCs nearly doubled when they were incubated with LDL under oxidizing conditions. We also found that oxLDL had a significant effect on collagen production by early passage (passage 2) aortic SMCs that exhibited a differentiated phenotype. Interestingly, oxLDL stimulated a more marked increase in collagen production by graft SMCs that exhibit a synthetic phenotype. The greater effect of oxLDL and LY-83583 on collagen production by graft SMCs than aortic SMCs suggested that graft SMCs are more susceptible to oxidative stress.

The mechanism by which oxLDL and LY-83583 stimulates collagen production may involve production of ROS. Superoxide is known to stimulate collagen synthesis by hepatocytes and fibroblasts in vitro (9, 17), and a prooxidant system also induces collagen gene expression and protein production in liver fat-storing cells (35). In addition, oxidative stress, as evidenced by increases in TBARS, stimulates collagen synthesis in the rat aorta in vivo (41). Because oxLDL stimulates reactive oxygen species production by SMCs (16), oxLDL may upregulate collagen production through a redox-sensitive pathway. Our finding that LY-83583, which stimulates superoxide production, increases SMC collagen secretion, supports the role of ROS in the stimulation of collagen production by oxLDL. Jimi et al. (20) showed that butylated hydroxytoluene abrogated the increase in collagen production by porcine aortic SMCs incubated with LDL under oxidizing conditions, again suggesting that oxidation products are responsible. Interestingly, H2O2 does not stimulate collagen production by graft or aortic SMCs (data not shown). This could reflect the inability of H2O2 to stimulate collagen production or its effectiveness in stimulating production of matrix metalloproteinases that could break down the newly synthesized collagen (42).

Another mechanism by which oxLDL stimulates collagen production may be through increased PDGF production. We have shown that graft SMCs produce high levels of PDGF (37) and that oxLDL induces PDGF gene expression and protein production in graft SMCs (1). Conflicting reports on the effect of PDGF on collagen production exist in the literature. PDGF is reported to increase production of collagen types I and III by venous SMCs (3) and upregulate collagen gene expression in murine mesangial cells (27) but suppress collagen production by human arterial SMCs (33). This may be related to culture density or the proliferation state, because PDGF stimulates proliferation of subconfluent myofibroblasts but stimulates collagen production in confluent cultures (18). Jimi et al. (20) report that antibodies to PDGF and transforming growth factor (TGF)-β did not decrease collagen synthesis in quiescent, subconfluent SMCs, but we show that an antibody to PDGF suppresses the oxLDL-induced collagen production by graft SMCs. The apparent discrepancy may be explained by experimental differences. Jimi et al. studied collagen production over 24 h under oxidizing conditions, whereas our studies extended to 72 h. Thus an oxidative stress may stimulate an early increase in collagen production, but oxLDL induces a later increase PDGF production (1) that stimulates collagen production. Furthermore, oxLDL induces PDGF production by graft SMCs but not aortic SMC (1), suggesting that PDGF may cause the increased collagen production by synthetic SMCs but not contractile SMCs.

The effect of PDGF on collagen production can be mediated by TGF-β, because PDGF stimulates TGF-β secretion (27, 36), and TGF-β is a potent stimulator of collagen production. TGF-β induces collagen gene expression directly and also stimulates collagen production indirectly by the induction of PDGF production. Lipid peroxidation products have been shown to upregulate TGF-β gene expression in macrophages (28), but in our studies, oxLDL had no effect on TGF-β production by graft SMCs (data not shown). Thus TGF-β is not the major mediator of the oxLDL-stimulated collagen synthesis.

Our study shows that oxLDL consistently stimulates collagen secretion by graft SMCs that are in a synthetic phenotype and has a smaller but significant effect on contractile aortic SMCs. The increase in collagen is not secondary to a decrease in collagenase activity detectable by in-gel zymography (data not shown). We postulate that oxLDL or other oxidative stresses induce collagen accumulation. An oxidative stress stimulates a prolonged increase in PDGF gene expression and protein production in graft SMCs that is more pronounced for SMCs of a synthetic phenotype. PDGF in turn increases collagen gene expression and protein production. This may stimulate the accumulation of collagen in intimal hyperplastic lesions and adversely impact prosthetic graft patency.
GRANTS

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