

# Genetically Engineered Biologically Based Hemostatic Bioassay

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(Received 2 July 2002; accepted 12 November 2002)

**Abstract**—Real-time direct measures of hemostatic parameters *in vivo* are required for optimizing the dynamic delivery of coagulation modifying pharmacotherapies. Typical sensors of physiologic functions *in vivo*, however, have only a restricted array of sensory inputs, and thus limited capacity to monitor thrombotic and hemostatic activity. To overcome this limitation we have developed a genetically engineered excitable cell line that can be potentially used for an implantable thrombin biosensor. Specifically, we have generated stem cell-derived cardiac myocyte aggregates overexpressing the human thrombin receptor, protease activated receptor-1 (PAR-1), which exploit the inherent electropotential input–output relationship of the cells to detect local changes in thrombin activity. *In vitro*, the signaling activity of PAR-1 cardiac myocytes was highly responsive to thrombin, inducing a sixfold increase in intracellular cAMP as compared with a twofold increase in control cells. *In vivo*, the engineered myocytes also detected alterations in local coagulation potential. Specifically, PAR-1 engineered cells implanted *in vivo* detected local increases in thrombin with a doubling in chronotropic activity compared with a 50% increase in control aggregates. Overall these studies demonstrate the potential of genetic engineering to expand the physiologic signals recognized by excitable cells, and may facilitate the translation of this approach for the real-time monitoring of hemostatic function *in vivo*. © 2003 Biomedical Engineering Society. [DOI: 10.1114/1.1537693]

**Keywords**—Tissue engineering, Stem cell, Coagulation, Thrombin, Biosensor.

## INTRODUCTION

Dynamic regulation of biological systems requires real-time assessment of relevant physiological events, such as changes in the hemostatic/coagulation cascades. Biosensors, which transduce biological actions or reactions into signals amenable to processing, are well suited for such monitoring. Unfortunately, no devices have yet been developed for the monitoring of hemostatic activity *in vivo*. Presently, real-time approaches to measure hemostatic parameters *in vivo* are limited.<sup>13</sup> An *in vivo* biosensor of the coagulation system could allow the dy-

namic monitoring of changes in thrombotic potential directly and thereby optimize the titration of appropriate pharmacotherapies.

Recently, we reported the development of a cardiac myocyte-based biosensor, which couples the detection of *in vivo* blood-borne physiological catecholamine inputs via endogenous receptor pathways of excitable cardiac myocyte aggregates to a functionally responsive electropotential output signal via changes in membrane depolarization dynamics.<sup>2,7</sup> As previous studies have demonstrated that the chronotropic function of cardiac myocytes can be altered through overexpression of endogenous cell surface receptors signaling through native G protein-coupled pathways,<sup>5,6</sup> we hypothesized that genetic engineering of cardiac myocytes to overexpress protease activated receptor-1 (PAR-1), a transmembrane cellular receptor for the coagulation enzyme thrombin (for review see Ref. 3) that can mediate signaling cascades in cardiac myocytes,<sup>12</sup> could expand the functional biosensory utility of embryonic stem (ES) cell-based *in vivo* biosensors to detect real-time changes in hemostatic parameters.

## EXPERIMENTAL PROTOCOLS

### *Genetic Engineering and Cellular Derivation*

Genetically engineered ES cell-derived cardiac myocytes were generated from E9 murine pluripotent ES cells (American Tissue Culture Company). ES cells were cultivated on a mitomycin C treated feeder layer of primary mouse embryonic fibroblasts in DMEM supplemented with nonessential amino acids, L-glutamine,  $\beta$ -mercaptoethanol, 20% fetal calf serum, and 100 IU leukemia inhibiting factor (LIF). ES cells ( $10^7$  cells) were electroporated with linearized cDNA (30  $\mu$ g) encoding “neomycin resistance and constitutively expressed” FLAG epitope-tagged human PAR-1, generous gift of S. R. Coughlin (University of California, San Francisco) or enhanced green fluorescent protein (GFP) gene (Clontech) in 1 mL PBS, at 250 V and 500  $\mu$ F. Cells were then plated into a 25 cm<sup>2</sup> flask and neomycin-resistant colonies were then selected and expanded by

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the addition of G418 ( $400 \mu\text{g mL}^{-1}$ ). Cardiac myocytes were then generated as previously described.<sup>11</sup> Briefly, droplets of cells ( $10^4$  cells in  $30 \mu\text{L}$  of culture media without LIF) were transferred onto the lids of 3 cm bacteriological petri dishes filled with PBS and cultivated for two days. The resulting aggregates were transferred from the hanging drops into 6 cm dishes, cultivated for five days, and then transferred to 12-well plates. Spontaneous chronotropic myocyte aggregates formed between 5 and 10 days after transfer and were subsequently employed in subsequent *in vitro* and *in vivo* assays.

#### Molecular Studies

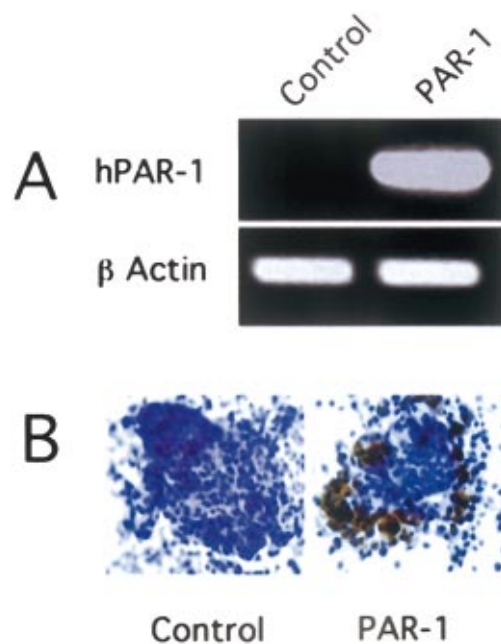
Genomic integration of the PAR-1 transgene in the selected ES cell clones was confirmed by PCR. Genomic DNA was extracted (DNeasy, Qiagen) from wild-type and transfected ES cell lines and PAR-1 were amplified (forward primer 5'CTTGGAGCCTACCTAGACTCA3' and reverse 5'TCCTAAGTTAACAGCTTTTG3'). Expression of the PAR-1 transgene was confirmed reverse transcriptase (RT) PCR analysis (Senscript Reverse Transcriptase, Qiagen) of RNA isolated from cardiac myocytes-derived from ES cells (RNeasy, Qiagen) (forward primer 5'CAGTTTGGGTCTGAATTGTGT3' and reverse 5'TGCACGAGCTTATGCTGCTGA3'). In addition, transgenic receptor expression in the transfected ES-derived cardiac myocytes was further confirmed by immunostaining with an anti-FLAG epitope monoclonal antibody (M1, Sigma), and developed with DAB (Iso-IHC Kit, Immunogenex) followed by hematoxylin counterstaining. GFP expression in controls was confirmed by epifluorescence (excitation 488 nm/emission 540 nm).

#### In Vitro Thrombin Biosensing

The ES-derived cardiomyocytes ( $10^6$  cell  $\text{mL}^{-1}$ ) were pretreated for 10 min in culture medium containing thrombin (American Diagnostics,  $2 \text{ IU mL}^{-1}$ ), isoproterenol (Sigma,  $10^{-9} \text{ M}$ ), and metoprolol (Ayerset,  $10 \text{ ng mL}^{-1}$ ). The normalized cardiac cAMP content ( $\text{fmol cAMP mg}^{-1}$  cardiac troponin T) was determined from ELISA measurements of cAMP (EIA kit, Biotrack) and antitroponin T (MS-295, Santa Cruz, CA). Statistical significance was determined by a student's t-test.

#### In Vivo Thrombin Biosensing

In order to determine the *in vivo* thrombin responsiveness of the genetically engineered cardiac myocytes aggregates, we employed a murine pinnal transplant model, which provides an electrically isolated and easily accessible environment to study the exogenous tissue, as previously described.<sup>2</sup> ES cell-derived cardiac myocyte aggregates were physically dissociated and suspended in PBS ( $5 \times 10^4$  cells in  $20 \mu\text{L}$ ) and implanted subcutane-



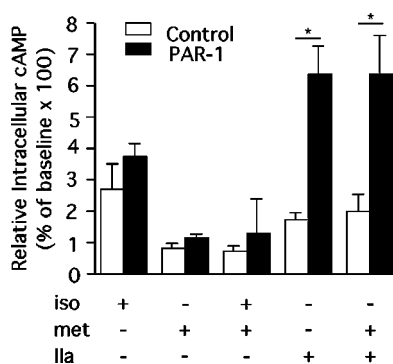
**FIGURE 1.** Genetic engineering of PAR-1 expression in ES cell-derived cardiac myocyte aggregates. (A) Representative RT-PCR for PAR-1 in cardiac myocytes derived from PAR-1 and control ES cells. (B) Representative immunostaining for FLAG-tagged PAR-1 (DAB) of cardiac myocyte aggregates derived from PAR-1 and control ES cells.

ously in the pinnae of 3-month-old C57B1/6 mice pretreated with PDGF AB. One week after transplantation, endogenous and allograft electrocardiographic (ECG) activity were measured at base line and after local delivery of thrombin ( $10 \text{ IU}/10 \mu\text{L}$  PBS) or vehicle alone injected subdermally at the base of the aggregates through a 33g needle. Electrocardiograms were acquired via an A-M Systems Model 1700 four-channel differential ac amplifier, bandpass filtered between 3.0 and 100.0 Hz, notch filtered at 60.0 Hz, amplified 1000 $\times$ , and sampled at 500 Hz by a National Instruments AT-MIO-16E-10 data acquisition board on a 266 MHz Pentium-II computer running real-time Linux and analyzed using custom Linux C++ software to measure two second mean interexcitation intervals so that the signal dynamics could be compared quantitatively at synchronized time slices as previously described.<sup>1</sup> Statistical significance was determined by the student's t-test.

## RESULTS

#### Genetic Engineering of Cell-Based Biosensors

In order to develop cell-based biosensors for hemostatic activity ES cell-derived cardiac myocytes were genetically engineered to express PAR-1. Molecular and protein assays confirmed the successful genetic engineering of the aggregates. RT-PCR of RNA isolated from the ES-cell-derived cardiac myocytes demonstrated that the



**FIGURE 2.** *In vitro* responsiveness of PAR-1 engineered ES cell-derived cardiac myocyte aggregates. Relative levels of intracellular cAMP in PAR-1 and control engineered cardiac myocytes (% of base line) in the presence of isoproterenol (iso;  $10^{-9}$  M), metoprolol (met;  $10$  ng  $\text{ml}^{-1}$ ), and thrombin (IIA;  $2$  IU  $\text{mL}^{-1}$ ) as well as combinations of isoproterenol with metoprolol and thrombin with metoprolol. \* $P < 0.05$  PAR-1 vs. control.

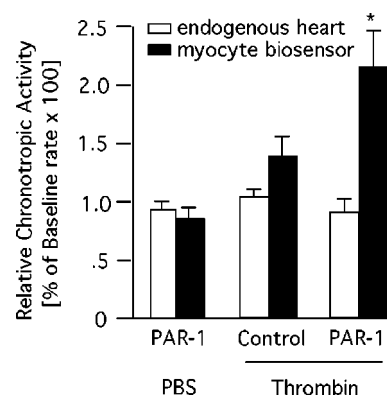
PAR-1 engineered cells expressed the PAR-1 transgene [Fig. 1(A)]. Moreover, immunostaining revealed that the transgenic receptor was present on the engineered cardiac myocytes [Fig. 1(B)].

#### *In Vitro Cell-Based Biosensor Thrombin Responsively*

The integrity of the PAR-1 transgenic receptor intracellular signaling pathways intracellular was confirmed *in vitro* (Fig. 2). Studies in the presence of isoproterenol, an adrenergic agonist, and metoprolol, an adrenergic antagonist, revealed that the engineered cells maintained responsiveness to endogenous catecholaminergic inputs. Importantly, PAR-1 cardiac myocytes cultured in the presence of thrombin demonstrated sixfold induction cAMP generation, as compared with a twofold induction in the control cells. Moreover, the engineered cellular response to thrombin was independent of adrenergic stimulation, suggesting the PAR-1 expressing cells could act as biosensors of local hemostatic activity.

#### *In Vivo Cell-Based Biosensor Thrombin Responsively*

In order to test the *in vivo* thrombin biosensor responsiveness the engineered cardiac aggregates were transplanted into the pinnae of host mice. The PAR-1 engineered cells specifically responded to delivery of thrombin with a twofold increase in chronotropic activity as compared with a less than 50% increase in control aggregates activity (Fig. 3). Delivery of vehicle alone had a minimal change in the chronotropic rates of the PAR-1 expressing cells. Similarly, the endogenous host heart rates were not significantly altered by delivery of thrombin or control vehicle.



**FIGURE 3.** *In vivo* thrombin biosensor responsiveness of PAR-1 engineered ES cell-derived cardiac myocyte aggregates. The relative chronotropic activities of myocyte aggregates and endogenous host hearts (% of base-line activity) in the presence of thrombin or vehicle alone (PBS). Thrombin resulted in a twofold increase in the rate of the PAR-1 engineered biosensors as compared with a less than 50% increase in control aggregates activity and did not significantly affect the endogenous heart rates. PBS resulted in minimal changes in rates of the aggregates and the endogenous heart rates. \* $P < 0.05$  PAR-1-thrombin vs. PAR-1-PBS and PAR-1 thrombin vs. control-thrombin.

## DISCUSSION

The results of our studies demonstrate the feasibility of expanding the physiologic inputs of a new class of *in vivo* biosensors. Importantly, we found that genetic engineering of the cell-based biosensor through the expression of the thrombin receptor allows for the real-time monitoring of the enzymatic components of the blood coagulation cascade. Moreover, the application of genetically plastic stem cell technology to detect specific blood-borne signals should facilitate the development and potential clinical translation of cell-based biosensors for the direct monitoring of other physiologic, as well as pathophysiologic, signals.

Our studies exploited the cellular signaling pathways endogenous to cardiac myocytes. Biosensors engineered to overexpress PAR-1 specifically detected thrombin both *in vitro* and *in vivo*, suggesting that this approach may allow the real-time monitoring of blood coagulation. Developing the utility of cardiac myocyte-based thrombin biosensors may require that the engineered cardiac myocyte aggregates interface with silicon chips or other defined biocompatible materials<sup>4,8,9</sup> as part of intravascular devices to measure changes in hemostatic function. Indeed, such approaches could allow for monitoring of vascular bed-specific thrombotic potential, which is not possible with present *in vitro* measurements of blood clotting parameters. Moreover, *in vivo* thrombin biosensors might enhance catheter-based treatments for coronary and peripheral arterial diseases by facilitating real-time titration of local anticoagulant therapies.

Overall, the present studies demonstrated that biologically based biosensor systems can be targeted to detect specific blood-borne signals. Developing the clinical utility of excitable cell-based biosensors may require that the cardiac myocytes be derived from autologous sources of stem cells such as the endogenous bone marrow.<sup>10</sup> Alternatively, molecular advances may facilitate the genetic engineering of precursor or preexisting excitable cells for the development of a long-term, physiologically tuned, functionally integrated bioprocessing interface.

### ACKNOWLEDGMENTS

This work was supported by grants from the American Heart Association 015034N (J.M.E.) and 0030028N (D.J.C.) and the National Heart Lung and Blood Institute PO1-HL-59312 (J.M.E.).

### NOMENCLATURE

ES embryonic stem  
PAR-1 protease activated receptor-1

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