Beta-Adrenoceptor Blockade Markedly Attenuates Transgene Expression From Cytomegalovirus Promoters Within the Cardiovascular System

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Objectives—The major immediate–early cytomegalovirus enhancer/promoter (MIECMV), widely used in cardiovascular gene therapy, contains several positively regulatory cAMP response elements (CRE). Catecholamine signaling via β-adrenoceptors might increase transgene expression from MIECMV, and if so, β-blockers may have a detrimental effect on the efficacy of clinical cardiovascular gene therapy strategies.

Methods and Results—Cultured smooth muscle cells were exposed to isoprenaline, atenolol, or propranolol, alone and in combination before infection with adenoviruses expressing β-galactosidase. β-galactosidase expression was assayed 72 hours later. Isoprenaline increased transgene expression from MIECMV up to 8-fold (P<0.001), but had no effect on a promoter containing no CRE. The effect of isoprenaline was inhibited by β-blockade and by specific CRE-decoy oligonucleotides. β-blockers did not reduce transgene expression below basal levels. After adenovirus-mediated porcine intracoronary gene transfer, however, β-blockade reduced β-galactosidase expression by up to 250-fold compared with non-β-blocked animals (P<0.01).

Conclusions—Enhancement of promoter activity by endogenous catecholamines is essential for high-level transgene expression from MIECMV within the vasculature. β-blocker-mediated suppression of transgene expression from MIECMV in vascular tissues has a significant bearing on clinical studies of cardiovascular gene transfer. This is the first described interaction to our knowledge between widely prescribed pharmaceuticals and a commonly used promoter of clinical transgene expression. (Arterioscler Thromb Vasc Biol. 2006;26:2267-2274.)

Key Words: β-adrenergic receptor blockers ♦ catecholamines ♦ gene therapy

By early 2006 >1100 clinical trials of potentially therapeutic gene transfer had been authorized.1 With 100 trials approved, vascular diseases represent the joint-second most common area for study of clinical gene transfer (http://www.wiley.co.uk/genmed/clinical/). Gene therapy is regarded as offering the potential for new approaches to many clinical conditions for which current treatments are inadequate, merely palliative, or nonexistent. Fundamentally, however, gene therapy is simply a new addition to the pharmacological armamentarium. Therapeutic transgenes are prodrugs that use normal cellular mechanisms to elicit the expression of therapeutically active peptides. Complex technical problems exist, making delivery of such nucleic acid “prodrugs” to their intended sites of action significantly more demanding than is the case for classical pharmacological agents, but like all other therapeutic interventions, there is the possibility of clinically significant interactions between gene therapy agents and coprescribed pharmacological therapies. One potential area for such interactions exists between prescribed drugs and regulatory elements within promoters of transgene expression.

Intracellular signaling after ligand-mediated activation of β-adrenoceptors is mediated by intracellular adenosine-3’, 5’-cyclic monophosphate (cAMP). Ligand-binding leads to the activation of adenylyl cyclase by G-proteins coupled to the cytosolic domain of the β-adrenoceptor. Activation of adenylyl cyclase increases cAMP synthesis, which leads to activation by protein kinase A of the transcriptional factor cAMP-response element-binding protein (CREB). The cAMP-response element (CRE) is an ubiquitous 8-base-pair-long palindromic DNA sequence that mediates the effects of cAMP on transcriptional activity.2 Once activated, CREB binds to CRE and stimulates assembly of the RNA polymerase complex which promotes transcription.2

The major immediate–early enhancer/promoter from human cytomegalovirus (MIEhCMV) is a commonly used promoter of transgene expression, particularly in vascular gene therapy. In a recent review of experimental vascular

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gene transfer, of the 69 studies cited in which the promoter used was identified, 57 (ie, >80%) used MIEhCMV to promote transgene expression.3 MIEhCMV contains several copies of CRE. Clearly there is potential for enhancement by β-adrenergic agonists of transgene expression under the transcriptional regulation of MIEhCMV and for inhibition of any such effect by antagonists of β-adreceptors (β-blockers). Because β-adreceptors are widely distributed throughout the vasculature and β-blockers are frequently prescribed in a range of cardiovascular diseases, such an interaction might prove very pertinent with respect to the clinical application of gene therapy to those pathologies for which β-blockers may be coprescribed.

To establish whether β-adreceptor-mediated signaling enhances transgene expression from MIEhCMV by activation of CREB, we studied the effect of the nonsubtype-specific β-adreceptor agonist isoprenaline on transgene expression in cultured smooth muscle cells (SMCs) infected with 1 of 3 recombinant adenoviruses. Ad5-lacZ contains Escherichia coli lacZ under transcriptional regulation of MIEhCMV.4 RAd36 contains lacZ regulated by the more potent homologue from murine cytomegalovirus (MIEmCMV).5 and RAd122 contains lacZ under regulation of the Rous sarcoma virus long terminal repeat promoter (RSV-LTR), which contains no CRE.6 We have studied the effects of the nonselective β-blocker propranolol, the β1-selective β-blocker atenolol and the β2-selective agonist IC1118,551 on transgene expression. β-blockers were studied alone and in combination with isoprenaline or forskolin, the latter of which directly activates adenyl cyclase.7 We have also investigated the effect of a specific CREB-binding decoy oligonucleotide on transgene expression.8 To determine to what extent endogenous catecholamines affect transgene expression in vivo, we subsequently studied the effects of atenolol and propranolol on β-galactosidase expression after intracoronary delivery of RAd36.

**Methods**

**Recombinant Adenoviruses and Oligonucleotides**

Viruses were generated by homologous recombination in 293 cells. Stock cultures were purified by cesium chloride gradient centrifugation and titrated by serial-dilution end-point assay.

Two phosphorothioate-oligonucleotides were purchased (Sigma-Genosys). The CRE-decoy sequence comprises 3 copies of the palindromic CRE sequence: 5’-TACGCTCA-TAGCCTGCA-TAGCCTGCA-3’. The control oligonucleotide was a 24-nt nonsense-sequence palindrome: 5’-CTAGCTAGCTAGCTAGCTAGCTAGCTAG-3’.

**SMC Culture**

Primary-cultured porcine coronary SMCs were established as previously described.9 Porcine and human (TCS Cellworks) coronary SMCs were seeded in 6-well plates and grown to 90% confluence, then rendered quiescent by exposure to 2%-serum medium for 72 hours. To establish the maximum concentrations of isoprenaline, atenolol, propranolol, IC1118,551, and forskolin, to which SMC could be exposed without toxicity, medium supplemented with one of each agent at progressive 2-fold increases in concentration, starting at 12.5 μmol/L, were added to SMCs in different wells. The medium was replaced with fresh supplemented medium daily for 72 hours. In subsequent experiments, quiescent SMCs were exposed to atenolol, propranolol, IC1118,551, isoprenaline, forskolin, isoprenaline + atenolol, isoprenaline + propranolol, isoprenaline + IC1118,551, forskolin + atenolol, forskolin + propranolol, or unsupplemented medium only for 24 hours before infection with Ad5–lacZ, RAd36, or RAd122 at multiplicities of infection of 50, 10, and 500, respectively. For each virus, 4 wells of SMCs were exposed to each agent or combination of agents. Freshly supplemented medium was added daily to infected SMCs until 72 hours postinfection, after which β-galactosidase activity was quantified by chromogenic assay in lysates of the cell monolayers, as described previously.5

**SMC Transfection**

To establish the maximum concentration of phosphorothioate-oligonucleotide to which cells could be exposed without inducing toxicity, SMCs were transfected with escalating doses of each oligonucleotide. Single doses of CRE-decoy or nonsense oligonucleotide were complexed with 20 μL of Lipofectamine 2000 transfection reagent (Invitrogen) to achieve a final concentration in the SMC medium of 125, 250, 500, or 1000 nM. Those SMC used for oligonucleotide-transfection studies were subsequently transfected, at the same time as adenovirus infection, by addition of a single dose of CRE-decoy or nonsense oligonucleotide complexed with 20 μL of Lipofectamine 2000 sufficient to achieve the maximum concentration in the SMC medium that elicited no toxicity.

**Intracoronary Gene Transfer**

Fifteen large white pigs (21 to 26 kg; Biological Sciences Facility, University of Manchester) underwent intracoronary delivery of RAd36. Procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and were authorized by the Home Office. Intracoronary gene transfer was performed using a stent-based gene transfer device. Collagen-bound CoverStents (Medtronic) were loaded with 2.5×107 IU of RAd36 immediately before procedure. Five pigs received 100 mg atenolol orally once daily for 96 hours before and 72 hours after procedure, 5 pigs received oral propranolol (80 mg twice daily) for the same period and 5 pigs received no β-blocker. All animals received 150 mg aspirin daily, commencing 24 hour before procedure. Anesthesia was induced by inhalation of 4% isoflurane and maintained, after endotracheal intubation, with 2.5% isoflurane. The heart rate of each animal was recorded electrocardiographically immediately postintubation. The left carotid artery was exposed and an 8-F guide catheter inserted; 5000 IU of heparin were injected before coronary angiography was performed. Vessel segments in the LAD (diameter 2.2 to 2.7 mm) were selected by intravenous bolus of pentobarbitone (100 mg/kg). Stented arterial segments were harvested and cut open longitudinally. The stents were carefully removed. Sections of artery lying outwith the stented segment were trimmed off and the adventitia carefully stripped off using ophthalmological tweezers. The central 5 mm of each segment were embedded in OCT medium and snap-frozen in liquid nitrogen (LN2) for X-gal staining. The remaining lengths of each infected vessel segment were frozen in LN2 before β-galactosidase assay.

**Transgene Expression In Vivo**

OCT-embedded segments were cut, using a cryostat, into as many 7-μm-thick sections as could be obtained. X-gal cytochemistry was performed on each section as described previously.5 The area of X-gal staining in each section was measured using a Leica Quantimet 600s digital analysis system. Frozen vessel segments were homogenized in LN2 using a mortar and pestle, then resuspended in lysis solution.5 β-galactosidase activity was assessed by chromogenic assay in vessel homogenates as described previously.5

**Statistical Analysis**

Multiple groups were analyzed by 1-way analysis of variance (ANOVA). Comparisons were made between groups by Newman-Keuls post-hoc test.
Results

Isoprenaline Increases β-galactosidase Expression Under Transcriptional Regulation of the Major Immediate-Early Cytomegalovirus Enhancer/Promoter in Cultured SMCs

The maximum concentration of isoprenaline to which cultured coronary SMC could be exposed for 72 hours was 50 μmol/L. The maximum concentrations of propranolol, atenolol, ICI118,551 and forskolin that coronary SMCs could survive for the same period were 50 μmol/L, 400 μmol/L, 25 μmol/L, and 50 μmol/L, respectively. Reagents were used at these concentrations in subsequent in vitro studies.

Cells exposed to isoprenaline revealed significantly greater transgene expression 72 hours after infection with Ad5-lacZ and RAΔ36. By comparison with cells exposed to unsupplemented medium, increases in β-galactosidase activity of 4.8- and 7.6-fold (both P<0.001) were observed in Ad5-lacZ-infected porcine and human coronary SMCs, respectively, after exposure to isoprenaline (Figure 1).

Exposure to isoprenaline increased β-galactosidase expression 3.4-fold in porcine coronary SMCs and 5.6-fold in human coronary SMCs infected with RAΔ36 (both P<0.001; Figure 2). Subsequent exposure of Ad5-lacZ-infected HCSMC to lower concentrations revealed significant enhancement of β-galactosidase expression with 6.25 μmol/L isoprenaline, although no effect was observed at 3.125 μmol/L (Figure 3a).

Cosupplementation of culture medium with atenolol or propranolol in addition to isoprenaline significantly inhibited isoprenaline-induced enhancement of transgene expression. Coexposure with propranolol reduced β-galactosidase activity in Ad5-lacZ- and RAΔ36-infected SMC to levels that did not differ from those observed in SMC exposed to unsupplemented medium (Figures 1 and 2). Exposure to atenolol in addition to isoprenaline decreased isoprenaline-induced enhancement by ~50% in porcine and human SMC infected with Ad5-lacZ (Figure 1). β-galactosidase expression was nonetheless significantly greater than that observed in SMC exposed to unsupplemented medium (both P<0.05). In cells
infected with RAd36, atenolol reduced the effects of isoprenaline by little >20% (Figure 2). Again, β-galactosidase expression was significantly greater in SMCs exposed to isoprenaline+atenolol than in SMC exposed to unsupplemented medium (both P<0.001). SMC exposed to atenolol or propranolol in the absence of isoprenaline expressed levels of transgene that did not differ significantly from cells exposed to unsupplemented medium (Figures 1 and 2). Subsequent exposure of Ad5-lacZ–infected HCSMC to ICI118,551 in addition to isoprenaline reduced β-galactosidase activity to the level observed in SMCs exposed to unsupplemented medium (Figure 3b).

Exposure to forskolin increased transgene expression by 1.6- to 4.6-fold in porcine and human coronary SMCs infected with either virus (all P<0.05; Figures 1 and 2). Transgene expression in SMCs exposed to atenolol or propranolol in addition to forskolin did not differ significantly from levels observed in SMCs from the same species infected with the same virus and exposed to forskolin alone (Figures 1 and 2). All cells exposed to forskolin, in the absence or presence of β-blocker, expressed more β-galactosidase than cells exposed to unsupplemented medium (all P<0.05).

Figure 2. β-galactosidase activity in lysates of cultured coronary SMCs 72 hours after infection with RAd36. In both (a) porcine and (b) human SMCs, isoprenaline and forskolin increase transgene expression from MIEhCMV. As with MIEhCMV, the effect of isoprenaline is antagonized by atenolol and propranolol, although propranolol is more efficacious in both species.

The Effect of Isoprenaline on Transgene Expression From MIECMV Is Mediated by CREB

Isoprenaline did not increase transgene expression in SMCs infected with a recombinant adenovirus in which expression of β-galactosidase was under transcriptional regulation of RSV-LTR (Figure 4a).

The maximum concentration of CRE-decoy oligonucleotide to which SMC could be exposed without toxicity was 500 nM. Both oligonucleotides were used at this concentration in vitro. Transfection with CRE-decoy inhibited isoprenaline-induced enhancement of β-galactosidase expression in Ad5-lacZ–infected SMC (Figure 4b), but did not affect levels of transgene expression in RAd122-infected SMC (Figure 4a). In contrast, transfection with nonsense oligonucleotide did not affect β-galactosidase expression in Ad5-lacZ-infected SMC (Figure 4b). CRE-decoy had no effect on levels of transgene expression in Ad5-lacZ–infected SMCs in the absence of isoprenaline.
**β-Blockers Significantly Decrease Transgene Expression Regulated by the Major Immediate–Early Murine Cytomegalovirus Enhancer/Promoter In Vivo**

Mean heart rates in pigs given oral β-blockers for 96 hours before gene transfer were significantly lower immediately postendotracheal intubation than in animals receiving no β-blocker (atenolol = 121 ± 17 beats/min; propranolol = 117 ± 6 beats/min; no β-blocker = 174 ± 9 beats/min; P = 0.02).

Expression of β-galactosidase in coronary arteries 72 hours after CoverStent-mediated delivery of RAd36 was substantially greater in animals receiving no β-blocker than in animals administered atenolol or propranolol. β-galactosidase activity in lysates of snap-frozen artery segments was ~80-fold greater (P < 0.001) in non-β-blocked animals and ~500-fold greater (P < 0.01) than in those receiving propranolol (Figure 5a). The mean area of staining with X-gal in cryostat-sections of infected arteries was ~80-fold greater (P < 0.001) in animals that received no β-blocker than in those that received atenolol and ~260-fold greater (P < 0.001) than in those that received propranolol (Figures 5b and 6). At least one X-gal–positive cell was observed in 60% of sections from non-β-blocked animals. In contrast only 2.9% of sections from atenolol-treated animals and 0.9% of sections from propranolol-treated animals contained one or more X-gal–positive cells (P < 0.001 by χ²).

**Discussion**

We have investigated the effect of the β-adrenoceptor agonist isoprenaline on the activity of the major immediate–early enhancer/promoter from human and murine cytomegalovirus as promoters of transgene expression in coronary SMCs. Exposure to isoprenaline at concentrations in excess of 3.125 μmol/L increased transgene expression in cultured...
SMCs infected with Ad5-lacZ or RAd36. Transgene expression from a promoter lacking CRE was not enhanced by isoprenaline, confirming that the enhancer effect of isoprenaline in Ad5-lacZ- and RAd36-infected SMCs is not the consequence of a nonspecific increase in cellular transcription. Concomitant β-blockade reduced the effect of isoprenaline in Ad5-lacZ-infected and RAd36-infected SMCs. Propranolol was a more potent antagonist of this effect than atenolol. The mode of action of both β-blockers (inhibition of β-adrenoceptor-mediated upregulation of adenyl cyclase) is verified by the lack of effect of either on forskolin-mediated enhancement of transgene expression.

Greatest isoprenaline-induced enhancement of transgene expression was observed in Ad5-lacZ-infected human SMCs. We therefore studied the effects in such cells of the β2-adrenoceptor-specific antagonist ICI118,551 and of a well-characterized CRE-decoy oligonucleotide that specifically interferes with CREB-dependent transcription.6 In keeping with the β2-adrenoceptor being the predominant subtype in vascular tissues,10 ICI118,551 completely abolished isoprenaline-induced enhancement of transgene expression.

Transfection with CRE-decoy at the time of Ad5-lacZ infection abolished isoprenaline-mediated enhancement of transgene expression. Control oligonucleotide did not affect isoprenaline-mediated enhancement, confirming that the effect of isoprenaline on MIECMV is dependent on the integrity of the CREB pathway.

These results suggest that endogenous catecholamines may have a significant effect in vivo on transgene expression regulated by MIECMV. Our observations after intracoronary gene transfer demonstrated that transgene expression from MIECMV within the vasculature is heavily dependent on endogenous catecholamine-mediated enhancement of promoter activity. This has a significant bearing on attempts to translate preclinical data into viable clinical gene therapies, particularly within the vasculature, as β-blockers are prescribed widely for myocardial ischemia, hypertension, and heart failure, all of which are targets for the clinical application of therapeutic gene transfer. We did not assay plasma β-blocker levels in our in vivo studies, as our interest lay in β-blockers as they would be used clinically. The significantly reduced heart rate immediately after intubation indicates clinically adequate β-blockade. Because the collagen membrane that invests the CoverStent (please see http://atvb.ahajournals.org) prevents direct contact between the blood and the luminal surface of the stented segment, the reduction in transgene expression is unlikely to be a consequence of changes in shear stresses resulting from reduced heart rate in β-blocked animals.

MIEhCMV has been used infrequently for gene transfer in vivo. In contrast, MIEhCMV is used widely in experimental studies of “therapeutic” gene transfer and has been used in many clinical studies of cardiovascular gene therapy. MIEhCMV was used to promote expression of vascular endothelial growth factor in the first human trial of arterial gene transfer.11 It has been used subsequently in studies of gene transfer to peripheral arteries.12,13 Coronary arteries,14 and ischemic myocardium.15–18 Several of these studies reported the number of patients concomitantly receiving β-blockers. Rajagopalan et al reported that 6% of patients received β-blockers (although 45% had an excellent clinical indication in the form of previous myocardial infarction).12 In the study by Losordo et al, 80% of patients took a β-blocker.16 Grines et al reported that β-blockers were among the most frequent concomitant medications17 and 90% of recipients of gene therapy in the study by Hedman et al received a β-blocker.14 Although some improvements in clinical end points were reported14,17,18 the effects of gene transfer were not striking. It is impossible to state that concomitant β-blockade in these studies is the reason for their limited clinical success. However, the potential for β-blockade to have influenced the outcome of such studies cannot be ignored.

Studies in vitro have shown that MIECMV is the most potent promoter of transgene expression in vascular tissues. It elicits greater transgene expression in SMCs than SMC-specific promoters.19,20 When compared with other viral promoters in SMCs (RSV-LTR, simian virus 40 [SV40] early promoter) MIEhCMV elicited most expression.19,21 Because the RSV-LTR and SV40 promoters are not cAMP-responsive, it is likely that an even greater advantage would be conferred on MIEhCMV in the presence of catecholamines. Our observations also show that β-blockers do not reduce transgene expression to sub-basal levels in SMC, in keeping with reports that CRE does not govern basal levels of activity of MIEhCMV in CMV-infected cells.52 The use of cAMP-insensitive promoters would therefore be unlikely to confer any advantage over MIEhCMV within the vasculature, as those that have been compared with MIEhCMV do not achieve greater basal levels of transgene expression in SMCs; unlike MIEhCMV, they would not permit increased transgene expression in response to endogenous catecholamines.
Our observations have implications that spread beyond the field of vascular gene therapy. MIEhCMV is a potent promoter in cells from nonvascular tissues. Clinical studies of gene therapy for cystic fibrosis, liver metastases, α1-antitrypsin deficiency, factor IX deficiency, and esophageal carcinoma have used MIEhCMV to promote therapeutic transgene expression. Furthermore, activation of MIEhCMV is the rate-limiting step in cytomegalovirus replication. Our observations suggest that β-blockade may restrict cytomegalovirus replication in humans, which is pertinent not only to the setting of clinical infection with cytomegalovirus but also might account for some of the cardioprotective effects of β-blockers in the setting of atherosclerosis, as cytomegalovirus is implicated in atherogenesis and is associated with increased mortality from coronary artery disease.

The requirement of the CRE for maximal transcriptional activity has also been demonstrated in herpes simplex virus-1, the human T-cell leukemia virus type 1, the Epstein-Barr virus BamHI W promoter, and the varicella-zoster virus immediate-early gene 62 promoter. In these viruses, too, β-blockade may act as adjunctive therapy to limit viral replication.

We have investigated the effects of β-blockers on transgene expression elicited by MIECMV in vivo. The finding that β-blockers severely inhibited transgene expression after intracoronary gene transfer has implications for all studies of clinical gene therapy. It is likely that the effect of β-blockade on transgene expression from MIECMV in vivo is just the first clinically significant interaction between pharmacological agents and promoters of transgene expression. The α1-adrenoceptor agonist phenylephrine, for example, acting through a cAMP-independent pathway, enhances MIEhCMV-regulated transgene expression in rat cardiomyocytes, raising the possibility that α-adrenoceptor antagonists might also have significant effects on transgene expression in vivo. Our findings stress the importance of the potential for interactions between gene therapy and other therapeutic modalities. To date, these have largely been neglected in the undertaking of clinical trials to assess the therapeutic potential of gene transfer.

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Disclosures
None.
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The CoverStent comprises a 15mm x 3.0mm BeStent (Medtronic) bound in a membrane of cross-linked type I collagen (open arrow), held in place by Teflon rings (arrows). Adenovirus solution is pipetted dropwise onto the membrane allowing virus adsorption to the collagen immediately before stent deployment.