# Different Inflammatory Response and Oxidative Stress in Neointimal Hyperplasia after Balloon Angioplasty and Stent Implantation in Cholesterol-fed Rabbits

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running title: CRP and oxidative stress in restenosis after ballooning and stenting.

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**Summary**

Inflammatory responses appear to play an important role in the occurrence of restenosis following coronary intervention. However, contribution of C-reactive protein (CRP) and oxidative stress to restenosis after balloon angioplasty and stent implantation remains unclear. The aim of this study is to examine this issue using hyperlipidemic rabbits. Rabbits were divided into two groups; fed with a 0.5% cholesterol diet and fed with a mixed 0.5% cholesterol and 0.5% probucol diet. Each group of rabbits underwent balloon injury and stent implantation in right and left iliac arteries, respectively. Eight weeks after intervention, we examined luminal stenosis, neointimal hyperplasia, immunoreactivity for macrophage, CRP and oxidized phosphatidylcholine (oxPC), and also expression of CRP mRNA. The degrees of neointimal hyperplasia and immunopositive areas (%) for macrophage, CRP and oxPC in the neointima were significantly higher after stent implantation than after balloon injury, but CRP mRNA was undetectable in either artery. Anti-oxidant probucol reduced angiographic stenosis, neointimal hyperplasia, macrophage- and oxPC-positive areas much more significantly after stenting. The results demonstrate that inflammatory response to the development of neointimal hyperplasia differs after balloon injury and stent implantation and that CRP deposition and oxidative stress might be involved more significantly in neointimal development after stent implantation.

**Introduction**

Restenosis after percutaneous coronary interventions (PCI) is an important clinical issue because large numbers of coronary interventions are required and their indication is expanding. Although the use of drug-eluting stent dramatically reduced the incidence of restenosis, restenosis still remains a crucial problem [1]. Restenosis after balloon angioplasty is considered to arise through a combination of inadequate or deleterious arterial remodeling and neointimal hyperplasia, whereas in-stent restenosis arises primarily from neointimal hyperplasia [2, 3]. Increasing evidence from both clinical and animal studies indicates that inflammation plays a pivotal role in restenosis after both types of intervention [4].

C-reactive protein (CRP) is an acute phase protein that can serve as a marker of inflammation. In prospective epidemiological studies, plasma levels of CRP predict future cardiovascular events [5] and recent clinical studies indicate that this protein is also a predictor for restenosis after PCI [6, 7]. We previously reported that positive immunostaining for CRP in initial culprit lesions could predict the outcome of directional coronary atherectomy (DCA) [8], and CRP was more involved in the pathogenesis of in-stent restenosis than in restenosis after DCA [9]. In addition, CRP has a pro-oxidative effect [10] and oxidation is implicated in atherogenesis and restenosis after PCI [11, 12]. These lines of evidence suggest that CRP and oxidized lipoprotein are directly involved in the development of restenotic lesion. However, expression and localization of these molecules in restenotic lesions has been not well examined.

The present study investigates the synthesis of CRP, the localization of CRP and

oxidized lipoprotein in neointimal hyperplasia after balloon injury and stent implantation in hyperlipidemic rabbits, and furthermore examines the effects of anti-oxidant probucol on neointimal growth and the inflammatory response.

## Materials and Methods

### Balloon injury and Stenting protocol

In this study, 24 male Japanese White rabbits weighing 3.1 to 3.3 kg were used in research protocols that were approved by the Animal Care Committee of University of Miyazaki (No. 2003-011). All animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

The rabbits were randomly separated into Group C (n = 12) that was fed with a diet containing 0.5% cholesterol and Group P (n = 12) that received a mixed diet containing 0.5% cholesterol and 0.5% probucol (Daiichi Pharmaceutical Co. Tokyo, Japan). All surgical procedures proceeded under aseptic conditions and general anesthesia was applied through an intravenous injection of pentobarbital (25 mg/kg, body weight). One week after starting the diets, an angioplasty balloon catheter (2.5-mm diameter, 9-mm length; QUANTUM, Boston Scientific, Golway, Ireland) was inserted via the carotid artery into the bilateral iliac arteries, inflated at 12 atm, and pulled back twice to denude the endothelium. Immediately thereafter, an ACS Multi-link Plus stent (2.5-mm-diameter, 15-mm-length; Guidant, Temecula, CA, USA) mounted over the balloon, was implanted in

the left iliac artery (30-second inflation at 10 atm, stent-to-artery ratio; 1.2:1 to 1.3:1), then the balloon catheter (without stent) was moved to the right iliac artery and balloon injury was performed (30-second inflation at 10 atm). Immediately and 8 weeks after stenting or balloon injury, the iliac arteries were imaged by angiography, and the luminal diameter was measured. Then the rabbits were killed with an overdose of pentobarbital (60 mg/kg, i.v.) 5 minutes after an injection of heparin (500 U/kg i.v.). The animals were perfused with 50 ml of 0.01 mol/L phosphate buffered saline, and then perfusion-fixed with 4% paraformaldehyde for immunohistochemical evaluation.

**Serum lipid and Peroxidation marker sampling and analysis**

Fasting blood samples were taken from the central ear artery of each rabbit before starting the cholesterol diet as well as 2, 4, and 8 weeks after injury. Serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) levels were measured using Eiken T-CHO (Eiken Kagaku, Tokyo, Japan), Cholestest LDL (Daiichi Kagaku, Tokyo, Japan), and Triglyceride G test (Wako Chemical, Osaka, Japan), respectively. Serum lipid peroxide was estimated as thiobarbituric acid-reactive substances (TBARS) using a lipoperoxide test kit (Wako Chemical, Osaka, Japan).

**Light microscopy and Immunohistochemistry**

The iliac arteries were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Sections (3 μm thick) were stained with hematoxylin and eosin/Victoria blue dye (HE/VB), and were examined immunohistochemically using

antibodies against alpha-muscle actin (HHF35, DakoCytomation, Glostrup, Denmark), rabbit macrophages (RAM11, DakoCytomation), CRP (CRP-8, Sigma, Saint Louis, Missouri, USA) and oxidized lipoprotein (DLH3) [13]. DLH3, a monoclonal antibody, reacts with oxidized phosphatidylcholine (oxPC) in oxidized lipoproteins. Regions ($mm^2$) of neointima, media, inside the external elastic lamina (EEL), and areas of positive immunoreaction (%) for smooth muscle cells, macrophages, CRP, and oxPC in the neointima in at least 5 cross sections of each injured area were measured using an image analysis system (Axio Vision 2.05 Carl ZEISS, Munchen, Germany). Two of the investigators (E. F. and K. H.) who were blinded to the treatment assignment performed the morphological analyses.

**Rabbit CRP mRNA amplification by the polymerase chain reaction**

The neointima of the bilateral iliac arteries were carefully separated from the media and adventitia. Liver and neointimal RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). All samples were amplified by the reverse transcription-polymerase chain reaction (RT-PCR) with oligonucleotide primers specific for rabbit CRP and rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences that amplified a 180 bp fragment of the rabbit CRP gene were as follows: forward, 5-AGGATCAGGATTCGTTTG-3; reverse, 5-CACCACGTACTTGATA-TGTC-3. The primer sequences that amplified a 154 bp fragment of the rabbit GAPDH gene were as follows: forward, 5-CGCCTGGAGAAAGCTGCTA-3; reverse, 5-CCCCAG-CATCGAAGGTAGA-3. The PCR profile consisted of 35 cycles of denaturation at 94℃

for 1 minute, annealing at 57℃ for 45 seconds, and elongation at 72℃ for 1 minute, followed by a final extension at 72℃ for 10 minutes. Each sample set included one control that was not reverse transcribed and another without a cDNA template. The PCR products were visualized by ethidium bromide staining on 2% agarose gels.

**Statistical analysis**

All data are presented as means ± S.E.M. An unpaired Student's *t* test and ANOVA with Bonferroni multiple comparisons performed comparisons between groups. A value of *P* less than 0.05 was considered significant.

**Results**

**Serum concentrations of TC, LDL-C, TG and lipid peroxide**

Table 1 shows the serum concentrations of TC, LDL-C, TG, and TBARS. These levels, except for TBARS of Group P, increased with times at 2, 4 and 8 weeks in both groups. Values of TC, LDL-C, and TG were lower in Group P than in Group C, but not statistically significant. Whereas TBARS level was significantly lower in Group P than in Group C. The weights of both groups of rabbits similarly increased (data not shown).

**Luminal stenosis after balloon injury and stent implantation**

Angiographs of rabbit iliac arteries obtained 8 weeks after balloon injury (right iliac artery) and stenting (left iliac artery) revealed marked luminal stenosis in Group C (Fig. 1A). The luminal diameters of the stenotic arteries did not significantly differ after

either procedure (Fig. 1C and D). Probucol (Group P) obviously prevented stenosis after each type of intervention (Fig. 1B, C and D), but the effect was more significant with respect to that after stenting (Fig. 1D).

**Neointimal Hyperplasia after Balloon Injury and Stent implantation**

Neointimal hyperplasia was histologically evident in the both arteries after balloon injury and stenting (Fig. 2A and B). Areas of the neointima after stenting were significantly larger than those after balloon injury, and probucol significantly inhibited the neointimal hyperplasia of both injured arteries (Fig. 2C). Areas inside the EEL were significantly different between after ballooning and stenting (Fig. 2D). The areas 8 weeks after ballooning were significantly smaller than those of immediately after ballooning, which indicates the constrictive remodeling. While there was no difference in the areas after stenting.

**Immunohistochemistry of Neointimal Hyperplasia**

The neointima generated after each type of intervention was composed of proliferating smooth muscle cells, macrophages, and extracellular matrix (Fig. 3). Although macrophages were present in the neointima produced after both procedures in Group C, more cells were evident after stenting and these were located relatively deeply in the neointima (Fig. 3 and Fig. 4A). Immunoreactions against CRP and oxPC were positive in stent-induced neointima, and were relatively colocalized to macrophage-positive areas, especially surrounding stent struts. In contrast, balloon-induced neointima contained very

few CRP- and oxPC- positive areas (Fig. 3). Probucol (Group P) significantly reduced neointimal hyperplasia and immunopositive areas for macrophages, and CRP after both types of intervention, and immunopositive area of ox-PC after stenting (Fig. 3 and Fig. 4C and D). Conversely, the amount of areas that were positive for smooth muscle cells was significantly increased in Group P (Fig. 3 and Fig. 4B).

**CRP mRNA expression in liver and neointima**

We detected CRP mRNA in the liver but not in the neointima 8 weeks after balloon injury or stenting (Fig. 5).

**Discussion**

The present study demonstrated that the inflammatory response during neointimal development differs after balloon injury and stent implantation. In addition, although the anti-oxidant prevented the development of stenosis after both types of vascular injury in the hyperlipidemic rabbits, it was more effective after stenting.

Restenosis is the process of luminal narrowing in an atherosclerotic artery after intervention such as balloon angioplasty and stenting. Increasing evidence indicates that the underlying mechanisms of restenosis are significantly regulated by inflammatory mediators and thus anti-inflammatory therapy might prevent restenosis after PCI [4]. Animal studies have shown that monocytes invade the forming neointima, and that a blockage of early monocyte recruitment with anti-inflammatory agents reduces the amount of late restenosis [14-16]. Moreno et al. [17] found a close positive correlation between the

numbers of macrophages in coronary plaques at the time of angioplasty and subsequent susceptibility to restenosis in a study of DCA samples. On the other hand, pathological observations of coronary stenting in humans found important relationships among inflammation, vascular injury, and neointimal growth [18, 19]. Our recent study demonstrated that the inflammatory response is more involved in the mechanism of in-stent restenosis than in restenosis after DCA [9]. Experimental studies of in-stent restenosis also showed prominent inflammatory infiltration in neointimal hyperplasia [14, 20], and a linear correlation between monocyte adherence and neointima [14]. The mechanisms of this obvious inflammatory response would be dependent on mechanical vascular disruption by stenting and an inflammatory response to stent materials. Horvath et al. [21] have demonstrated that inflammatory responses differ after balloon injury and stent implantation in normolipidemic animals. In their model, balloon injury induced a transient phenomenon consisting predominantly of an influx of neutrophils, whereas the inflammatory response to stent implantation was more prolonged and the accumulation of macrophages was sustained. Because macrophages were rich in neointima after stenting, cytokines (such as tumor necrosis factor-α, interleukin-1, and transforming growth factor β) and growth factors (such as platelet-derived growth factor and insulin-like growth factor I) produced by macrophages might enhance inflammatory response and were involved in neointimal development [22].

Immunoreactivity against CRP was significantly more abundant in the neointima after stenting. Not only is CRP a powerful predictor of future ischemic heart disease [5], but recent clinical studies also indicate that it is a reliable marker of restenosis

after stenting [7, 23]. Although the mechanism remains unclear, CRP can actively stimulate the classical complement pathway and enhance phagocytic activity as an important mediator of innate immunity [24, 25]. CRP stimulates endothelin-1 and some inflammatory cytokines leading to the activation of smooth muscle cells [26, 27]. Moreover, CRP induces upregulation of matrix metalloproteinase in macrophages [28, 29]. CRP immunoreactivity was observed in stent-induced neointima, and was relatively colocalized to macrophages. Although the biological activity of CRP in the neointima can not be evaluated in this study, these findings suggest that CRP deposition is closely associated with inflammatory responses and plays an important role in neointimal growth after stenting. CRP is present within human atheromatous plaques, and is primarily localized to macrophages and extracellularly within the lipid core [9, 30]. Yasojima et al. [30] reported that higher levels of CRP mRNA were expressed in human atherosclerotic lesions than in the liver. However, we found little CPR mRNA in coronary atherosclerotic lesions of a patient with unstable angina [31]. Recent animal study by Sun et al. [32] clearly demonstrated that CRP protein found in atherosclerotic lesions is derived from the circulation rather than synthesized *de novo* by vascular cells. The present animal study also found that CRP immunoreactivity was relatively colocalized to macrophages and the surrounding extracellular matrix, whereas CRP mRNA was undetectable by RT-PCR in the neointima after ballooning and stenting. The results suggest that most of the CRP deposited in neointima is not produced by neointimal cells but is rather derived from plasma. The discrepancy might be due to differences between restenotic and atherosclerotic lesions as well as among animal species.

Probucol significantly prevented the development of stenosis after each type of intervention, but the effect was more obvious after stenting. Oxidative stress is involved in the development of atherosclerosis and restenosis [11, 12] and balloon injury indeed leads to the immediate release of oxygen species [33]. Ox-PC, a component of oxidized low density lipoprotein, is a marker of local oxidative stress. Moreover, oxidized lipoproteins modulate inflammatory responses in atherosclerotic lesions by expression of inflammatory genes, and are capable of inducing specific monocyte adhesion to endothelial cells [34, 35]. The accumulation of macrophages and ox-PC surrounding stent struts suggests that persistent oxidative stress and inflammatory response could be involved in the neointimal growth after stenting. Therefore, powerful antioxidants might represent a promising treatment for restenosis after coronary intervention. We and others have shown that probucol prevents neointimal hyperplasia in injured vessels of hyperlipidemic animals [36-38], and reduces restenosis in patients who have undergone PCI [39-42]. The preventive effect of probucol after stent implication has not been thoroughly addressed. The recent Canadian Antioxidant Restenosis Trial (CART-1) demonstrated that both probucol and AGI-1067, a metabolically stable modification of probucol, improve luminal dimensions at the site of stent placement 6 months thereafter [43]. The cellular mechanisms were not identified in that clinical study.

The present study found that the immunopositive area of oxPC in stented vessels was obviously decreased in Group P compared with Group C. Levels of TBARS in Group P were reduced to below the baseline. Group P showed lower serum lipid levels than Group C, but remains hyperlipidemic condition. We therefore consider that the

TBARS reduction would depend mainly on the anti-oxidative properties of probucol, which are more important for reducing neointimal hyperplasia after stent implantation than for lowering lipid levels.

The anti-restenotic mechanism of probucol remains somewhat obscure, but anti-oxidants prevent endothelial dysfunction, lipid oxidation and macrophage activation [44]. Furthermore, probucol significantly inhibits platelet-derived growth factor-A expression in injured arteries [45]. This would limit smooth muscle cell proliferation, migration and extracellular matrix production. In addition, the present study discovered that probucol suppresses CRP deposition in the neointima, although the mechanism remains unknown. These factors together might prevent neointimal growth and modify vascular remodeling leading to luminal narrowing.

In conclusion, our results indicated that the inflammatory responses to neointimal hyperplasia differ after stent implantation and balloon injury. Stent-induced neointimal hyperplasia is richer in macrophages, CRP and oxPC. CRP deposition and oxidative stress might be involved more significantly in neointimal development after stent implantation.

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**Figure legends**

Figure 1. Representative angiographs of rabbit iliac arteries 8 weeks after balloon injury and stent implantation.

Angiographs of Groups C (A) and P (B). The lumen of bilateral arteries injured by balloon catheter (white arrows) and stent implantation (black arrows) is significantly narrow in Group C, but absent or minimal in Group P. Luminal diameters of the arteries before, immediately after, and 8 weeks after balloon injury (C) or stent implantation (D). n=6 each, *P<0.001, **P<0.05.

Figure 2. Representative light micrographs of rabbit iliac arteries 8 weeks after balloon injury and stent implantation.

Neointima after balloon injury (A) and stent implantation (B). Areas of neointima/media ratio 8 weeks after ballooning (C). n=6 in each group, *P<0.05, **P<0.001, ***P<0.05. Areas inside the external elastic lamina immediately after and 8 weeks after stenting (D). n=6 in each group, *P<0.001.

Figure 3. Representative immunohistomicrographs of rabbit iliac arteries 8 weeks after balloon injury and stent implantation. St, Stent strut.

Figure 4. Immunopositive areas (%) for macrophage (A), smooth muscle cells (B), CRP (C) and oxPC (D). n=6 in each group, * P<0.001, ** P<0.05

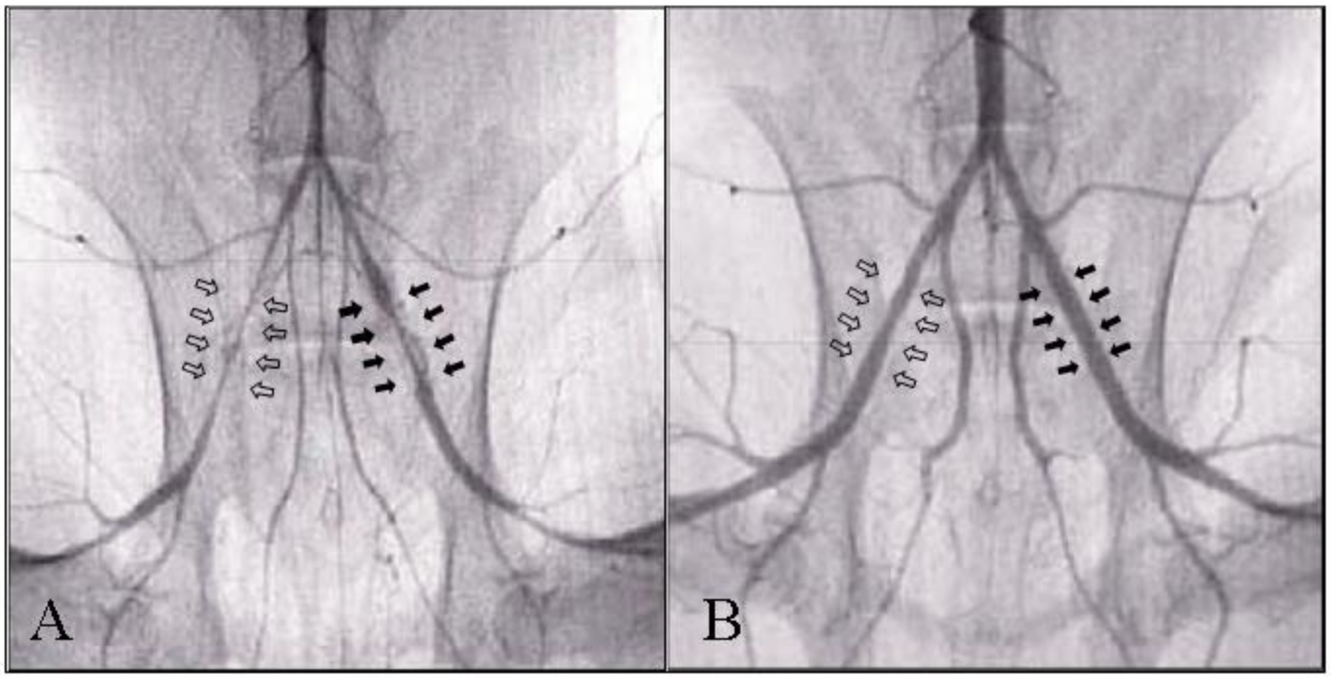
Figure 5. Expression of rabbit CRP mRNA in liver and iliac artery.

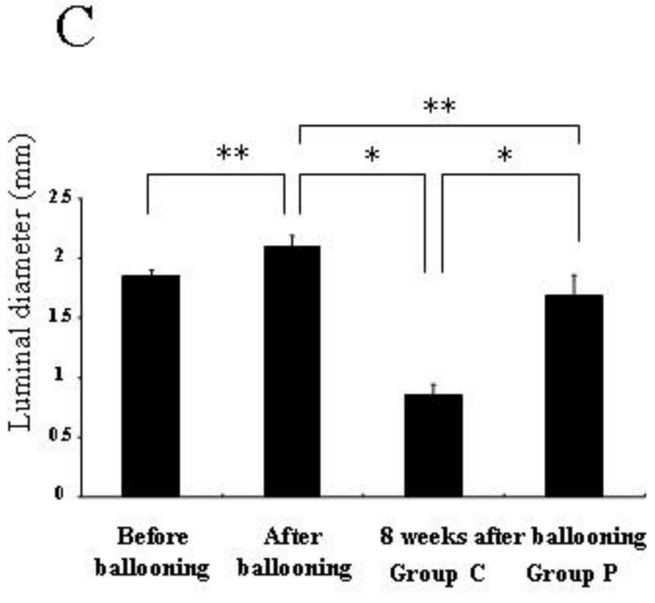
Lane 1 represents 100 bp DNA ladder; Lane 2 and Lane 3, liver; Lane 4 and 5, iliac artery 8 weeks after ballooning; Lane 6, iliac artery 8 weeks after stenting, respectively. Band at 180 bp corresponds to rabbit CRP. CRP mRNA is detected in the liver (line 3), but not in the neointima of iliac arteries after balloon injury (line 5) and stenting (line 6). The results were similar in the all samples (n=3 per group).

**Table 1. Serum Lipids and TBARS data at Baseline, 2 weeks, 4 weeks and 8 weeks in two groups**

| | Baseline | 2 weeks | 4 weeks | 8 weeks | |
|---|---|---|---|---|---|
| Total cholesterol (mg/dl) | | | | | |
| Group C | 24.0±3.6 | 466.7±49.7 | 462.8±83.5 | 909.8±138.9 | |
| Group P | 24.2±3.3 | 321.0±64.0 | 334.2±63.2 | 659.5±148.3 | |
| LDL-cholesterol (mg/dl) | | | | | |
| Group C | 4.2±1.1 | 297.3±32.9 | 234.7±42.8 | 401.3±61.6 | |
| Group P | 5.5±0.8 | 193.9±36.8 | 188.8±30.9 | 299.2±57.1 | |
| Triglyceride (mg/dl) | | | | | |
| Group C | 89.4±13.6 | 129.5±21.7 | 220.9±48.2 | 355.2±78.3 | |
| Group P | 95.7±13.1 | 103.0±24.2 | 138.5±29.8 | 168.8±31.6 | |
| TBARS (nmol/ml) | | | | | |
| Group C | 3.52±0.26 | 3.60±0.29 | 3.86±0.42 | 3.96±0.75 | |
| Group P | 3.54±0.37 | 2.42±0.22 | 2.98±0.26 | 3.00±0.42 | * |

Value are mean ±S.E.M., n=6 in each groups, * $P<0.05$ vs. Group C


A
B


C
Luminal diameter (mm)
2.5
2
1.5
1
0.5
0
**
**
*
*
Before ballooning
After ballooning
8 weeks after ballooning
Group C
Group P

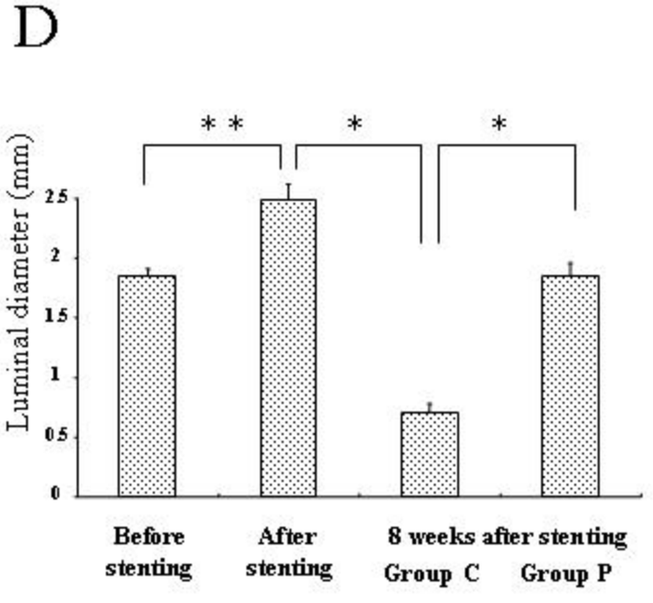

D
Luminal diameter (mm)
2.5
2
1.5
1
0.5
0
* *
*
*
Before stenting
After stenting
8 weeks after stenting
Group C
Group P

Figure 1

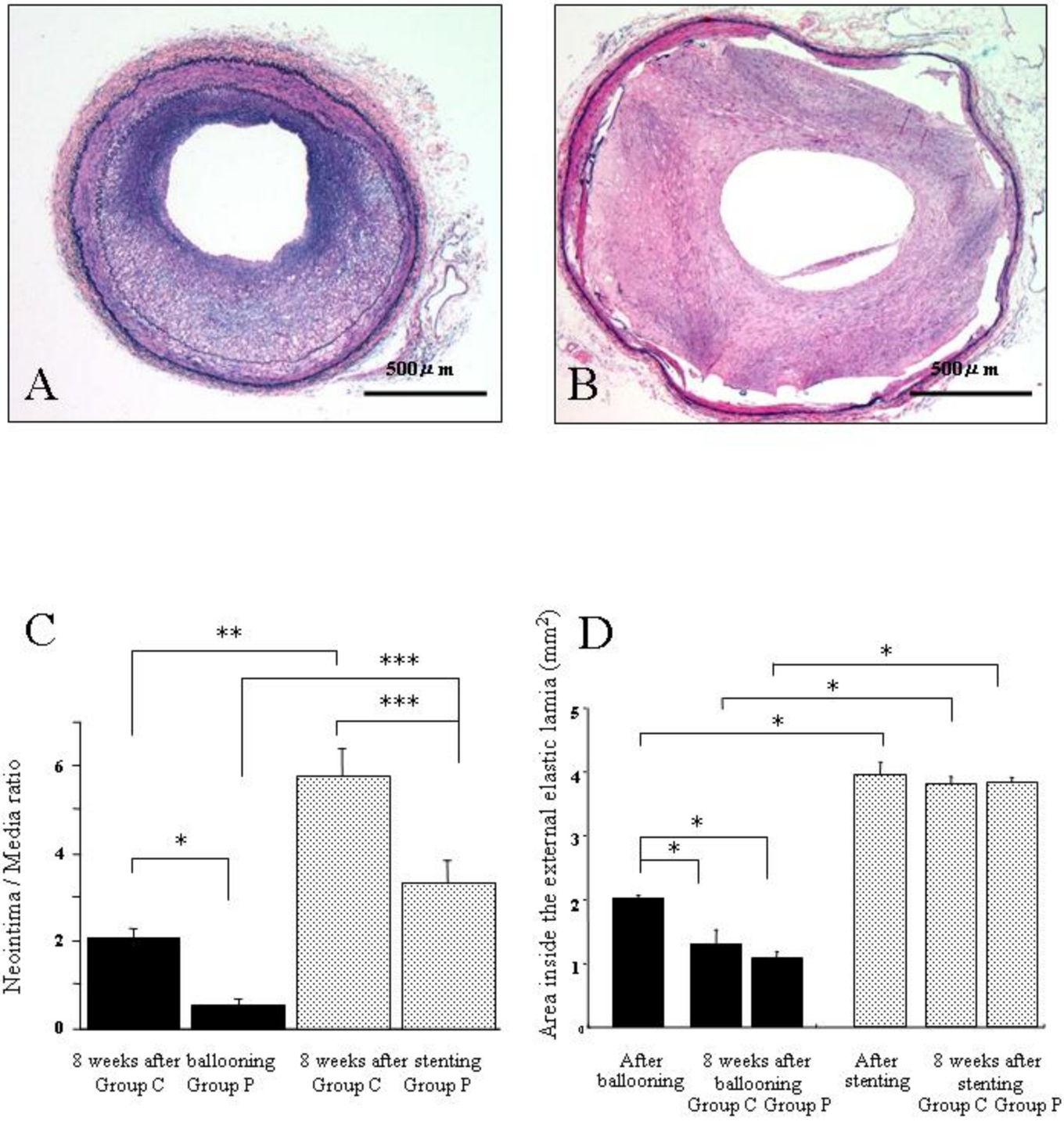

A
500 μ m
B
500 μ m
C
**
***
***
*
Neointima / Media ratio
0
2
4
6
8 weeks after ballooning
Group C
Group P
8 weeks after stenting
Group C
Group P
D
*
*
*
*
*
Area inside the external elastic lamia (mm2)
0
1
2
3
4
5
After ballooning
8 weeks after ballooning
Group C
Group P
After stenting
8 weeks after stenting
Group C
Group P

Figure 2

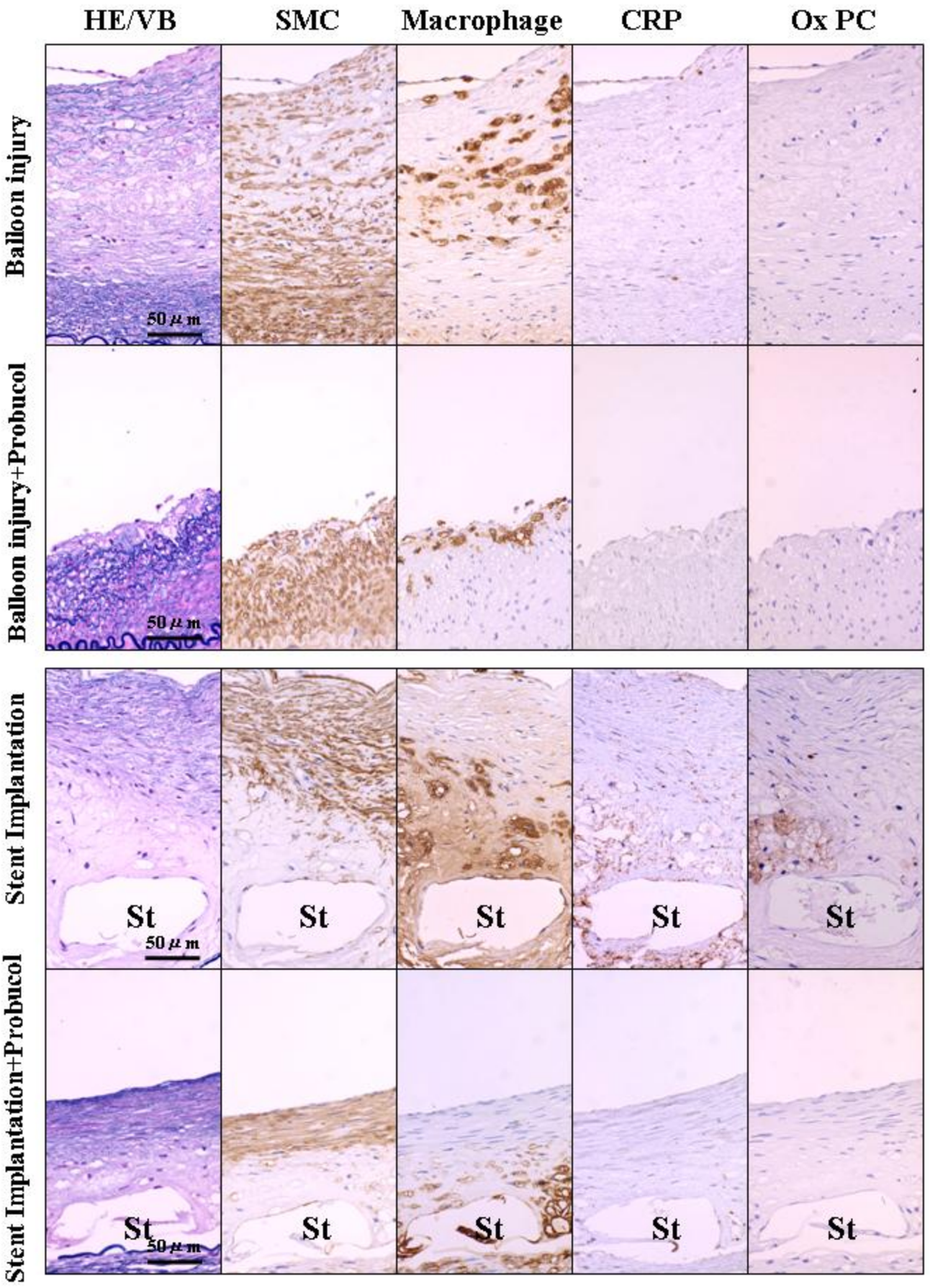

HE/VB
SMC
Macrophage
CRP
Ox PC
Balloon injury
Balloon injury+Probucol
Stent Implantation
Stent Implantation+Probucol
50 μm
St

Figure 3

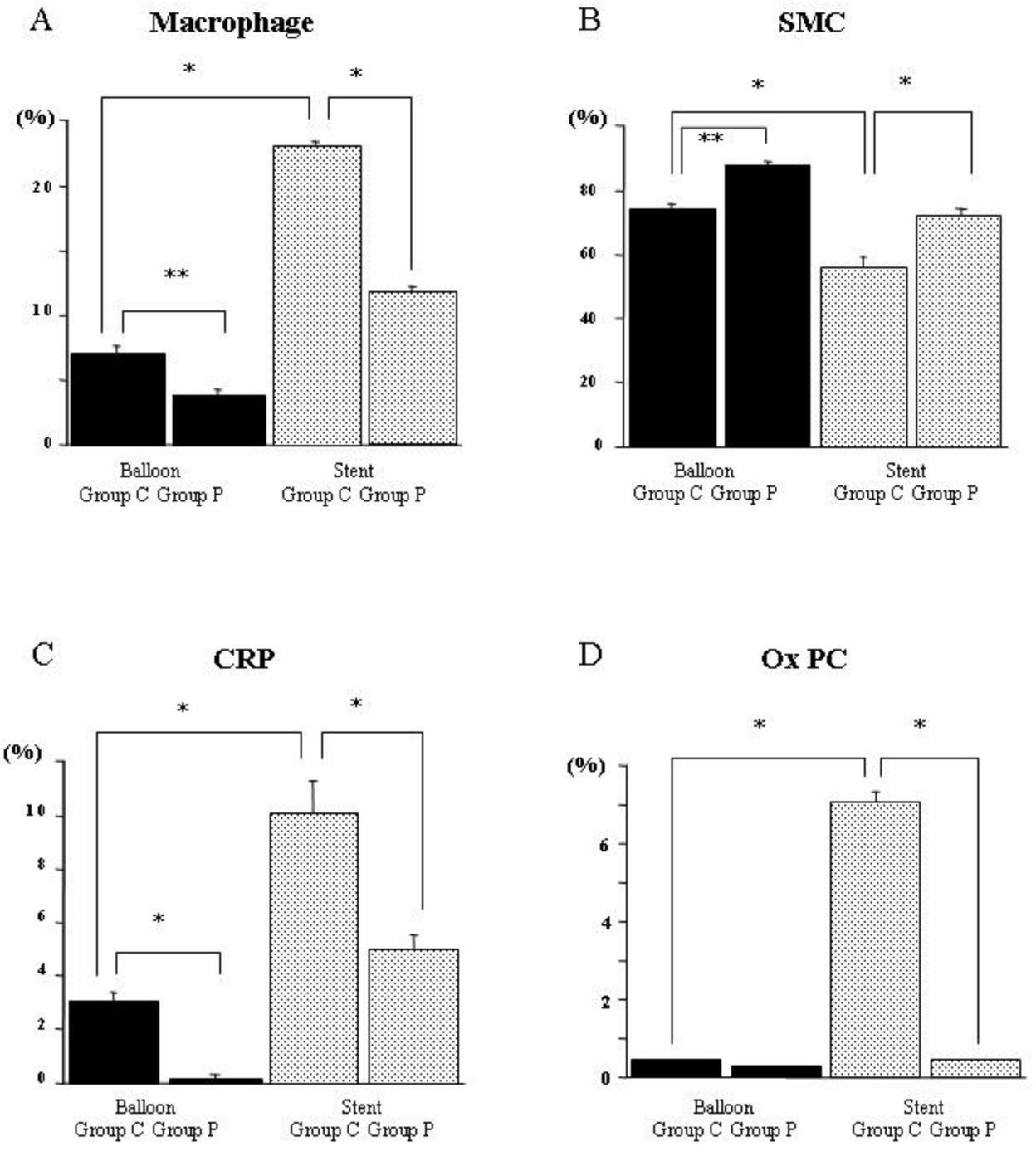

A
Macrophage
(%)
20
10
0
*
*
**
Balloon
Group C Group P
Stent
Group C Group P
B
SMC
(%)
80
60
40
20
0
*
*
**
Balloon
Group C Group P
Stent
Group C Group P
C
CRP
(%)
10
8
6
4
2
0
*
*
*
Balloon
Group C Group P
Stent
Group C Group P
D
Ox PC
(%)
6
4
2
0
*
*
Balloon
Group C Group P
Stent
Group C Group P

Figure 4

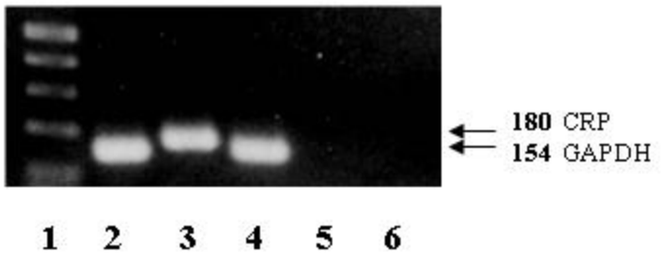



Figure 5