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Local Delivery of 17-Beta-Estradiol Modulates Collagen Content in Coronary Porcine Arteries after PTCA and Stent Implantation

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Key Words
Restenosis · Vascular smooth muscle cell · Estrogens · Extracellular matrix · Collagen · Local delivery

Abstract
Background: Percutaneous transluminal coronary angioplasty (PTCA) and stent implantation are associated with intimal hyperplasia and extracellular matrix (ECM) accumulation, resulting in restenosis. We showed that local delivery of 17-beta-estradiol (17βE) reduced restenosis following PTCA and stent implantation by 47 and 23%, respectively. Because estrogens decreased type I and type III collagen synthesis in vitro, we hypothesized that local delivery of 17βE may influence intimal hyperplasia formation by modulating ECM expression. ***Methods:*** Porcine coronary arteries underwent PTCA or stenting and were randomly assigned to 17βE or placebo. After 28 days, animals were sacrificed for histology and collagen type I and III content analysis. ***Results:*** Both collagen subtypes increased in the media by 1.7 to 2.6-fold after PTCA and by 15.7 to 16.1-fold after stenting, as compared to PTCA segments. In the neointima, the ratio of collagen type III to type I was 2.7 in stented arteries and only 0.3 in PTCA arteries. In the neointima of 17βE-treated animals, collagen type I (but not type III) content upregulation was limited by 53% after PTCA and by 74% after stenting. ***Conclusion:*** Local delivery of 17βE reduces restenosis, in part by decreasing the density of collagen type I in the neointima in PTCA and stented arteries.

Introduction

Percutaneous coronary intervention (PCI) is an efficient technique to treat symptomatic atherosclerosis. However, the major limitation of PCI is related to restenosis, which occurs in 20–40% of patients within the first 6 months [1]. Stent, and especially drug-eluting stent (DES), implantations substantially reduce the angiographic restenosis rate [2, 3]. On the other hand, even with DES, restenosis remains a problem in some patients and lesion subgroups, e.g., diabetics or bifurcation stenosis. In-stent restenosis is mostly related to vascular smooth muscle cell (VSMC) migration and proliferation with extracellular matrix (ECM) synthesis [4]. ECM regulates various important events, such as remodeling, vascular cell proliferation and growth-factor production, which can contribute to the development of restenosis [5]. ECM tissue accumulation after coronary intervention occurs within 2 weeks and up to 3 months after either PTCA alone or PTCA followed by stent implantation [6]. The new ECM mainly consists of collagen (type I and type III) which can represent 25% of total protein and more than 50% of vascular protein upon angioplasty and stenting [7, 8].

The cardioprotective effects of estrogens have been well described in postmenopausal women. Estrogens induced beneficial effects on blood vessels by modulating the lipid profile and coronary artery relaxation. Previous studies in postmenopausal women have suggested that

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hormone replacement therapy prevents negative long-term outcomes of PTCA [9]. Moreover, in vivo experiments have demonstrated that estrogen deficiency is correlated with increased risk of atherosclerosis and neointimal formation after balloon injury [10]. Vascular effects of estrogens have been well studied but still little is known about how estrogens influence blood vessel structure and remodeling after vascular intervention. In vitro studies have shown that 17-beta-estradiol (17βE) reduces procollagen and collagen type I and III production by VSMC [11]. Concomitantly, clinical studies have reported that hormone replacement therapy can regulate the collagen to elastin ratio in human vascular tissues, suggesting that the downregulation of ECM might play a role in the reduction of atheromatous plaque size [12]. However, to the best of our knowledge, there have been no data reported on the role of estrogens on collagen type I to type III ratio after PTCA or stent implantation. In order to better understand the mechanisms by which a single delivery of 17βE modulates neointimal hyperplasia, we evaluated its effects on collagen type I and III content in porcine coronary arteries after PTCA and stent implantation.

Materials and Methods

Animal Preparation

Twenty three juvenile farm swine (11 immature females and 12 castrated males) weighing 20–25 kg were used as previously described [13]. The protocol was approved by the Montreal Heart Institute Animal Care and Ethical Experimentation Committee. A day before the procedure, each animal received 650 mg of acetylsalycylic acid and 30 mg of nifedipine orally. Prior to the procedure, animals were premedicated with intramuscular injection of 6 mg/kg of a mixture of tiletamine hydrochloride and zolazepam hydrochloride, they were given 0.05 mg of atropine and underwent general anesthesia with a mixture of enriched oxygen and isoflurane (1–1.5%). The femoral artery was then cannulated percutaneously, and an 8 French arterial sheath was introduced. Xylocaine (100 mg) and heparin (250 U/kg) were administrated intra-arterially. Activated coagulation time was maintained at >300 s throughout the procedure, with supplemental heparin given as required.

Procedure

Balloon angioplasty was performed with 3 inflations of 30 s at 10 atm pressure, with a 30 s interval between inflations. The balloon size was selected to provide a balloon to artery ratio of 1.1–1.2:1 and PTCA was performed adjacent to major side branches to facilitate identification during harvesting. Following two 30 s balloon inflations at 10 atm of 3 coronaries (the left anterior descending, circumflex, and right coronary arteries), each animal was randomized to receive either local delivery of 100 μg/kg of 17βE [number of arteries treated (n) = 28], 200 μg/kg of 17βE (n = 14) or vehicle [2-hydroxypropyl-β-cyclodextrin (HPCD); n = 27]. The HPCD-coated 17βE (Sigma) or vehicle, in a total volume of 5 ml, was delivered using the InfusaSleeve Catheter (LocalMed, Inc., Palo Alto, Calif., USA) at a driving pressure of 10 atm supported by balloon pressure of 6 atm, as previously described [14]. After the infusion, a slotted tubular stent (7 mm long; Palmaz-Schatz, Johnson & Johnson) was then deployed at a higher pressure of 14 atm for 30 s to achieve a stent to artery ratio of 1.3–1.4:1 and to provide optimized stent expansion as performed under clinical conditions.

Morphometry

All animals were euthanized under general anaesthesia at day 28 post-procedure, as previously described [13]. Briefly, following cross clamping of the descending thoracic aorta, the heart was perfusion-fixed in vivo with 10% formalin PBS-buffered solution and the coronary arteries were harvested immediately. PTCA and stented segments were stored in 10% formalin PBS-buffered solution for 24 h. PTCA segments were dehydrated with increasing concentrations of alcohol, treated with xylene and embedded in paraffin, and then cut into slices 6 μm thick with a microtome (Olympus Cut 4060E). Stented segments were processed by a modification of the technique described by Wolf et al. [15]. Sections of 8 μm thickness were prepared using a motorized microtome (Olympus) with a D-profile tungsten knife (Delaware Diamond Knives, Inc, Wilmington, Del., USA). Sections were then mounted on gelatin-coated slides for histological staining.

Morphometric Analysis

Morphometric analyses were performed under Verhoeff's staining condition. Measurements were made by digital planimetry. We analyzed and averaged the results from a minimum of 3 sections for each injured segment with PTCA and 2 sections from each stented segment that macroscopically demonstrated maximum lumen narrowing from proximal and distal halves. The areas of external elastic lamina, internal elastic lamina, lumen, neointima [internal elastic lamina – lumen] and media [external – internal elastic lamina] were obtained. Histological injury score was determined as previously defined by Schwartz et al. [16]. Vessel injury at each stent wire site was scored on a scale from 0 to 3, where 0 = endothelium denuded, 1 = internal elastic lamina lacerated, 2 = media lacerated, and 3 = external elastic lamina lacerated.

Collagen Density

Each cross-section of the coronary arteries was stained with picrosirius red to evaluate collagen type I and type III expression, as previously described [17]. Histological quantification of collagen was performed by capturing color images that were subjected to a series of natural density filters to insure a linear relationship between the intensity of the light and the gray level. Collagen type I, classified as orange/red pixels, and collagen type III, classified as green pixels, were individually selected and changed to white color using Adobe Photoshop 6.0. The modified color images were then transformed into monochrome with a 256-level gray scale using Image J (National Institutes of Health, Bethesda, Md., USA). Noncollagenous and unselected collagenous components were depicted by gray levels 1–255, and selected collagen (white pixels) was defined by gray level zero. Expression of collagen type I (orange/red) and type III (green) was evaluated by the relative number of pixels in the media and the neointima region divided by the total area of each region, by adjusting the threshold permitting a binary analysis.

Table 1. Morphometric analyses of porcine coronary arteries

Characteristics	PTCA + VHC	PTCA + 17βE	Stent + VHC	Stent + 17βE	p value
Segment analysed	17	24	7	12	
EEL area, mm^2	3.47 ± 0.20	3.83 ± 0.30	7.55 ± 0.88	8.26 ± 0.56	
IEL, mm^2	2.86 ± 0.18	3.06 ± 0.29	6.69 ± 0.81	6.99 ± 0.53	
Lumen area, mm^2	2.00 ± 0.17	2.19 ± 0.23	2.75 ± 0.64	2.75 ± 0.41	
Media area, mm^2	0.62 ± 0.06	0.77 ± 0.07	0.86 ± 0.14	1.07 ± 0.14	N/S
Neointima area, mm^2	0.85 ± 0.10	0.44 ± 0.04[a]	4.42 ± 0.28	4.05 ± 0.24	<0.05[a]
Mean injury score	1.38 ± 0.13	1.81 ± 0.10	1.50 ± 0.22	1.72 ± 0.16	N/S
Morphometric stenosis, %	30.57 ± 3.60	16.47 ± 2.98[a]	70.93 ± 4.94	55.08 ± 4.94[b]	<0.05[a, b]
VSMC density, cells/mm^2					
Media	5,278 ± 577	4,585 ± 227	4,599 ± 119	4,265 ± 156	N/S
Neointima	3,433 ± 202	3,811 ± 255	2,745 ± 106	3,005 ± 130	N/S

Unless otherwise indicated, figures are mean ± SEM. EEL = External elastic lamina; IEL = internal elastic lamina; VHC = vehicle; N/S = not significant.

[a] vs. PTCA + VHC. [b] vs. STENT + VHC.

Statistical Analysis

Values are expressed as mean ± SEM. To reach a 0.05 significance, a sample size of 3 animals per group (3 arteries per animal) was estimated to have a power of 80% to detect a 50% reduction in collagen type I expression in 17βE treatment groups as compared with the vehicle group. Statistical comparisons of morphometric and immunohistochemical data among groups were performed by ANOVA followed by Bonferroni's test correction for multiple comparisons. Pearson's correlation coefficient was used to determine the linear relationship between variables. Values were considered significant at $p < 0.05$.

Results

Follow-Up Post-Procedure

Following the procedure, animals recovered and gained weight steadily. Two animals died, one at 48 h and one at 72 h post-PTCA and local delivery. Autopsy on these animals revealed occlusive thrombus at the PTCA site. They were excluded from the analysis, bringing the number of animals studied to 21. Three coronary arteries developed extensive dissections following accidental sliding of the wire after predilation; drug delivery and stent implantation were not performed, and these arteries were excluded from analysis. During the local delivery, heart rate, electrocardiogram and blood pressure were noted, with no changes.

Morphometric Analyses

Morphometric analyses were performed on each coronary artery. The extent of the injury was similar among all arteries that underwent either PTCA or stenting. Surface area of the media was similar for both procedures. However, a greater percentage of stenosis was observed in stented arteries (70.9%) than those that underwent PTCA (30.6%). Overall, the effects mediated with low (100 μg/kg) and high doses (200 μg/kg) of 17βE were similar and no significant differences were observed between the two groups. A possible explanation for these results is that saturation of estrogen receptors with the lower dose prevented any additional effects of a higher dose of 17βE. Thus, to maximize the power analysis of our study, data from animals treated with 17βE (100 and 200 μg/kg) were pooled. When compared with control vehicle-treated groups, arterial segments treated with local delivery of 17βE showed a significant reduction of morphometric area stenosis, from 30 to 16% for PTCA and from 71 to 55% for stent, which represented a decrease of 47 and 23%, respectively (table 1). Previous work from our laboratory has demonstrated that local delivery of 17βE significantly decreases the number of proliferating cell nuclear antigen (PCNA)-positive VSMC after PTCA or stent implantation [13, 14]. Based on these results, we evaluated VSMC density in the media and the neointima at post-procedure day 28. In both media and neointima regions of PTCA segments, VSMC density was slightly higher than the equivalent regions of stented arteries. However, the data did not reach statistical significance. Treatment with local delivery of 17βE did not significantly change VSMC density in the media and the neointima for either procedure, suggesting a potential role of estrogen on ECM expression (table 1).

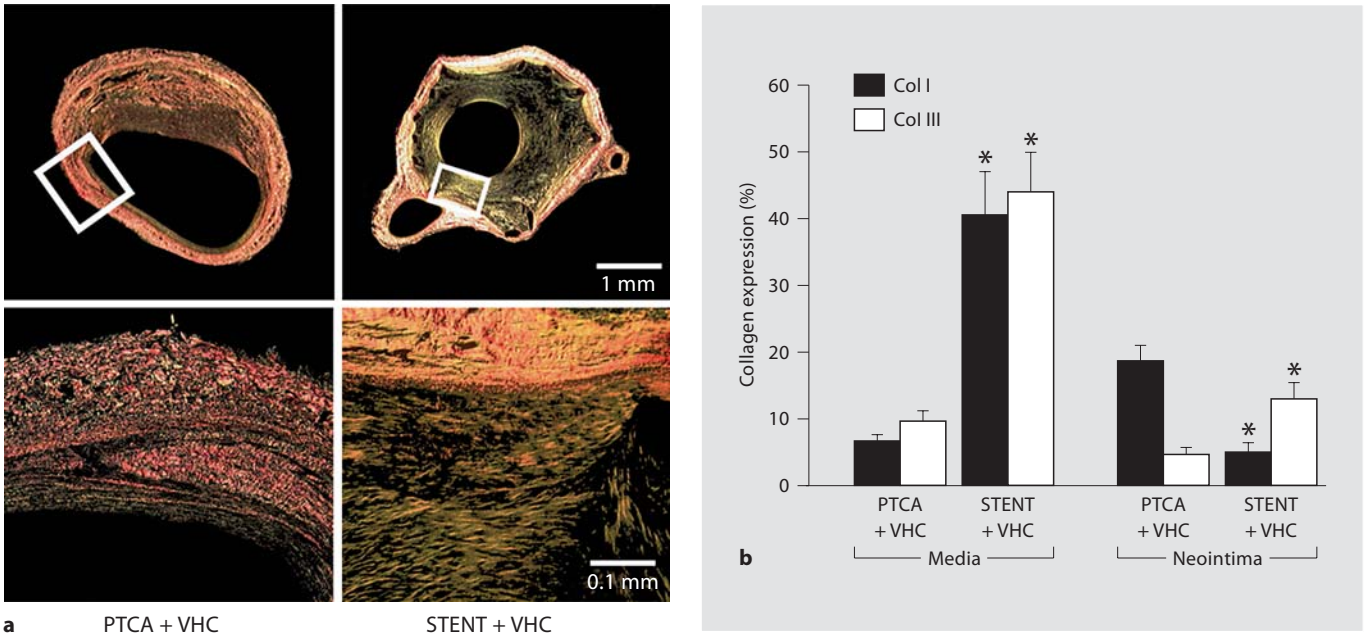

Fig. 1. a Picrosirius red-stained sections of coronary arteries 28 days post-PTCA or stenting. Magnification: ×20 (upper panels) and ×200 (lower panels). Expression of collagen type I is in red and type III is in green. **b** Expression of collagen types I and III in the media and neointima. The values are means ± SEM for each treatment. * $p < 0.05$ as compared to PTCA + vehicle (VHC).

Collagen Type I and Type III Content in PTCA and Stented Segments

In control segments, collagen content was relatively low. Collagen type I occupied only 2.4% whereas collagen type III represented 2.6% of the total surface area (data not shown). However, after PTCA collagen type I and collagen type III area fractions in the media increased by 1.7- and 2.6-fold, respectively. As compared to the PTCA segments, stent implantation increased collagen type I and type III levels in the media by 16.1- and 15.7-fold, respectively (fig. 1). On the other hand, in the neointima, the collagen type III to type I ratio was 0.3 in PTCA segments, compared to 2.7 in stented arteries (fig. 1). Our results indicated that, in contrast to PTCA, collagen type III is more predominant in the neointima 28 days after stent implantation.

Effects of Local Delivery of 17βE on Collagen Type I and Type III Levels

We previously demonstrated that local delivery of 17βE prevented restenosis formation after PTCA and stent implantation [13, 14]. Therefore, we evaluated if these effects of local delivery of 17βE were in part due to the reduction of collagen type I and type III content. Treatment with local delivery of 17βE following PTCA reduced the level of collagen type I in the media by 72% and type III by 85%, compared to segments unexposed to 17βE (fig. 2). However, in the neointima, only collagen type I content was decreased (by 53%) under 17βE treatment (fig. 2). Based on our results, we were able to establish a positive correlation between collagen type I levels and injury score. As expected, elevated density of collagen type I in the media (fig. 3a) and neointima (fig. 3b) correlated with severity of injury. The reduction, compared with untreated arteries, of collagen type I by local delivery of 17βE was observed in the media (fig. 3a) as well as in the neointima (fig. 3b) in the case of moderate and severe injury scores. In stented segments, levels of collagen type I and type III were increased in both the media and neointima (fig. 4). Local delivery of 17βE reduces by 63% the induction of collagen type I content in the media and by 74% in the neointima, as compared with the vehicle-treated segments. However, collagen type III density was not significantly changed by 17βE treatment (fig. 4). In the same series of experiments, we observed that increased levels of collagen type I in the media (fig. 5a) and the neointima (fig. 5b) of stented segments correlated positively with injury scores. As for PTCA, re-

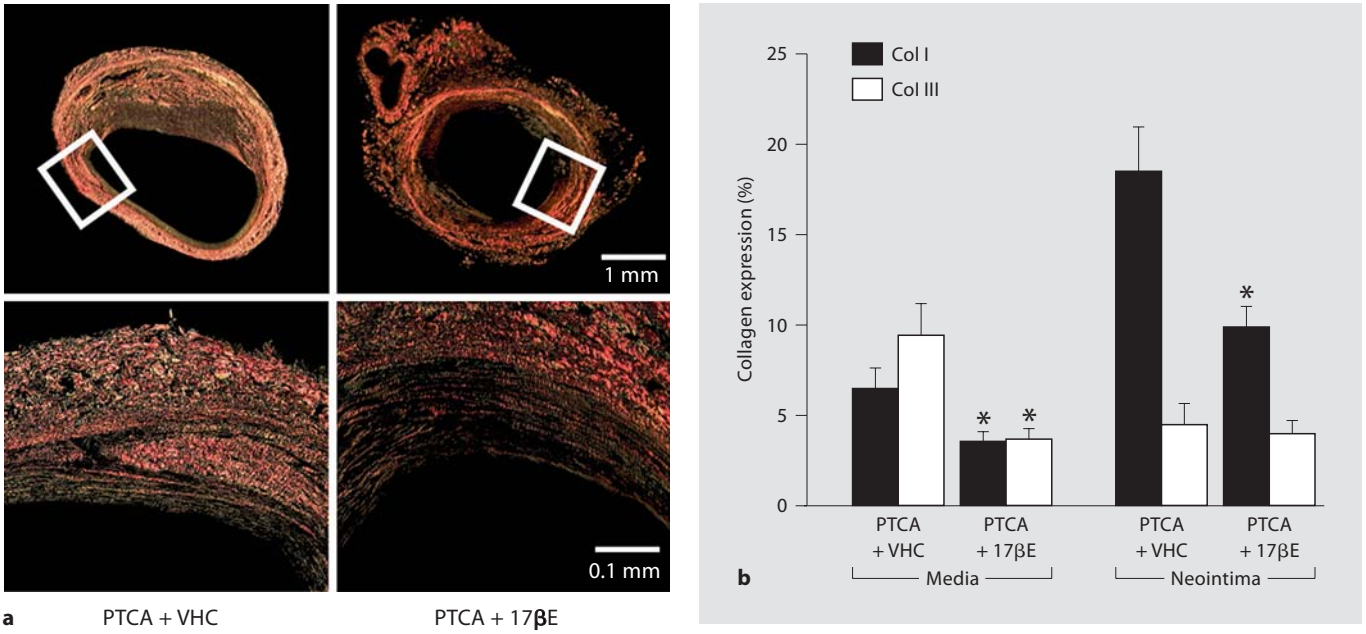

Fig. 2. a Picrosirius red-stained sections of coronary arteries 28 days post- PTCA with or without local delivery of 17βE. Magnification: ×20 (upper panels) and ×200 (lower panels). **b** Expression of collagen types I and III in the media and neointima. The values are means ± SEM for each treatment. * $p < 0.05$ as compared to PTCA + vehicle (VHC).

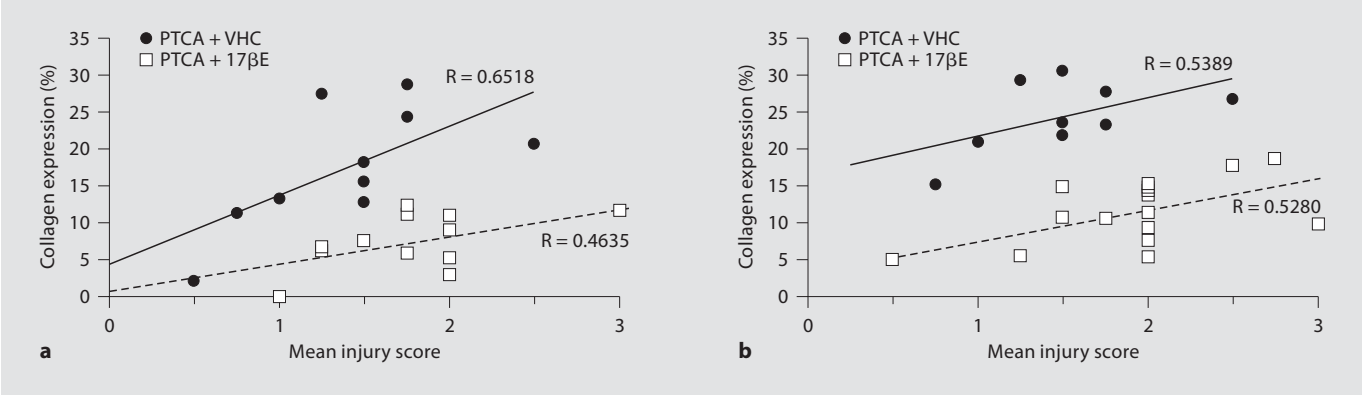

Fig. 3. The correlation between collagen type I expression and injury score in the media (**a**) and in the neointima (**b**) with or without local delivery of 17βE after PTCA. VHC = vehicle.

duction in collagen type I expression in 17βE-treated stented segments was observed with moderate and severe, but not mild, injury scores in both regions (fig. 5a, b). Local delivery of 17βE did not reduce collagen type III content in either region of dilated or stented arteries. Thus, no correlation was found between the levels of collagen type III and injury score with the single bolus delivery of 17βE (data not shown).

Discussion

Restenosis results from a healing and remodeling response to vascular injury. As determined by histological analysis of lesion specimens obtained by atherectomy, VSMC proliferation and ECM deposition increased after stent implantation. Although little is known of the exact mechanisms of ECM accumulation after PTCA and stent

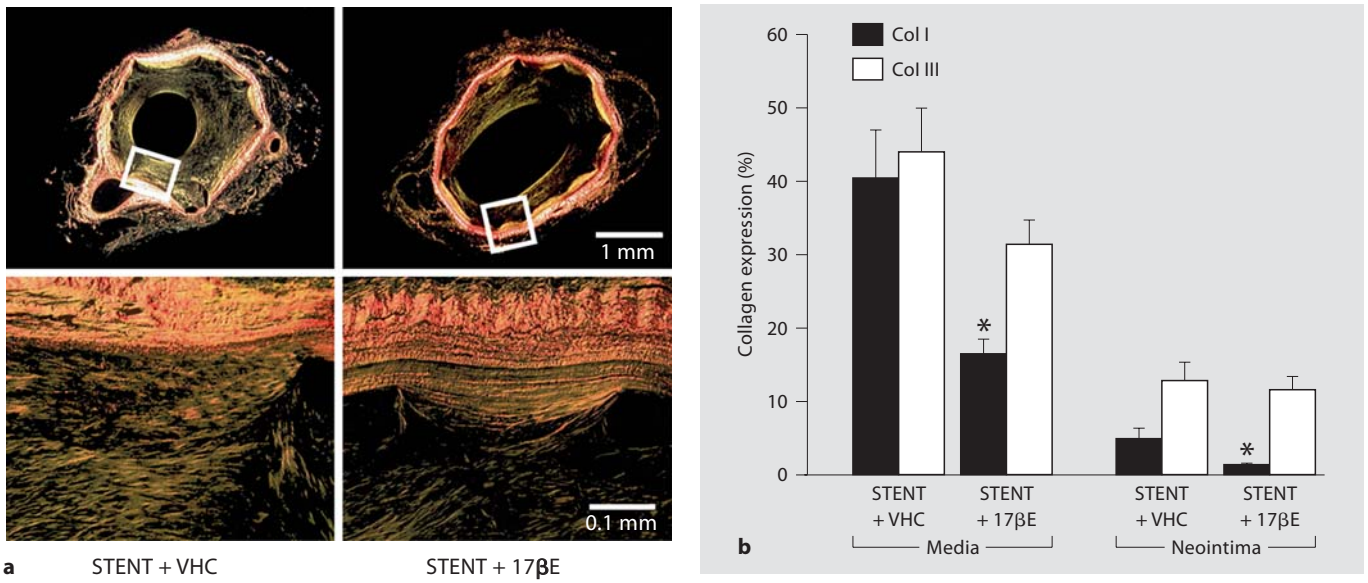

Fig. 4. a Picrosirius red-stained sections of coronary arteries 28 days after stent implantation with or without local delivery of 17βE. Magnification: ×20 (upper panels) and ×200 (lower panels). **b** Expression of collagen types I and III expression in the media and neointima. The values are means ± SEM for each treatment. * $p < 0.05$ as compared to Stent + vehicle (VHC).

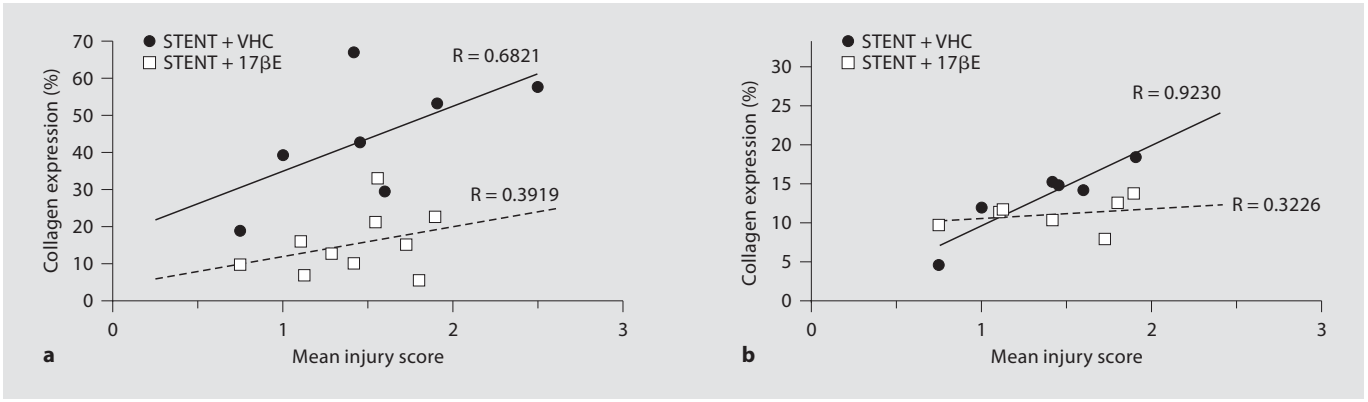

Fig. 5. The correlation between collagen type I expression and injury score in the media (**a**) and in the neointima (**b**) with or without local delivery of 17βE after stenting. VHC = vehicle.

implantation, the use of several techniques and DES has considerably reduced the rate of restenosis. However, recent studies reported an increased rate of late thrombosis events with DES [18]. Therefore, new strategies are required for better long-term prognosis and restenosis prevention. Our group previously demonstrated that local delivery of a single bolus of 17βE reduced restenosis after PTCA and stent implantation by reducing PCNA-positive VSMC numbers and the inflammation process [13, 14]. In this study, we reported a significant difference in the ratio of collagen type III to type I in the neointima between PTCA (where collagen type I predominates) and stent implantation (mostly collagen type III). We also demonstrated that local delivery of 17βE significantly reduced levels of collagen type I, but not type III, in the neointima after both PTCA and stent implantation.

ECM Composition and Restenosis

ECM production plays an important role in the physiopathology of restenosis, which modifies the new and pre-existing ECM by exaggerated ECM synthesis. In a normal vessel, contractile VSMC have limited capacity to produce new ECM proteins [19]. Previous studies suggested that PTCA induced a 4- to 10-fold increase in collagen synthesis [20]. In contrast, another group indicated that collagen content in iliac arteries is significantly decreased in restenotic versus nonrestenotic vessels after angioplasty in the atherosclerotic rabbit model [21]. The use of different models and arteries can explain these opposing results. In our study, we observed a significant increase of collagen type I and type III levels in the media and the neointima after PTCA and stent implantation. Our results indicate that collagen type III is the predominant collagen subtype in the neointima 28 days after stenting, which is in contrast to PTCA segments, where intimal hyperplasia mostly contain collagen type I. Collagen type III has previously been associated with immature thin collagen fibres, as compared to mature thick collagen type I fibres [22]. According to these studies and our results, we suggest that stabilization of the vascular healing process may not be fully complete 28 days after stent implantation in porcine coronary arteries. A study has shown that porcine coronary arteries stented for 2–6 months have reduced levels of collagen type III and an upregulation of collagen type I [23]. This observation was also reported in human stented coronary arteries, with clear changes in collagen content in the neointima over time. Lesions of <18 months had a high content of collagen type III while older lesions were mainly enriched by collagen type I expression [24].

Effects of Local Delivery of 17βE on Collagen after PTCA and Stenting

Animal and epidemiological studies provided evidence that hormone replacement therapy could insure cardioprotection in postmenopausal women. Indeed, estrogen replacement may offer beneficial effects against clinical coronary events after PTCA in postmenopausal women [9]. Previous experiments showed that estrogen replacement therapy improved long-term outcomes after PTCA in this group. Moreover, in a porcine model, estrogen administered intramuscularly inhibited VSMC migration, proliferation and neointima formation [25, 26]. In contrast to these studies, which used systemic estrogen treatment, we provide new evidence on the potential role of estrogens using local delivery of a single bolus. Furthermore, unlike antiproliferative and antichemotactic agents such as rapamycine and paclitaxel, which may delay arterial healing by reducing re-endothelialization and increase the risk of late thrombosis events [18, 27], estrogens promote vascular healing by favouring endothelium regeneration after the injury. To better understand the effects of 17βE on restenosis, we evaluated the density of collagens type I and III following a single bolus of 17βE. Our results showed that local delivery of 17βE after PTCA partially inhibited the upregulation of both collagen types in the media while modulating downwards collagen type I expression in the neointima. In comparison, local delivery of 17βE after stent implantation prevented collagen type I accumulation in both regions but had no significant impact on collagen type III level. We have previously reported that local delivery of 17βE reduced PCNA-positive VSMC in PTCA segments. The reduction of collagen type I density by local delivery of 17βE may be attributed to the lower number of synthetic VSMC, rather than inhibition of collagen synthesis. However, our data demonstrated that 28 days following PTCA or stenting, the total number of VSMC did not differ significantly from 17βE-treated segments, as compared to vehicle segments in both regions (table 1), which suggests a direct role for estrogen in the collagen synthesis/degradation process. Previous experiments reported that collagen type I increased significantly after ovariectomy and was normalized with estrogen treatment [28]. Estrogens could act on collagen expression through different mechanisms. Various studies have demonstrated that estrogens induce cyclic adenosine monophosphate production, which reduces collagen synthesis in cultured human VSMC [29]. In contrast, a recent study observed an increase of collagen fraction in the media and neointima of estrogen-treated rabbits as compared to control [30]. Our study showed opposite results, which can be explained by various factors such as the animal model used (pig vs. rabbit), the injury time course (28 vs. 14 days), and the arteries used (coronary vs. iliac).

The effects of estrogens are mainly mediated by estrogen receptors (ER). Two ERs have so far been identified, ERα and ERβ, which are both expressed and functional in cardiovascular tissues. A negative correlation was previously established between ERα expression and collagen content in pre- and postmenopausal women [31]. It is known that through ERα estrogens activate endothelial nitric oxide synthase, and as a result increase NO release. Previous studies have reported that inhibition of NO production is marked by upregulation of collagen type I and that NO donors added directly to the VSMC decrease col-

lagen type I expression [32]. Consequently, more studies will be required to determine the precise mechanism by which 17βE impacts on collagen I accumulation in dilated or stented arteries, either directly or indirectly through NO release, and if this regulation is ER-mediated.

Limitations

The ECM protein accumulation in the restenosis lesion is determined by a dynamic interplay between matrix synthesis by VSMC and degradation by matrix metalloproteinases. A previous study using cultured aortic smooth muscle cells from atherosclerosis-susceptible (C57BL6/J) mice has shown that estrogens decreased collagen type I synthesis without affecting matrix metalloproteinase activity [33]. However, another group has stated that estrogen treatment is also associated with increased collagen degradation and impacts on the synthesis of procollagen types I and III [34]. In the present study, we did not evaluate the specific effects of 17βE on collagen degradation. At this point, we cannot confirm if local delivery of 17βE inhibited collagen synthesis or increased collagen degradation by activation of matrix metalloproteinases. Further studies will be required to answer these questions. Our model of over-expansion injury of juvenile swine also has some limitations since healthy pigs do not have flow-limiting natural atherosclerosis and extreme injury is not induced in proper stent procedures in humans. Therefore, the relevance of our study to human clinical medicine needs further experiments using natural atherosclerosis stenosis in pigs.

Conclusion

In summary, we showed that after PTCA and stent implantation, the level of collagens type I and type III increased in the media and the neointima. We also showed that collagen type III is predominantly present in the neointima after stenting, compared to PTCA. The ability of local delivery of 17βE to prevent neointimal hyperplasia was previously shown after PTCA and stent implantation. In our study, we demonstrated that local delivery of 17βE in PTCA and stented arteries decreased collagen type I content in the neointima and intimal hyperplasia. Therefore, we propose another pathway by which single bolus of 17βE reduced restenosis formation.

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