EXPERIMENTAL STUDIES

In-Stent Restenosis: Contributions of Inflammatory Responses and Arterial Injury to Neointimal Hyperplasia

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Objectives. We examined the relative contributions of inflammation and arterial injury to neointimal formation in a porcine coronary overstretch restenosis model.

Background. Previous studies established that stents cause neointimal proliferation proportional to injury. Although inflammation has been postulated to be a major contributor to restenosis after angioplasty, there is a paucity of data on the relation between inflammation and subsequent neointimal formation.

Methods. Twenty-one pigs underwent balloon injury followed by implantation of oversized, tubular, slotted stents (stent/artery ratio 1.2:1) in the left anterior descending coronary artery. Morphometric analysis of the extent of injury (graded as injury score 0 to 3) and inflammation (graded as inflammation score 0 to 3) 1 month later was assessed and correlated with neointimal formation.

Results. An inflammatory reaction was observed in 20 of 21 pigs, and significant positive correlations were found between the degree of arterial injury and the extent of the inflammatory reaction (r = 0.80, p < 0.01) and between the extent of inflammatory reaction and the neointimal thickness (r = 0.75, p < 0.01), neointimal area (r = 0.53, p = 0.01) and percent area stenosis (r = 0.66, p < 0.01) within the stents. Importantly, there were areas with inflammation only in the absence of injury, and vice versa, that were also associated with neointimal hyperplasia.

Conclusions. These data suggest that the inflammatory reaction plays an equally important role as arterial injury in neointimal formation after coronary stenting, and that anti-inflammatory approaches may be of value to reduce in-stent restenosis.

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inserted. Continuous hemodynamic and surface electrocardiographic monitoring was maintained throughout the procedure. After systemic heparinization (300 U/kg intravenously), control angiograms of the left coronary arteries were performed with a JL 3.5 guiding catheter (SciMed Life Systems, Inc.) and using nonionic contrast agent (Optiray 320, Mallinckrodt Medical Inc.) in two orthogonal views. Before stent implantation, all animals underwent balloon injury (Sleek, Cordis Corp., balloon/artery ratio 1.2:1) for 1 min with a 0.014-in. (0.035-cm) guide wire in the left anterior descending coronary artery. Then, the balloon catheter was removed and one 15-mm Palmaz-Schatz stent (Johnson & Johnson Interventional Systems Co.) was manually crimped on it. The stent was deployed in the mid left anterior descending coronary artery by inflating the balloon to nominal pressure (8 atm) for 1 min at the injury site. Repeat angiograms were obtained immediately after stent implantation. Then, all equipment was removed and the carotid artery was ligated. All animals received 325 mg of aspirin and 250 mg of ticlopidine orally daily until death. Meticulous attention was undertaken during the procedure to avoid contamination of any transcatheter device. Four weeks later the animals underwent repeat angiography in the same orthogonal views before death and had perfusion fixation of the coronary arteries for histologic analysis.

**Evaluation of angiographic stenosis.** Quantitative analysis of initial and follow-up angiograms was performed with an electronic hand-held caliper (Brown and Sharpe) using the guiding catheter for calibration and consisted of the following measurements: 1) mean reference diameter ([proximal + distal] reference diameters/2); 2) mean diameter of the stent at full expansion; 3) stent/artery ratio ([2]/[1]); 4) minimal stent diameter at follow-up; and 5) percent diameter stenosis at follow-up: 

\[
\text{Stenosis} = \left( \frac{\text{Diameter at follow-up} - \text{Reference diameter}}{\text{Reference diameter}} \right) \times 100
\]

All initial and follow-up measurements were made during end-diastole in the same left anterior oblique projection.

**Histopathologic analysis.** The perfusion-fixed hearts were harvested and sent to the study pathologist after overnight fixation with 10% buffered formalin. Then, the specimens were embedded in methylmethacrylate and sections were cut with the low speed diamond wafer mounted to the Buehler Isomet saw (Buehler Ltd.), leaving the stent wires intact in the cross sections to minimize potential artifacts from removal of stent wires. Fifty- to 100-μm sections were obtained at about 1 mm apart and stained with methylchromatin. Measurements were carried out with the Sigmascan software (Jandel Scientific) through an optical microscope integrated to a digitizing tablet.

Morphometric analysis of the neointima from histology slides was done by an independent observer. Histologic evaluation included the degree of injury (13), inflammatory score (as outlined subsequently) and morphometric analysis of neointimal area, neointimal thickness and percent area stenosis. The measurements were made on four cross sections from each stent, visually estimated to be the most narrowed segments that would correspond to the minimal lumen diameter by angiography, and were averaged for each stent. Arterial injury at each strut site was determined by the anatomic structures penetrated by each strut. A numeric value from 0 (no injury) to 3 (most injury) was assigned, as previously described by Schwartz et al. (12): 0 = no injury; 1 = break in the internal elastic membrane; 2 = perforation of the media; and 3 = perforation of the external elastic membrane to the adventitia. The average injury score for each segment was calculated by dividing the sum of injury scores by the total number of struts at the examined section. According to the injury severity, neointimal thickness was measured over the struts in the area with the most severe cross-sectional stenosis and averaged for all struts found in that cross section. Neointimal percent area stenosis was calculated as 100 × \((\frac{\text{Stenotic lumen area}}{\text{Original lumen area}})\).

**Evaluation of inflammation scores and neointimal reaction.** The inflammation score took into consideration the extent and density of the inflammatory infiltrate in each individual strut. With regard to the inflammatory score for each individual strut, the grading is as follows: 0 = no inflammatory cells surrounding the strut; 1 = light, noncircumferential lymphohistiocytic infiltrate surrounding the strut; 2 = localized, moderate to dense cellular aggregate surrounding the strut noncircumferentially; and 3 = circumferential dense lymphohistiocytic cell infiltration of the strut. The inflammatory score for each cross section was calculated in the same manner as for the injury score (sum of the individual inflammatory scores, divided by the number of struts in the examined section). Then, the correlation between the cross-sectional inflammatory score and neointimal formation variables was calculated.

In addition, to determine the relative contributions of inflammation and injury to neointimal formation, individual struts from each stent were analyzed separately and neointimal thickness was calculated with corresponding inflammation scores in the absence of injury, or vice versa (injury without inflammation, both or none). This analysis provided insight into the independent contribution of inflammation or injury in the absence of the other to neointimal hyperplasia.

**Statistical analysis.** All data are presented for each animal and for individual struts as mean value ± SD. Statistical analysis was made using the SAS statistical package. Means of nominal values were compared between groups using analysis of variance. To examine the correlations between the measured histologic variables, regression analysis was applied for each set of measured variables. Multiple regression analysis was applied to assess the impact of injury and inflammatory scores (taken as independent variables), and their interaction-term (injury score × inflammation score), on neointimal thickness (taken as the dependent variable). A logarithmic transformation has been applied to the regression model owing to large-scale values of the dependent variable relative to the independent ones. A p value <0.05 was considered statistically significant.
Results

Angiographic findings. Twenty-one animals underwent successful implantation of one Palmaz-Schatz coronary stent in the left anterior descending coronary artery. All pigs survived until death 4 weeks later. The mean vessel size before stent implantation was 2.84 ± 0.29 mm, and the stent/artery ratio was 1.15 ± 0.13:1. The worst angiographic stenosis was 48.5 ± 23.7% at follow-up, with 11 of 21 lesions manifesting ≥50% diameter stenosis.

Neointimal response to injury. Histologic study revealed neointimal formation and lumen stenosis of varying magnitude within all 21 examined stents. Table 1 lists planimetry-determined measurements of neointimal area, percent area stenosis, neointimal thickness and the corresponding injury score for the arterial segments. A significant correlation was found between the degree of arterial injury and neointimal formation, measured either as neointimal thickness (r = 0.76, p < 0.01), neointimal area (r = 0.61, p < 0.01) or percent area stenosis (r = 0.73, p < 0.01). Representative linear fit curves for these correlations are presented in Figure 1. Figure 2A is a histologic example of the neointimal response to minimal injury in the absence of an inflammatory reaction.

Neointimal response to inflammation. All the examined sections, except for one, revealed various amounts of inflammatory infiltrate surrounding at least one or more of the struts. The inflammatory reaction consisted of groups of pale histiocyte cells adjacent to the struts with occasional multinucleated foreign body giant cells. A rim of small, dark lymphocytes was also noted at the periphery, along with fibroblasts and capillaries. Neutrophils sometimes infiltrated the granulomatous reaction, forming microabscesses. In contrast, eosinophils and plasma cells were not prominent. There was also proliferation of fibrocollagenous tissues in association with the neovascularization of the struts, which together resemble granulation tissue in normal wound healing. Hemorrhage around the strut was also present and appeared to be associated with strut neovascularization.

A significant correlation was found between the degree of inflammation and neointimal formation, measured either as neointimal thickness (r = 0.75, p < 0.01), neointimal area (r = 0.53, p = 0.01) or percent area stenosis (r = 0.66, p < 0.01). Representative linear fit curves for these correlations are presented in Figure 3. These results are very similar to the relation between the degree of injury and neointimal formation in the examined arteries. Figure 2 shows examples of neointimal response to varying degrees of inflammatory reaction.

Inflammatory response to injury. The inflammatory reaction was more severe in those struts with perforation of the internal or external elastic membrane toward the adventitia.

![Figure 1. Linear fit curves for correlations between the degree of vascular injury (injury score) and neointimal thickness (upper panel), neointimal area (middle panel) and percent area stenosis (lower panel).](image-url)
Figure 2. Histologic examples of the neointimal response to inflammation or injury, or both, and representative types of inflammatory cells. A, Inflammatory score = 0: There is normal neointima over the strut, with normal complement of smooth muscle with no histiocytes or lymphocytes around. No mural injury or neovascularization of the strut is present. B, A schematic representation of the histologic sections to signify the position of the stent struts in relation to the arterial wall structures. EEM = external elastic membrane; IEM = internal elastic membrane. C, Inflammatory score = 1: There is a light, widespread lymphohistiocytic infiltrate adjacent to the lumen surface of two adjoining struts. Compression of the media is present without mural injury. D, Inflammatory score = 2: The moderate, localized cellular aggregate of mononucleated and multinucleated histiocytes along with dispersed lymphocytes adjacent to the lumen surface of the strut (arrows). E, Inflammatory score = 3: There is dense, circumferential inflammatory cell infiltration of the strut, with neovascularization and loss of the internal elastic membrane. F, Inflammatory score = 3: Each of the two struts is surrounded by the mixed lymphohistiocytic cell infiltrates, with the paler staining histiocytes immediately adjacent to the struts. The internal elastic membrane is intact, and no significant mural injury caused by the strut is noted. G, There is perforation of the internal elastic membrane and tunica media of the artery, with only grade 1 inflammatory reaction around the strut. A, C to G, ×160, reduced by 50%.
Extension of the inflammatory process to the adventitia was frequent at these sites of perforation. Fragments of disrupted internal elastic membranes were also isolated by the histiocytes and other inflammatory cells. However, in some animals, significant mural injuries were only associated with very mild lymphocytic infiltrates with an otherwise normal neointima abutting against the struts. There was no fibrosis in the intima or the media. Certain animals with significant mural injuries caused by the struts showed sparse lymphohistiocytic reaction but had fibrosis and neovascularization. This most likely represents the hypocellular phase of the healing process, with expected disappearance of the histiocytes and then the lymphocytes from the area of injury. In several sections, granuloma formation and multinucleated giant cells were noted, indicating a foreign body reaction.

A significant correlation was found between the degree of vascular injury (taken as the independent variable) and the inflammatory score (the dependent variable) \( (r = 0.80, p < 0.01) \). The linear fit curve for this correlation is presented in Figure 4. Although in a single isolated specimen the struts had significant arterial injury without inflammation (Animal 12 in Table 1), the overall correlation between the two variables was excellent.

**Neointimal formation in response to either inflammation or injury only.** In evaluating each individual strut, it is interesting to note that there were struts with neointimal formation from inflammatory reaction only in the absence of injury, or vice versa, suggesting that either inflammation or injury by itself may cause neointimal formation. A subgroup analysis of these individual struts derived from 10 stents is presented in Figure 5. In the absence of arterial injury (injury score = 0, \( n = 17 \)), neointimal thickness was \( 430 \pm 277 \) µm with an inflammatory score of \( 1.3 \pm 0.6 \). There were other struts without evidence of inflammation (inflammatory score = 0, \( n = 11 \)), but they had a neointimal thickness of \( 599 \pm 177 \) µm with an injury score of \( 1.8 \pm 0.4 \). When there were both inflammatory and injury changes (\( n = 58 \)), the neointimal reaction was greatest (neointimal thickness \( 775 \pm 269 \) µm; \( p = 0.02 \) for overall comparison by analysis of variance), suggesting that both injury and inflammation may contribute independently to...
neointimal formation. There were also struts without evidence of either inflammation or injury (n = 36), but neointimal thickness was 271 ± 231 μm, most likely due to the initial balloon injury.

**Multiple regression analysis.** Multiple regression analysis was applied to assess the contributions of injury and inflammation scores and their interaction term (injury score times inflammation score) to neointimal thickness (the dependent variable). Using logarithmic transformation, the model yielded the following regression equation:

\[
\text{log(Neointimal thickness)} = 5.11 + 0.64 \times (\text{Injury score}) + 0.58 \times (\text{Inflammation score}) - 0.23 \times (\text{Injury} \times \text{Inflammation scores})
\]

The regression model yielded a significant positive correlation between neointimal thickness and the tested variables (r = 0.85, p < 0.001). According to this model, each of the scores had a significant independent contribution to the regression equation (p = 0.003 for injury score, p = 0.007 for inflammation score and p = 0.019 for the interaction term). This equation signifies that both injury and inflammation are independently correlated with neointimal formation. However, because injury and inflammation are interrelated processes, their combined effect on neointimal formation is less than an additive one, although it is still greater than the effect derived by each factor alone.

**Discussion**

This study evaluated the relative contributions of inflammatory reaction and arterial injury to neointimal response after oversized stent implantation in porcine coronary arteries. The major findings of this study were as follows: 1) inflammatory reaction of the arterial wall was frequently observed at 1 month after stent implantation. 2) The inflammatory reaction was mainly composed of histiocytes, lymphocytes, granuloma formation and neutrophils in the most severe inflammatory forms. 3) A significant positive correlation was found between the degree of arterial injury and the resultant neointimal formation. 4) There was a strong correlation between the extent of inflammatory reaction and the amount of neointimal formation within the stents. And 5) interestingly, both inflammatory changes and the arterial injury can cause a neointimal reaction independently, even in the absence of the other. Finally, the combination of inflammation and injury caused the greatest amount of neointimal formation.

**Arterial injury and inflammation in neointimal hyperplasia.** Our study confirms the relation between arterial injury by stent struts and subsequent neointimal hyperplasia (11–13). In addition, they corroborate the findings by other investigators supporting the association between inflammation and release of cytokines with subsequent neointimal hyperplasia (13–18). Karas et al. (13) identified reactive inflammatory infiltrates with granuloma formation surrounding stented wires in a pig coronary restenosis model, which implied a foreign body reaction in response to the stent. Importantly, tissue specimens retrieved by directional atherectomy from in-stent restenosis in the peripheral arteries of 10 patients disclosed inflammatory infiltrates composed of macrophages and leukocytes in all specimens, in addition to extensive smooth muscle cell proliferation and organized thrombus (19).

Further support for the role of inflammation in neointimal formation comes from experimental restenosis models, in which immunosuppressive drugs were effectively applied. Local periadventitial application of dexamethasone caused a marked inhibition of neointimal proliferation after balloon injury in a rat model (20). A recent experimental study by Lucas et al. (21) found that inhibition of the inflammatory reaction caused by a virus-encoded serine proteinase inhibitor could delay vascular lesion formation after balloon injury in rabbits. In contrast, an exaggerated inflammatory response and exuberant neointimal reaction were found when supposedly “biocompatible” polymers were impregnated on stents and implanted in porcine coronary arteries (22).

**Inflammation and the paradigm for in-stent restenosis.** Our data suggest that in this pig model, inflammatory reaction plays an important role in neointimal formation after coronary stenting. The observed correlation found between the extent of inflammation and the neointimal response suggests a potential causality between these two variables. According to the results of this study, it seems that oversized stent implantation causes acute vessel wall injury, which contributes to the extent of inflammatory reaction at the stented site. Thus, a deeper arterial laceration causes a greater inflammatory reaction. In contrast, inflammation might also arise in the absence of arterial injury, probably in response to the foreign metal struts. Neointimal formation results from these vascular events being proportional to the degree of arterial injury or to the extent of inflammatory reaction, or both, independently or in combination.

Scott et al. (23) suggested a central role of the adventitia in neointimal formation after balloon overstretch injury, which was associated with local inflammatory adventitial responses. The adventitial reaction to the presence of a stent is probably dependent on the extent of mechanical disruption as well as a local “foreign body” response to the metallic prostheses.

**Clinical implications.** To prevent excessive neointimal formation within stents, a combined strategy might be beneficial to minimize the degree of arterial injury and to suppress the extent of inflammatory reaction. According to this paradigm, measures expected to increase vessel wall injury (such as aggressive balloon injury before stenting, stent oversizing or balloon dilation at very high pressures during stent implantation, or a combination of these) might be associated with more inflammatory reaction and a higher tendency for in-stent restenosis. In contrast, measures to minimize the amount of vessel wall injury and/or to suppress the extent of vascular inflammatory reaction after stenting might be effective in decreasing the amount of neointimal formation within stents. Furthermore, stent design, its geometric configuration and the amount of surface metal coverage might also affect the amount
of vessel wall injury and inflammation, and hence neointimal formation after implantation (24,25). For example, using a self-expanding stent without balloon injury in a pig model, we found that neointimal reaction was associated with a favorable response without a peak reaction at 4 to 8 weeks, despite evidence of a continuing increase in the injury scores and further expansion of the stent over time (26).

**Study limitations.** It remains to be established whether our findings in normal nonatherosclerotic porcine coronary arteries stimulated with oversized stents for neointimal proliferation could be extrapolated to human clinical scenarios with preexisting atherosclerosis and stent sizes matched to the reference vessel. Although atherosclerotic plaques in humans are heterogeneous and might differ in their responses to mechanical injury and inflammatory reaction, the morphologic characteristics of neointimal reaction to stent injury is similar in the pig model and the human coronary arteries (11,12). Of note, however, unstable atherosclerotic plaques in humans might contain high contents of inflammatory cells (27), which might further aggravate the neointimal response after stent implantation. Our study did not examine the early inflammatory response, as there is little neointimal formation at that time. Nevertheless, the degree of inflammation may have been much greater earlier, which might have influenced the study results.

**Conclusions.** In this porcine stent restenosis model, inflammatory reaction was frequently observed at 1 month after tubular, slotted stent implantation. A significant correlation was frequently observed at 1 month after tubular, slotted stent implantation. A significant correlation could be extrapolated to human clinical scenarios with preexisting atherosclerosis and stent sizes matched to the reference vessel. Although atherosclerotic plaques in humans are heterogeneous and might differ in their responses to mechanical injury and inflammatory reaction, the morphologic characteristics of neointimal reaction to stent injury is similar in the pig model and the human coronary arteries (11,12). Of note, however, unstable atherosclerotic plaques in humans might contain high contents of inflammatory cells (27), which might further aggravate the neointimal response after stent implantation. Our study did not examine the early inflammatory response, as there is little neointimal formation at that time. Nevertheless, the degree of inflammation may have been much greater earlier, which might have influenced the study results.

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