Localization of c-Myb and Induction of Apoptosis by Antisense Oligonucleotide c-myb After Angioplasty of Porcine Coronary Arteries

D.L. Lambert,† N. Malik, L. Shepherd, J. Gunn, S.E. Francis, A. King, D.C. Crossman, D.C. Cumberland, C.M. Holt

Abstract—Previous studies have shown that inhibition of the proto-oncogene c-myb inhibits neointimal formation in various animal models. However, the temporal and spatial expression of c-Myb in the vessel wall after injury is not known, and the mechanism of action of antisense oligonucleotide (AS-ODN-c-myb) inhibition remains unclear. One potential effect of cell cycle dysregulation by inhibition of c-myb is an increase in the rates of apoptosis. In this study, c-Myb expression after percutaneous transluminal coronary angioplasty (PTCA) injury and induction of apoptosis after AS-ODN-c-myb treatment were determined. Immunohistochemistry and cellular phenotyping were used to localize c-Myb expression in porcine coronary arteries at various time intervals after PTCA. In vitro, the effects of AS-ODN-c-myb on the apoptosis of porcine vascular smooth muscle cells (PVSMCs) and endothelial cells were determined by using a cell-death ELISA and time-lapse video microscopy. In vivo, local delivery of AS-ODN-c-myb was performed after PTCA of pig coronary arteries, and apoptosis was quantified at 6 hours. c-Myb is induced in pig coronary arteries after angioplasty, with maximal expression in inflammatory cells at 18 hours and in vascular smooth muscle cells at 3 to 7 days. In vitro, AS-ODN-c-myb enhanced PVSMCs (6.8±0.8% [P<0.001] versus 0.5% serum) but not endothelial cell apoptosis (1.4±0.5% [P=NS] versus 0.5% serum). In vivo, 6 hours after porcine coronary angioplasty and delivery of AS-ODN-c-myb, the proportion of apoptotic cells within the media was 4.2±0.8% (PTCA alone), 2.3±0.2% (PTCA+vehicle), and 9.0±1.1% (PTCA+AS-ODN-c-myb; P<0.05 versus PTCA alone and P<0.01 versus PTCA+saline). c-Myb is expressed after PTCA of pig coronary arteries, and AS-ODN-c-myb induces apoptosis of PVSMCs in vitro and medial cells in vivo. (Arterioscler Thromb Vasc Biol. 2001;21:1727-1732.)

Key Words: angioplasty ■ proto-oncogenes ■ antisense ■ apoptosis

Restenosis remains a major clinical problem after percutaneous transluminal coronary angioplasty (PTCA).1–3 Restenosis is the result of intimal hyperplasia, elastic recoil, and vessel wall remodeling.4 Vascular smooth muscle cell (VSMC) migration, proliferation, inflammation, and extracellular matrix deposition all contribute to intimal hyperplasia.5–8 In addition, as well as proliferation, apoptosis has been identified in human and experimental restenosis, and, together, these processes are important in the regulation of neointimal hyperplasia.9–12

The proto-oncogene c-myb is a transcription factor, originally described as a hematopoietic factor, with roles in cell proliferation and differentiation as well as apoptosis.13–17 An important role for c-Myb is its function as a progression factor at the G1-S boundary, and expression of the proto-oncogene c-myb is also necessary for proliferation of VSMCs.13,18 We have previously demonstrated upregulation of mRNA for c-myb in a porcine coronary angioplasty model, with peak expression 18 hours after PTCA.19 In addition, we and others have shown that antisense oligonucleotide to c-myb (AS-ODN-c-myb) reduces porcine VSMC (PVSMC) proliferation in vitro19,20 and neointimal hyperplasia in vivo.18,19,21 In another study, AS-ODN-c-myb was seen to reduce proliferation as assessed by determination of proliferating cell nuclear antigen–positive cells 7 days after local delivery to pig carotid arteries;22 thus, AS-ODN-c-myb has been assumed to exert its in vivo effects via cell-cycle dysregulation, resulting in reduced rates of proliferation. However, it is now known that cell-cycle dysregulation may occur under certain circumstances, and especially when proliferation is the background driving force, result in apoptosis.23 Induction of apoptosis after c-myb dysregulation has been suggested by Piacentini et al14 and Schmitt et al,24 who demonstrated that transfection of rat, rabbit, and human
VSMCs with dominant-negative c-myb gene constructs caused not only a decrease in proliferation but also induced apoptosis. Therefore, inhibition of c-Myb induces apoptosis, and this may subsequently reduce neointimal hyperplasia; however, this possible mechanism of action of AS-ODN-c-myb has not previously been investigated.

Therefore, the aims of the present study were as follows: (1) to determine the temporal and spatial distribution of c-Myb after PTCA in a pig coronary artery model, (2) to determine whether AS-ODN-c-myb induced apoptosis of vascular cells in vitro, and (3) to determine whether modulation of apoptosis occurs in vivo after local delivery of AS-ODN-c-myb to porcine coronary arteries.

Methods

An expanded version of Methods can be accessed at http://atvb.ahajournals.org.

Porcine Coronary Angioplasty Model
Porcine coronary angioplasty was performed according to an appropriate UK home office license by using methods previously described by Gunn et al. The hearts were explanted at varying time intervals, and the angioplastied arteries were excised and processed as previously described by Malik et al. Arterial blocks showing maximum balloon injury, defined as maximum disruption in the internal elastic lamina as evidence of injury, were selected for further analysis.25,26

Immunohistochemistry for c-Myb

c-Myb immunostaining was performed on transverse paraffin sections of control and angioplastied pig coronary arteries obtained at various different time intervals after the procedure. Positive immunostaining for c-Myb was semiquantified by using a grading system from 0 to ++++.27 The region of trauma was defined as the cross-sectional area of the artery adjacent to the breached internal elastic lamina, including remnants of the media, adventitia, and loose connective tissue. The specific cell phenotype expressing c-Myb was identified by double immunostaining for c-Myb together with a phenotypic marker.

Determination of Apoptosis

Apoptosis was determined in PVSMCs and pig aortic endothelial cells (PAECS) in vitro by using cell-death ELISA and time-lapse video microscopy (TLVM). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) was performed on histological sections of angioplastied pig coronary artery as previously described.10

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<th>Time After Injury</th>
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Scores are average values obtained from 3–6 vessels per time point. See Methods for definition of grading system.

*Highest possible score is 18.

Results

Time Course of c-Myb Expression

Western analysis revealed a single band at 75-kDa molecular mass against pig thymus, thus indicating specificity of this antibody for porcine c-Myb. In control uninjured arteries, very low levels of immunopositivity for c-Myb was identified within the media and adventitia (Figure 1A, Table). At later time points, when visible, positive staining for c-Myb was characterized by brown nuclear staining (Figure 1B and 1C). One and 6 hours after PTCA, positive staining for c-Myb was observed within the adventitia, and this appeared to localize within regions containing an inflammatory infiltrate (Figure 1D). At 18 hours, strong positive staining was detected within the media, and adventitial immunostaining was still observed in regions with an inflammatory infiltrate. Three days after injury, adventitial staining was more pronounced, and c-Myb staining was detected in microvascular endothelium and adjacent inflammatory cells (Figure 1E). Luminal endothelial and neointimal cells, when present, also expressed c-Myb. At 7 days, the staining pattern was similar to the 3-day specimens, with c-Myb staining present within the media and neointima (Figure 1B and 1C) and α-actin–positive cells within the adventitia. At 14 days, the distribution of c-Myb was similar to that at 7 days but was less intense, and at 28 days, minimal c-Myb staining was observed.

Colocalization of c-Myb With Phenotypic Markers

At 6 hours, c-Myb that was colocalized mainly within inflammatory cells was localized within areas of media and overlying thrombus at sites of trauma and within the adventitia (Figure 1D). Cell types that were not stained with the phenotypic markers used were also positive for c-Myb. These may have been fibroblasts. At 18 hours, colocalization of c-Myb with inflammatory cell phenotype was still evident, and in addition, positive cells were also identified within the media, where colocalization with α-actin was seen (Figure 1F). Maximal VSMC c-Myb expression was identified at 3 to 7 days (Figure 1B and 1C), and at these time points, c-Myb was also detected within adventitial microvascular endothelium (Figure 1E). At 7 days, the adventitial cells expressing c-Myb were now α-actin positive. At 28 days, minimal c-Myb expression was detected; therefore, colocalization experiments were not performed.

Immunohistochemistry for c-Myb

c-Myb immunostaining was performed on transverse paraffin sections of control and angioplastied pig coronary arteries obtained at various different time intervals after the procedure. Positive immunostaining for c-Myb was semiquantified by using a grading system from 0 to ++++. The region of trauma was defined as the cross-sectional area of the artery adjacent to the breached internal elastic lamina, including remnants of the media, adventitia, and loose connective tissue. The specific cell phenotype expressing c-Myb was identified by double immunostaining for c-Myb together with a phenotypic marker.

Determination of Apoptosis

Apoptosis was determined in PVSMCs and pig aortic endothelial cells (PAECS) in vitro by using cell-death ELISA and time-lapse video microscopy (TLVM). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) was performed on histological sections of angioplastied pig coronary artery as previously described.
Figure 1. A. Transverse histological section of control unangioplasted pig coronary artery immunostained for c-Myb. Note the minimal positive staining. i indicates lumen; m, media; and a, adventitia. Original magnification ×20. B, Seven days after angioplasty. Numerous c-Myb-positive cells can be seen within the media (m, arrowhead) and are also present within the intima (i, brown). Arrow indicates internal elastic lamina. Original magnification ×100. C, High-power view of boxed area, shown in panel B, 7 days after angioplasty. Numerous c-Myb–positive cells can be seen within the media (m, arrowhead) and are also present within the intima (i, brown). Arrow indicates internal elastic lamina. Original magnification ×100. D, Six hours after angioplasty showing area of media. VSMCs are positive (red, arrows). Original magnification ×100. E, Three days after angioplasty. Numerous c-Myb–positive cells can be seen within the media (m, arrowhead) and are also present within the intima (i, brown). Arrow indicates internal elastic lamina. Original magnification ×100. F, Eighteen hours after angioplasty showing area of media. VSMCs are α-actin positive (red), and some of these cells are c-Myb positive (brown, arrow). Original magnification ×100.

Cell Isolation
In vitro PVSMCs displayed smooth muscle cell morphology and were stained positively for α-smooth muscle actin. PAECs showed cobblestone morphology when confluent and were α-smooth muscle actin negative and DBA-lectin positive.

Time-Lapse Video Microscopy
Viewed by TLVM over 24 hours, a percentage of PVSMCs showed morphological features characteristic of apoptosis, including membrane blebbing, cell shrinkage, nuclear condensation, and detachment (data not shown). The apoptotic index, derived from TLVM, was 1.8±0.3% in 0.5% serum (n=12, Figure 2). The apoptotic index of AS-ODN-c-myb-treated PVSMCs was 6.8±0.8% (P<0.001 versus 0.5% serum, n=6). The sense-treated PVSMC apoptotic index was 2.2±0.3% (P=NS versus 0.5% serum, n=6). Thus, AS-ODN-c-myb increased apoptosis 3.8-fold over 0.5% serum and 3.1-fold over sense-treated controls. PAEC apoptotic indices were 1.4±0.2% for 0.5% serum and 1.4±0.5% for AS-ODN–treated cells (P=NS, n=6; Figure 2).

Cell-Death ELISA
Untreated PVSMCs in 0.5% serum had an apoptotic enrichment factor over the 5% serum “no death” control of 3.2±0.5. Sense-treated PVSMCs showed similar results: 2.8±0.4 (P=NS versus 0.5% serum, n=6; Figure 3). AS-ODN-c-myb treatment increased the apoptotic enrichment factor to 7.5±0.8 (P<0.005 versus 0.5% serum and sense SN-ODN, n=6). This represented a 2.4-fold increase over 0.5% serum–treated cells and 2.7-fold increase over SN-ODN-c-myb–treated cells. Staurosporine treatment of PVSMCs, used as a positive control, induced death of all cells by 8 hours, as determined by light microscopy, with an apoptotic enrichment factor of 41.2±1.1 (n=3). In contrast to PVSMCs, there was no significant effect of apoptosis on PAECs, after treatment with either AS-ODN (0.8±0.1 [fold increase over 0.5% serum–treated cells]) or SN-ODN (0.7±0.2% , n=6; both P=NS; Figure 3).
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analyzed, the percentage of apoptotic cells was 5.0% in the balloon-injured vessel showing brown staining and characteristic nuclear condensation. The majority of TUNEL-positive cells were located within the media and adventitia. Other investigators have described this phenomenon in vivo. AS-ODN-c-myb increased PVSMC apoptosis in vitro 3-fold. In contrast, there was no effect of SN-ODNs on PVSMCs in vitro. AS-ODN-c-myb did not alter the apoptosis of PAECs in vitro. In vivo, local delivery of AS-ODN-c-myb increased apoptosis 2-fold at 6 hours after angioplasty within the media and, to a lesser extent, within the adventitia of pig coronary arteries.

TUNEL Assay
Control vessels with no angioplasty showed very few TUNEL-positive cells with a labeling index of 0.5%. Cells were considered apoptotic only when they showed TUNEL-positive staining and the morphological characteristics of apoptosis, such as nuclear condensation and cytoplasmic shrinkage. Negative control slides without TdT enzyme showed no TUNEL-positive cells (Figure 4A). Cells were classified as medial if they were located within the internal and external elastic laminae. The majority of apoptotic cells were detected within the media and adventitia (Figure 4B). At 6 hours after angioplasty, when the whole artery (ie, intima, media, and adventitia) was shown, there were TUNEL-positive cells within the media and, to a lesser extent, within the adventitia (Figure 4B). At 6 hours after angioplasty, when the whole artery (ie, intima, media, and adventitia) was shown, there were TUNEL-positive cells within the media and, to a lesser extent, within the adventitia (Figure 4B).

Figure 5. Percentage of TUNEL-positive (apoptotic) cells within the media and whole artery of pig coronary arteries obtained 6 hours after balloon injury alone, after balloon injury with delivery of AS-ODN-c-myb, or after delivery of saline. **P<0.01 vs PTCA alone, *P<0.05 vs PTCA + saline, and †P<0.05 vs PTCA + saline.

Colocalization of TUNEL With c-Myb and Phenotypic Markers
Colocalization experiments revealed that a proportion of cells that were positive for mac-387 were also TUNEL positive (Figure 4C and 4D). When present, very occasional apoptotic cells were detected within the endothelium, as indicated by double staining for TUNEL and von Willebrand factor antigen (Figure 4E). However, the majority of TUNEL-positive cells were α-actin positive, although not all medial VSMCs were TUNEL positive.

Discussion
The present study has demonstrated that c-Myb protein is absent in normal pig coronary arteries but is induced after PTCA, with expression occurring at 6 to 18 hours in the media and adventitia and particularly in regions of inflammatory infiltration. Peak expression was at 3 to 7 days within α-actin-positive cells and returned to basal levels by 28 days. Furthermore, inhibition of c-Myb with the use of AS-ODN-c-myb increased PVSMC apoptosis in vitro, and proliferation after balloon injury occurs at 3 days, a temporal association between the rise of c-Myb levels (which begins 6 hours after injury) and these processes has been established, and it is possible that c-Myb plays a role in determining the balance between cell proliferation and apoptosis occurring in the vessel wall after angioplasty. The majority of c-Myb was localized within the media and adventitia, with localization at areas of trauma within inflammatory cells and VSMCs. Early inflammation was localized to areas of trauma as previously described. At 7 days after injury, α-actin–positive cells were identified within the adventitia. Other investigators have described this phenomenon of adventitial cells expressing smooth muscle cell character-
istics, and it has been suggested to occur as a response to injury of the vessel wall. Previous work by our own group has shown that the transport catheter is capable of delivering AS-ODNs to the media and, to a lesser extent, to the adventitia of pig coronary arteries. Thus, delivery of AS-ODN-c-myb with use of this device would be targeted to regions of c-Myb expression.

Inhibition of c-Myb and Apoptosis In Vitro

It is known that cell cycle dysregulation may lead to apoptosis, and recent evidence suggests a role for c-myb in the control of apoptosis. AS-ODN-c-myb, antigens, and dominant-negative c-myb constructs have all been shown to increase apoptosis in various cell lines. Recently, Schmitt et al showed a reduction in rat and rabbit VSMC proliferation and induction of apoptosis in vitro after exposure to c-myb dominant-negative constructs, supporting the findings of the present study, in which AS-ODN-c-myb induced apoptosis of porcine VSMCs. In contrast, apoptosis of PAECs was not enhanced, indicating that as previously suggested, cell fate is modulated in a cell type-specific manner. In addition, it suggests that endothelial cells may be spared and that early regrowth may occur after AS-ODN-c-myb treatment in vivo, with beneficial effects on arterial healing after PTCA.

Inhibition of c-Myb and Apoptosis In Vivo

Antisense oligonucleotides to c-myb have been used to inhibit neointimal hyperplasia after PTCA in a number of animal models. Given the well-characterized involvement of c-myb in VSMC proliferation and the in vitro antiproliferative effect of AS-ODN-c-myb, it was previously assumed that in vivo effects of AS-ODN-c-myb occurred via inhibition of VSMC proliferation; however, the data obtained in the present study suggest that they may also modulate early events, such as apoptosis. Several authors have postulated that the delivery of agents that modulate apoptosis after PTCA may inhibit neointimal formation and restenosis. Indeed, Pollman et al demonstrated that antisense to bcl-x (anti-apoptotic) mRNA after PTCA increased apoptosis 4-fold and resulted in a 50% decrease in lesion size at 7 days. Our data support the effects of increasing apoptosis after angioplasty, localized to mainly VSMCs and also inflammatory cells and very occasionally to endothelial cells, on the reduction of neointima. Interestingly, a recent clinical trial (Investigation by the Thoraxcenter on Antisense DNA given via the transport catheter reduces neointimal hyperplasia in pig coronary arteries at 28 days) has previously been demonstrated that heparin inhibits DNA synthesis and that this is associated with a reduction in c-Myb mRNA levels. Because heparin was used as an anticoagulant in the in vivo experiments performed in the present study, these findings may be of relevance. The present study and a previous study by our group have demonstrated upregulation of c-Myb in angioplastated vessels, with all procedures performed in the presence of heparin. Therefore, it is unlikely that the dose of heparin used in the present study is interfering with the observations made.

In the present study, in vitro studies were performed on pig aortic VSMCs, whereas in vivo studies used coronary arteries. However, it has recently been suggested that coronary artery smooth muscle cells exhibit unique structural and functional characteristics. In addition, cell heterogeneity may include the presence of VSMC subpopulations with unique differentiation patterns that may affect their susceptibility to apoptosis. However, the in vitro findings obtained in the present study have been validated in vivo, thus suggesting that induction of apoptosis via c-Myb antisense occurs independently of vascular tissue type.

Some studies have suggested that AS-ODN-c-myb may exert nonspecific effects. The AS-ODN-c-myb used in the present study has previously been shown by our group to reduce c-myb mRNA and protein levels with no effect on other proto-oncogene mRNA levels. Furthermore, S1 nuclease digestion, performed in our previous study, demonstrated the binding of ODNs to nucleic acid. Thus, the effects demonstrated in the present study are likely to be due to specific rather than nonspecific ODN effects. In addition, the ODN used was unmodified, thus decreasing the possibility of nonspecific binding as a result of phosphorothioation.

Summary and Conclusions

In conclusion, the present study has demonstrated, for the first time, expression of c-Myb protein after balloon injury of pig coronary arteries, its time course of expression, location within the vessel wall, and specific cellular source. In addition, the present study suggests a new mechanism of action at the cellular level for AS-ODNs targeting c-myb in vascular cells. Tissue homeostasis involves a balance between cellular proliferation and apoptosis. During the healing response after vascular injury, these interactions will be modified. AS-ODN-c-myb is well documented to modulate VSMC proliferation. In the present study, we have reported that AS-ODN-c-myb increases PVSMC apoptosis in vitro, and in a porcine coronary balloon angioplasty model, apo-
ptosis is induced in vivo after local delivery of AS-ODN-c-myb.

Acknowledgments

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References

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