Introduction

Many reports have detailed chronologic changes in blood vessels after stent implantation in animal models. In 1985, stents were implanted in canine aortas by Palmaz et al. They reported that thrombotic adhesion around stent wires 5 days after the implantation and complete obstruction 3 weeks later. They also reported arteriosclerotic changes after stent implantation sites in rabbit aortas and these changes gradually increased in the neointima after 1 week. Recently, Farb et al. reported a strong positive correlation between stenting areas and the degree of neoangiogenesis. These findings suggest a correlation between the neointima and inflammation, and regulating inflammatory cell invasion of the neointimal is important to prevent post-stent stenosis.

Background: Stenotic changes after stent implantation for coarctation of the aorta remain a major problem. There are only a few studies examining pathological vascular changes of the great arteries after stenting in the pediatric population.

Aim: Using immunohistochemistry, we investigated the involvement of chronic inflammation in in-stent stenosis of abdominal great arteries after stent implantation in juvenile pigs.

Methods: Five pigs, aged 4-5 weeks with body weights ranging from 8-10 kg, were used for this study. Stents were implanted in the abdominal great artery. Abdominal arteries with stents were later collected and stained with hematoxylin and eosin. Immunological staining was also performed for the intima, using vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), cyclooxygenase 2 (COX-2) and peroxisome proliferators activated receptor gamma (PPARγ) antibodies.

Results: Only one of five samples showed stenotic change due to neointimal proliferation. Neutrophil recruitment in the neointima was confirmed by H & E stain at the site of stenosis. VEGF, IL-8, COX-2 and PPARγ expressions in the neointima were also increased at the site of stent implantation compared with those in normal tissue.

Conclusion: Our data suggest that chronic inflammation is involved in the pathogenesis of in-stent stenosis after stent implantation. Management of inflammation may be important to prevent stenotic change after stenting in pediatric cardiology.

Key words: animal, stent, stenosis, inflammation, pediatric cardiology
Among the pediatric population, angioplasty with stent implantation is a common treatment of congenital heart diseases, such as coarctation, pulmonary artery stenosis and patent ductus arteriosus dependent diseases. However, there are only a few pathological studies investigating intravascular stenting in pediatric cardiology. In this study, we used juvenile porcine models to investigate the mechanism of in-stent stenosis. As the mechanism of in-stent stenosis is considered an inflammatory change, we examined the expression of inflammatory and anti-inflammatory factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), cyclo-oxygenase 2 (COX-2) and peroxisome proliferators-activated receptor gamma (PPARγ), and demonstrated their involvement in in-stent stenosis in a juvenile porcine model by using immunohistochemistry.

Methods

Animal preparation

All experiments and animal handling were conducted so as to minimize stress and discomfort to animals. Experiments were approved by the Subcommittee on Research Animal Care of Juntendo University School of Medicine, Tokyo. Five pigs, aged 4–5 weeks and weighing 8–10 kg, were used in this study. All pigs were fed normal chow and given aspirin at a dosage of 5 mg per day from 1 week before the first catheterization. The pigs were anesthetized intramuscularly and intravenously with xylazine and ketamine for catheterization. Continuous electrocardiographic monitoring was performed during the procedure, so there was no need for endotracheal intubation during this procedure.

Stenting and angiographic technique

After sterilizing the skin, a 6 or 7 French sheath was placed in the right femoral artery. Before catheterization, heparin (150 IU/kg) was injected. Single-plane cutfilm aortograms were performed in the frontal position using a 5 or 6 French angiography catheter to inject contrast medium (60% urografin) at a dosage of 5 ml for 1 to 2 seconds before stent implantation to measure the diameter of the abdominal aorta. Stents (Palmaz 8 mm × 3 cm) were mounted onto a balloon catheter. The diameter of the balloon was chosen to be 110 to 120 % of the size of the abdominal artery, based on the angiographic measurement. Repeated catheterization and angiography were performed 1, 2 and 3 months after stent implantation. After the procedure, all catheters were removed, the cut-down wound was repaired, and the animals were allowed to move after the recovery. All of these animals received oral aspirin at a dosage of 5 mg/kg/day after the procedure.

Tissue preparation

The pigs were sacrificed by a lethal dose of sodium pentobarbital (80 mg/kg) at the age of 3 months. The stenting sites were separated into two pieces. For the light-microscopic examination, 15% phosphate-buffered formalin was perfused for fixation. Sequential 4- to 5-μm-thick transverse sections were cut and fixed to glass slides with hematoxylin–eosin staining. For immunohistologic examination, the stent was carefully removed under stereoscopic microscope observation. Therefore, we did not use the cross section for immunohistologic examination. Samples were frozen in OCT compound using liquid nitrogen and stored at -80°C until processing.

Immunohistochemistry

The Vectastain Elite ABC system (Vector Laboratories, Burlingame, Calif., USA) was used for immunohistochemistry. Sections were deparaffinized, incubated with 0.3% hydrogen peroxide in methanol for 30 minutes, and blocked with 5% horse serum. After washing with phosphate-buffered saline (PBS), the sections were incubated with primary antibodies for 24 hours at 4°C in a moisture chamber. The slides were washed and incubated with biotinylated secondary horse antibodies (1:2,000; Vector Laboratories) for 2 hours. The sections were visualized with diaminobenzidine substrate followed by counterstaining with hematoxylin. Negative controls were carried out with nonimmune serum instead of primary antibody. The immunohistochemical analyses of VEGF, IL-8, COX-2 and PPARγ were performed with anti-porcine VEGF (V-3) rabbit, COX-2 murine, IL-8 rabbit and PPARγ antibodies.
Results

Angiography
Only one out of five pigs showed stenotic change at the site of stent implantation 1 month after the implantation (Figure-1). The stenotic lesion had developed gradually by the 2-month and 3-month check-ups, as examined by angiography.

Histology
Neointima was confirmed at the site of stent implantation by stereoscopic microscopy (Figure-2). Under optical microscopic observation, a fissure could be seen in the tissue sample that reached the internal elastic membrane and the tunica media. The smooth muscle cells proliferated from the tunica media to inside of the endothelial cells and formed neointima. Inflammatory cell infiltration was confirmed in neointima around the site of stent implantation (Figure-3).

Immunohistochemistry
Neither neointima nor inflammation occurred at the surface of vessels where no stent was placed (Figure-4). The expression of VEGF, IL-8, COX-2 and PPARγ were minimal in these vessels. However, at the site of stent implantation, the expression of VEGF, IL-8, COX-2 and PPARγ were significantly increased in proliferated neointima and media. These immunoreactivities were prominent around the struts (Figure-5).

Discussion
Balloon-expandable stents have been used in the vascular system since the mid-1980s. Animal studies in 1994 demonstrated the feasibility of stents in treating experimental coarctation. Stents support the integrity of the vessel wall after balloon dilatation by opposing the recoil of the elastic vascular stenosis and reapplying the torn intima to the media. Stents also provide a homogeneous framework for smooth endothelial growth along the aortic wall that reduces the risk of thrombosis, neointimal hyperplasia and subsequent re-stenosis.

Despite those mechanisms to prevent re-stenosis, re-coarctation after balloon angioplasty has been seen in 11% of patients. Lezo et al. reported...
that although the stent prevents recoil, different degrees of late intimal hyperplasia were observed in 17% of patients after stent implantation for the coarctation, causing re-stenosis in 8% of them during the short-term follow-up period. Our results are consistent with those studies. Although we have only five cases, one of them (20%) showed neointimal hyperplasia.

The mechanism of stenosis after stent implantation is principally neointimal hyperplasia, as stents resist arterial remodeling. The initial consequences immediately after stent placement are deendothelialization, dissection into the tunica media and stretching of the entire artery. Next is a cellular proliferation phase. Growth factors are subsequently released from platelets, leukocytes and smooth muscle cells (SMCs) and stimulate the migration of SMCs from the media into the neointima. Despite the widespread use of intracoronary stents, in-stent stenosis remains a major clinical problem. Although recent randomized studies have shown that the Palmaz-Schatz stent reduces re-stenosis significantly compared with balloon angioplasty, it has not eliminated the possibility of in-stent stenosis, especially in complex subsets of lesions with small vessels.

Recent reports suggest that acceleration of reendothelialization induced by VEGF is thought to be an important factor in the prevention of stent stenosis. VEGF is an endothelial cell-specific mitogen with a known ability to promote endothelial cell migration. In this study, VEGF expression was detected in the neointima after the stent implantation, suggesting VEGF may play an important role in the development of stenotic change after stent implantation.

Accumulation of macrophages and T lymphocytes contributes to the inflammatory reaction.
confirmed its expression at the site of neointima but not in the normal vessel. These results suggest COX-2 plays an important role in the subsequent development of stenotic change after stent implantation.

PPARs are ligand-activated transcription factors that have begun to receive considerable attention in studies of vascular biology. In vivo and in vitro works suggest that these transcription factors exhibit multiple protective effects within the vessel walls, such as anti-inflammatory, anti-atherogenic and anti-hypertensive effects. PPARγ is expressed in both endothelial and muscle cells of the vessels. We found increased PPARγ expression in the neointima after stent implantation. This result suggests PPARγ may protect vessels from stenotic changes after stent implantation.

Previous animal studies have established a significant correction between the degree of arteriosclerosis and after balloon angioplasty. Therefore, COX-2 is thought to limit disease progression and the development of neointimal hyperplasia, potentially by accelerating regeneration of the endothelium. To explore the role of COX-2 in lesion development after stent implantation, we examined the expression of COX-2 in the porcine artery and confirmed its expression at the site of neointima but not in the normal vessel. These results suggest COX-2 plays an important role in the subsequent development of stenotic change after stent implantation.

Figure 5 Panel A to D shows cross-section of a porcine artery immunostained with A anti-vascular endothelial growth factor (VEGF) antibody, B anti-IL-8 antibody, C anti-COX-2 antibody and D anti-PPARγ antibody in proliferated neointima. The expression of all anti-bodies are especially strong around the stent struts and sporadic in the media (arrows) compared with negative control (×100).
Conclusion

Our study showed that the neointimal proliferation with the chronic inflammation made the stenotic lesion after stent implantation. Chronic inflammation may be involved in the pathogenesis of in-stent stenosis after stent implantation. Management of the inflammation may be important to prevent stenotic change after stenting in pediatric cardiology.

References

10) Foley JB, Penn IM, Brown RI, et al: Safety, success, and restenosis after elective coronary implantation of the Palmaz-Schatz stent in 100 patients at a single cen-


背景：大動脈縮窄症に対するステント留置術後の狭帯性変化は、いまだに重要な問題である。しかし、小児の大動脈縮窄症に対するステント留置術後の、血管病理学的変化に関する研究は少ない。
目的：免疫染色を用い、腹部大動脈へのステント留置術後の狭帯の機序を組織学的に検討すること。
方法：生後4〜5週、体重8〜10kgの5頭のブタを使用した。ステント留置術2ヶ月後の腹部大動脈を摘出し、ヘマトキシリンエオジン染色を行った。またvascular endothelial growth factor（VEGF）、interleukin─8（IL─8）、cyclo─oxygenase 2（COX─2）およびperoxisome proliferators activated receptor gamma antibody（PPARc）を用いて免疫染色を行い、その免疫組織学的変化を比較検討した。
結果：5例頭中1頭に狭帯性変化を認めた。この狭帯性病変部において、好中球の集積が存在する新生内膜の増殖を認めた。またVEGF、IL─8、COX─2およびPPARcの強い発現が新生内膜に認められた。
考察：ステント留置術後の動脈の狭帯性変化には、組織の損傷に対する免疫学的反応に由来する慢性炎症が関与している可能性が示唆された。ステント留置術後再狭帯を防ぐためには、この炎症性変化を抑制することが重要であると思われた。

キーワード：動物実験、ステント、狭帯、炎症、小児循環器学