

■ Multi-Model Monitoring and Process Diagnosis for Sustainable Biological Operation

On-line monitoring of a batch bioprocess is a crucial task to produce high-quality products with safety. Small changes in concentrations or flows may have large effects on the biological reaction kinetics, leading to batch-to-batch variations in effluent quality and microorganism growth. Such influent variations cause bioprocesses to evolve over time as the microorganisms adapt to the changing operating conditions. These factors lead to changes in the microbial community and multiple operation modes within a bioreactor. To handle these problems in a bioreactor, Yoo and co-workers propose a local batch monitoring method with multiple probabilistic models in order to monitor a batch bioprocess with several operating conditions and to identify when a process has shifted to a new operating condition. Their findings show that the local monitoring approach can give more reliable and higher resolution monitoring results than the global model. *Page 687*

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■ Using Your Head for VFA Analysis

Biogas formation by anaerobic digestion of waste streams continues to be studied and evaluated as a fossil fuel replacement technology. Accurate measurement of the variables associated with these bioprocesses—pH, alkalinity, gas production or composition, volatile fatty acid (VFA) concentration—enable bioengineers to optimize a given process. Since the presence of certain VFAs is an indicator of stress in a process, analytical methods that detect and identify individual VFAs within a given sample, and not just total VFA concentration, are valuable. Although both online and offline methods that employ gas chromatography to measure individual VFAs exist, they often involve extensive sample preparation. The Angelidaki group describes an online sampling system coupled with head space gas chromatography (HSGC) to rapidly and reliably analyze individual VFA components. Since a filtration step is not invoked, their method reduces maintenance related to membrane fouling and is applicable to both solid waste and viscous samples. *Page 712*

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■ Neovasculature for the Healing Heart

Recent attempts at rebuilding the myocardium using stem cells have yielded disappointing results. The lack of a supporting vasculature for the highly oxygen dependent differentiating stem cells may in part explain these disappointing findings. Concerns over possible side effects have hampered

attempts at revascularizing the infarcted myocardium using systemic delivery of pro-angiogenic compounds such as vascular endothelial growth factor (VEGF). In this issue of B&B, Scott and co-workers present a novel approach for preferentially delivering drug carrying immunoliposomes to infarct tissue by targeting adhesion molecules, such as P-selectin, which are upregulated in the vasculature of infarcted myocardium. Local delivery of pro-angiogenic compounds, such as VEGF and/or angiopoietin, to the infarct area could initiate the growth of neovasculature supporting the growth of stem cells which in turn could lead to improved cardiac function. In addition, these investigators present data indicating that this targeted treatment can be given over an extended period of time by targeting different adhesion molecules which are upregulated at various time points post-infarction. Previously, these investigators have shown that selective delivery of immunoliposomes, containing antivascular drug combretastatin and targeted to upregulated adhesion molecules in irradiated tumor vasculature, results in significant tumor growth delay in mice. *Page 795*

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■ A Baculovirus Titration System for High-Throughput Applications

The numbers of proteins being targeted for structural and functional proteomics are increasing and consequently, the major expression systems are tasked with trying to meet this demand. As a result, different high-throughput expression strategies are being used to automate various steps in the protein production pipeline. The baculovirus expression vector system (BEVS) is no exception to this, but has proved difficult to adapt to a parallel process. However, recent developments in baculovirus technology, such as *flashBAC*TM (Oxford Expression Technologies, UK), have brought the BEVS back into the spotlight and it is now fully compatible with automated platforms. In this study, the Hitchman group describes a QPCR-based method to rapidly quantify recombinant virus stocks, removing another major bottleneck in the BEVS and further complementing its automation. By correlating virus DNA with plaque assay titre, they have developed an equation that determines unknown titres based on cycle threshold values. They show that this method is both accurate and reproducible, and removes the need for time consuming and technically demanding cell culture-based techniques. *Page 810*

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Targeted Delivery of Antibody Conjugated Liposomal Drug Carriers to Rat Myocardial Infarction

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ABSTRACT: Immunoliposome (IL) targeting to areas of inflammation after an acute myocardial infarction (MI) could provide the means by which pro-angiogenic compounds can be selectively targeted to the infarcted region. The adhesion of model drug carriers and ILs coated with an antibody to P-selectin was quantified in a rat model of MI following left coronary artery ligation. Anti-P-selectin coated model drug carriers showed a 140% and 180% increase in adhesion in the border zone of the MI 1 and 4 h post-MI, respectively. Radiolabeled anti-P-selectin ILs injected immediately post-MI and allowed to circulate 24 h showed an 83% increase in targeting to infarcted myocardium when compared to adjacent non-infarcted myocardium. Radiolabeled anti-P-selectin ILs injected 4 h post-MI and allowed to circulate for 24 h showed a 92% increase in accumulation in infarcted myocardium when compared to adjacent non-infarcted myocardium. Targeting to upregulated adhesion molecules on the endothelium provides a promising strategy for selectively delivering compounds to the infarct region of the myocardium using our liposomal-based drug delivery vehicle.

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KEYWORDS: adhesion molecules; P-selectin; myocardial infarction; targeted drug delivery

Introduction

Myocardial infarction (MI), which is the end result of the occlusion of coronary arteries, is a leading cause of death in the United States and other industrialized countries. Each year, approximately 1.5 million people suffer an MI and close to one third of them die (Thom et al., 2006). A transmural MI involves necrosis of cardiomyocytes and local inflammation and proteolysis of the extracellular matrix, vasculature, and nerves. After the acute episode of infarction, a scar will form in an attempt to repair the necrotic area. The infarct scar, which is avascular and composed predominately of fibrillar collagen, is enclosed by a border zone which is still somewhat nourished by a vasculature. Another prominent feature of a MI is inflammation, which occurs in the sub-acute phase, in which endothelial cells express adhesion molecules for leukocytes on their surfaces (Sakhalkar et al., 2003). Leukocytes in the blood stream express ligands for these adhesion molecules enabling them to selectively adhere to the inflamed endothelial cells. Adhesion molecules, including ICAM-1, P-selectin, E-selectin, and $\alpha_v\beta_3$, have been reported to be upregulated on endothelium in response to ischemia and inflammatory mediators in several tissues

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(Burch et al., 2002; Goetz et al., 1997; Hickey et al., 1999; Kanwar et al., 1997).

Coronary bypass surgery, stents, and balloon dilations are all classical but very invasive treatments for treating heart disease. However, the development of a less invasive treatment capable of preventing further damage (caused in part by the inflammatory response) and promoting repair of damaged tissue would likely improve patient outcomes. Bold innovative strategies are needed to prevent the appearance of chronic cardiac failure following MI. "Engineering" lost myocardium and its supporting vasculature, in approaches such as bone marrow-derived cell therapy (BMDSC), may represent such an initiative. Of necessity, rebuilt myocardium must include a vascular network able to nourish it under diverse metabolic demands. For new cardiac cells to grow, the creation of new vascular networks must also be initiated in the infarcted region. Ideally, this would occur before the phase of scar formation. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been found to promote angiogenesis in ischemic cardiac muscle after direct injection (Hughes et al., 2004). However, VEGF therapy, especially if administered systemically, has many observed and potential side effects including, but not limited to, unintended angiogenesis in distant tissues, increased microvascular permeability, development of new or enhancement of pre-existing neoplasms, and increased atherosclerotic plaque mass and instability (Epstein et al., 2001; Henry et al., 2003; Rosengart et al., 1999). Altered endothelial surfaces present an opportunity for selectively delivering drugs to inflamed tissue. For example, large anti-ICAM-1 microbubbles have been used in myocardial contrast echocardiography (MCE) in clinical setting to detect regions of acute cardiac transplant rejection (Weller et al., 2003). This modality could be adapted to selectively deliver drugs to areas of inflammation via upregulated adhesion molecules. Conventional liposomes, which have been used clinically (Kacimi et al., 1996; Kogaki et al., 1999), can be further developed as a biocompatible vector for a less invasive treatment for damaged myocardium.

Utilizing the targeting system by which leukocytes are attracted to inflamed areas in the body (Park et al., 2002; Smith et al., 2002), we have developed a methodology by which liposomal drug carriers can be selectively targeted to the infarcted regions of the heart. Therefore, the inflammation present in the border zone (the tissue immediately surrounding the necrotic region of the left ventricle wall) is what will be targeted by the liposomal drug delivery system developed in our laboratory. This approach can be used to deliver pro-angiogenic compounds in order to initiate the growth of new vessels capable of infiltrating the infarcted myocardium to regenerate the lost blood supply post-MI. By attaching ligands to various adhesion molecules to the surface of liposomes, we can determine which ligands present the most promising targeted delivery system for drugs to the post-MI myocardium.

Materials and Methods

Animal Model of MI

An MI model in 6-week-old male Sprague-Dawley (SD) rats (Harlan Laboratories, Frederick, MD), weighing ~250 g, was used in these experiments (Wang et al., 2005). In brief, following induction of anesthesia with isoflurane, an anterior transmural MI was induced by ligation of the left anterior descending coronary artery (LADA) with a 4-0 silk suture (Fisher Scientific Co.) as previously reported (Wang et al., 2005). The MI was detected by evidence of elevated ST segment and the appearance of Q waves on ECG, grossly visible necrosis of the left ventricular free wall, which was characterized by pallor and softening of the cardiac tissue, and by microscopic evidence of necrotic cardiomyocytes and acute inflammation (Sun and Weber, 1996). One minute before euthanasia, a fluorescent dye, (DiOC₇, 3,3'-Diheptyloxacarbocyanine iodide, Molecular Probes, Carlsbad, CA) was injected via tail vein to later identify perfused vessels in the cardiac tissue which was then used to identify the location and extent of the MI in each animal. The protocol was approved by the institution's Animal Care and Use Committee.

Preparation of Ligand Coated Model Drug Carriers

Ligand coated polystyrene microspheres (Duke Scientific, Fremont, CA) were used as model drug carriers (Prabhakarpandian et al., 2001). Red fluorescing polystyrene microspheres (2 μm in diameter) were coated with an IgG_{2a} mouse monoclonal antibody (mAb) RMP-1 to rat P-selectin (Kulidjian et al., 2002; Walter et al., 1997; Yuan et al., 2005). Blue fluorescing polystyrene microspheres (2 μm in diameter) coated with mouse IgG1, Kappa (Sigma-Aldrich) were used as a control.

The microspheres were prepared by washing three times with sodium bicarbonate buffer (0.1 M at pH 9.2) then overnight incubation with sodium bicarbonate buffer containing recombinant protein A (0.3 mg/mL, Zymed Co.) (Yuan et al., 2003). The protein A coats the microspheres providing a surface for the ligand to attach to the microsphere. The microspheres were then washed three times with Hank's Balanced Salt Solution (HBSS) (Fisher Scientific Co.) + 1% bovine serum albumin (BSA) (Fisher Scientific Co.) and then incubated for 20 min with HBSS + 1% BSA to block any non-specific binding sites on the surface of the microsphere not occupied by protein A. Finally, the ligand (i.e., anti-P-selectin or mouse IgG) was added at a concentration of 100 $\mu\text{g}/\text{mL}$. Similar procedures were used to attach other ligands to microspheres. Using published techniques (Burch et al., 2002) the surface density of antibodies on the microspheres was found to be approximately 6,400/ μm^2 and the total number of antibody molecules per microsphere was found to be approximately 81,000. The specific adhesion of antibody coated microspheres to activated endothelium under shear flow has been

confirmed at physiologically relevant flow rates using an *in vitro* flow chamber and has been confirmed in several *in vivo* models (Burch et al., 2002; Kiani et al., 2002; Sakhalkar et al., 2003; Yuan et al., 2005).

Targeting Ligand Coated Model Drug Carriers to Infarct Tissue

After a predetermined amount of time post-MI, antibody coated model drug carriers (2×10^8 microspheres of each color) were re-suspended in 0.1 mL of DiOC₇ (Burch et al., 2002) and then injected via tail vein and allowed to circulate in the blood stream for 1 min (Burch et al., 2002). The animal was euthanized and the heart was quickly removed and washed with a saline solution. The heart was covered with optimal cutting temperature (O.C.T., Fisher Scientific) and quickly frozen using a dry ice/2-methylbutane bath, and sectioned (10 μ m thick) using a cryostat (Lecia CM3050 S).

The area of infarction was identified by the absence of DiOC₇ staining of perfused vessels in the left ventricle wall (Wang et al., 2005). Images were taken (Nikon Eclipse TE200, ImagePro 6.0) in both the border zone (directly adjacent to the necrotic band in left ventricle wall) and non-infarcted myocardium (taken from right ventricle wall) with two different fluorescent filters (one for red fluorescing antibody coated model drug carriers and one for green fluorescing IgG coated model drug carriers).

Immunohistochemistry

Four hours after surgical induction of MI, another set of tissue was fixed in 10% buffered formalin, the dissected tissue was embedded in paraffin and sectioned in a microtome at 4 μ m in thickness. Sections were placed on electromagnetically charged slides (Superfrost plus, Fisher Scientific Co.) and then stained with hematoxylin and eosin for routine histological evaluation. Immunohistochemistry was performed using the avidin–biotin–peroxidase system according to the manufacturer's instructions (Vector Laboratories). Our modified protocol included deparaffination in xylene, re-hydration (100%, 90%, 70% alcohol, followed by water), antigen retrieval with citrate buffer pH 6.0 at 95°C for 25 min and endogenous peroxidase quenching with 20% H₂O₂ in methanol for 30 min. After rinsing with phosphate buffered saline (PBS), the sections were blocked with normal rabbit serum for 1 h at room temperature and incubated with a primary antibody (goat anti-P-Selectin, C-20, 1:250 dilution, Santa Cruz Biotechnology), overnight at room temperature in a humidified chamber. After washing with PBS, a rabbit anti-goat biotinylated secondary antibody was incubated for 1 h at room temperature, followed by incubation with avidin–biotin complexes (Vector Laboratories, Burlingame, CA) for 1 h. Finally, sections were developed with diaminobenzidine (Sigma-Aldrich), counterstained with hematoxylin and mounted with Permount (Fisher Scientific Co.).

Preparation of Immunoliposomes (ILs)

The long circulating liposomes were composed of hydrogenated soy phosphatidyl choline (HSPC, 50 mole %), cholesterol (45 mole %), and distearoyl phosphatidyl choline-PEG 2000 conjugate (DSPE-PEG, 5 mole %). Lipid ratios of 1–10 mole % DSPE-PEG were also tested in our liposome formulations. While, increasing the ratio of DSPE-PEG lowered the rate of liposome uptake by the liver and spleen, it was found that increasing the mole % of PEG above 5% (the level used in our final formulation) destabilized the liposome membrane. For preparation of the long circulating liposomes with attached ligands, a part of DSPE-PEG (2 mole %) was replaced with the DSPE-PEG-Maleimide functional lipid (Nallamotheu et al., 2006).

Cholesteryl (4-¹⁴C) oleate at a concentration of 0.4 μ Ci/ μ mol of lipid was used for radiolabeling of liposomes. Appropriate amounts of lipids were dissolved in chloroform:methanol (9:1 v/v) in a round bottom flask. The solvent was evaporated under reduced pressure and constant rotation (Rotovapor R-200, Buchi) to form a thin lipid film. Multilamellar liposomes were prepared by hydrating dry lipid films in 50 mM phosphate/150 mM NaCl- buffer pH 6.5, at a lipid concentration of 50 mM. The resulting multilamellar vesicles (MLV) were then sized by repeated extrusion (Lipex, Vancouver, BC, Canada) through polycarbonate membranes (Nucleopore) of gradually decreasing pore size (0.6, 0.4, 0.2, and 0.1 μ m) to prepare small unilamellar vesicles (SUV) approximately 100 nm in diameter. After extrusion, the liposomes were stored at 4°C until used (Nallamotheu et al., 2006).

To prepare ILs using IgG_{2a} mAb RMP-1 to rat P-selectin, the antibody was first modified with succinimidyl acetylthioacetate (SATA) to introduce thiol groups (Derksen and Scherphof, 1985). The thiolated antibody was then coupled with maleimide groups on the liposome surface at pH 6.5. Molar ratio of Ab-SH to maleimide was 1:40 using an Ab-SH concentration of 0.3 mg/mL. The reaction (carried out at room temperature) was stopped after 24 h by the addition of N-ethylmaleimide at a molar ratio of 1:24. Following the coupling reaction, unassociated ligand was removed by gel filtration on a Sepharose CL-4B column (Nallamotheu et al., 2006). The number of mAbs attached to each liposome was estimated by testing the protein content in the formulation after separation of the uncoupled mAb with a BCA protein assay (Pierce Biotechnology, Rockford, IL) (Pattillo et al., 2005). Using this value and the phospholipid content, the number of mAb on each liposome was calculated to be approximately 80 mAbs.

Biodistribution of ILs in an MI Rat

The MI was induced as previously described in the rat model. Immediately after the chest was closed following the LADA ligation surgery and before the animal recovered from anesthesia, 0.1 mL of 10 mM radiolabeled anti-P-selectin coated ILs was injected via tail vein and allowed to

circulate for 24 h. At the end of this 24 h period, the blood was flushed from the animal by injecting saline with a syringe into the left ventricle of the heart and making a small opening with microscissors in the right ventricle. The infarcted region in the left ventricle wall was isolated from the rest of the cardiac tissue and analyzed separately from the remaining non-infarcted myocardium.

The liver, spleen, and a sample of blood (taken with a heparinized syringe from the heart immediately before flushing the blood) were also removed from each rat. The organs were dissolved in 2 mL of solubilizer liquid (Solvable, PerkinElmer, Boston, MA) in a 50–60°C water bath. EDTA di-sodium salt solution, 0.1 mL of 100 mM concentration, was added to each vial to reduce foaming. Once fully dissolved, the samples were bleached with 0.3 mL of 30% hydrogen peroxide in a drop-wise fashion until each sample became clear. The scintillation vials were again incubated for 1 h in a 50–60°C water bath until the samples turned bright yellow. After the vials cooled to room temperature, Ultima Gold (Perkin-Elmer) was added to prepare the samples for analysis in a liquid scintillation counter (Tri-Carb 2800TR). The concentration of liposomes injected was normalized by body weight.

Targeting Radiolabeled ILs to Infarct Tissue

Radiolabeled ILs, 0.1 mL at a concentration of 10 mM, were injected via tail vein and allowed to circulate a predetermined amount of time. Afterwards, blood was flushed from the animal by injecting saline with a syringe into the left ventricle of the animal and making a small opening with microscissors in the right ventricle. The organs, blood, and heart tissue were removed as previously described. The tissue was processed as previously described for measurement in the scintillation counter.

Statistics

Data are presented as mean \pm standard error of the mean (SEM). The level of model drug carrier adhesion between various treatment groups was tested using ANOVA and Student–Newman–Keuls (SNK) was used to test between the means. In the IL experiments, the statistical comparisons between counts per minute (CPM) in the normal tissue and CPM in the adjacent infarcted tissue were done with a paired *t*-test. The differences were considered to be significant if $P < 0.05$.

Results

Targeting Anti-P-Selectin Coated Model Drug Carriers to Infarct Tissue

DiOC₇, which stains perfused vessels, readily demarcates the area between perfused vessels and non-perfused vessels. This

border zone of the infarcted myocardium is the transition area directly adjacent to the necrotic region. The level of adhesion of anti-P-selectin coated model drug carriers in the border zone of the infarcted myocardium (Fig. 1, red) was much higher when compared with the adhesion of IgG coated model drug carriers in the same region of the tissue (Fig. 1, green) from images taken using different fluorescent filters.

Anti-P-selectin coated model drug carriers injected 1 h post-MI showed a significant ($P < 0.001$) increase in adhesion (140%) in the border zone as compared to non-infarcted regions in the same heart (Fig. 2). The IgG coated model drug carriers injected 1 h post-MI did not show a significant ($P = 0.469$) increase in adhesion in the border zone as compared to non-infarcted regions in the same heart. Anti-P-selectin coated model drug carriers injected 4 h post-MI also showed a significant ($P < 0.001$) increase in adhesion (180%) in the border zone as compared to non-infarcted regions in the same heart (Fig. 2). IgG coated model drug carriers injected 4 h post-MI did not show a significant ($P = 0.424$) increase in adhesion in the border zone as compared to non-infarcted regions in the same heart. These results suggest that the model drug carriers coated with anti-P-selectin are preferentially adhering to the border zone of the infarcted heart 1 and 4 h post-MI and not to normal tissue in other parts of the myocardium. No preferential adhesion of anti-P-selectin coated model drug carriers injected 24 h post-MI was observed in the border zone of the infarcted myocardium (Fig. 2).

Immunohistochemical Analysis of P-Selectin Upregulation in Infarcted Tissue

The area of infarction was histologically identified by the presence of myocytolysis with coagulative necrosis, edema,

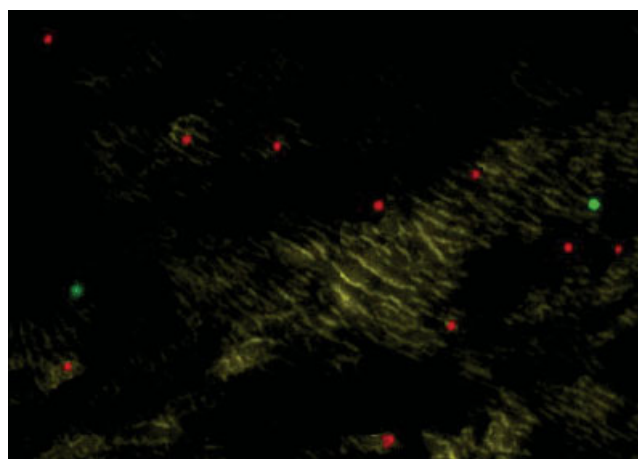


Figure 1. Anti-P-selectin coated model drug carriers (red) adhere preferentially to the infarcted myocardium as compared to IgG coated model drug carriers (green) in the same tissue 4 h post-MI. Muscle tissue autofluorescence is pseudocolored in yellow.

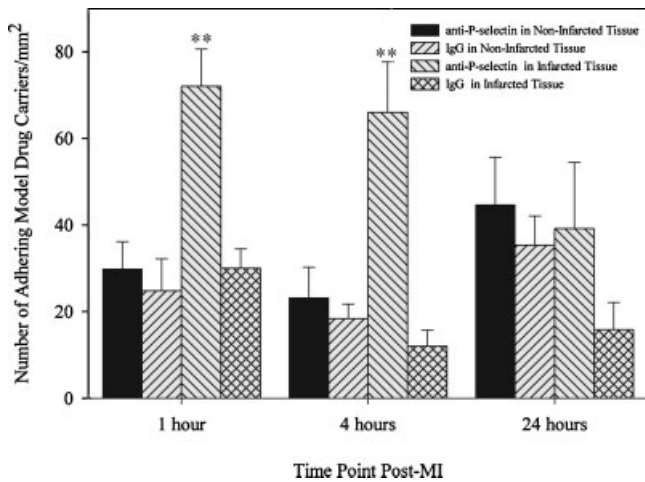


Figure 2. Anti-P-selectin coated model drug carriers were found to adhere preferentially to myocardium in the border zone near the infarcted area at 1 and 4 h post-MI (140% and 180%, respectively). Mean \pm SEM; $n=5$ animals per group, ** $P < 0.01$ indicates significant differences in anti-P-selectin adhesion in infarcted tissue compared to all other groups within each time point.

which caused separation between cardiac cells, areas of hemorrhage and incipient neutrophilic infiltrates (Fig. 3, Panel B) as compared to the normal myocardium, in which myocardial cells are well preserved, closely packed and present cytoplasmic striations (arrow in Panel A, Fig. 3). In the marginal zone between the normal tissue and the necrotic tissue, prominent blood vessels showed robust endothelial cell expression of P-selectin (Fig. 3, brown staining in Panel D) as compared to the weak immunoreactivity seen in endothelial cells from blood vessels in the normal heart (Fig. 3, Panel C). This further suggests P-selectin as a potential target for selectively delivering drug carriers to tissue post-MI.

Biodistribution of Anti-P-Selectin Coated ILs in an MI Rat

As shown in Figure 4, there was a significant ($P=0.01$) increase in the accumulation of anti-P-selectin conjugated ILs in infarcted myocardium compared to normal myocardium. In agreement with the literature (Allen, 1997; Lian and Ho, 2001; Torchilin et al., 1992) anti-P-selectin coated ILs injected immediately post-MI and allowed to circulate for 24 h accumulated in the spleen at a much higher per gram basis than in the other tissues tested. The liver accumulated a much higher total number of liposomes due to its large size but had a similar concentration of liposomes on a per gram basis as the liposomes still circulating in the blood after 24 h.

Targeting Anti-P-Selectin Coated ILs to Infarcted Tissue

Next the accumulation of radiolabeled anti-P-selectin coated ILs in the infarcted tissue was compared with the

accumulation of the radiolabeled anti-P-selectin coated ILs in the remaining non-infarcted tissue at different time periods of post-MI.

Anti-P-selectin coated ILs injected immediately post-MI and allowed to circulate for 24 h showed a significant ($P=0.01$) increase in adhesion (83%) to the infarct region as compared to the non-infarcted myocardium (Fig. 5). Anti-P-selectin coated ILs injected 4 h post-MI and allowed to circulate for 24 h showed a significant ($P=0.04$) increase in adhesion (92%) to the infarcted region as compared to the non-infarcted myocardium (Fig. 5). These findings indicated that anti-P-selectin mAb can be used to selectively target infarct tissue post-MI using our liposomal drug carrier. In a smaller group of animals ($n=3$), anti-P-selectin coated ILs were injected immediately post-MI and allowed to circulate for only 4 h. This experiment showed a significant ($P=0.03$) but small increase in adhesion (34%) to the infarcted region as compared to the non-infarcted myocardium (Fig. 5).

RGD conjugated ILs (RGD targets the $\alpha_v\beta_3$ integrin) were used as a control. The RGD conjugated IL control was tested at the peak anti-P-selectin conjugated ILs time point of 0–24 h (injected immediately post-MI and allowed to circulate for 24 h). No significant difference was observed between the accumulation of RGD conjugated ILs in the infarct region as compared to the accumulation of RGD conjugated ILs in the normal region of the myocardium. This control was not repeated at the other time points due to the cost involved in making the radiolabeled ILs.

Targeting to Other Adhesion Molecules Post-MI (Data Not Shown)

In addition to targeting upregulated P-selectin in myocardium post-MI, several other adhesion molecules were tested as potential targets. Anti-ICAM-1 coated model drug carriers injected 24 h post-MI showed a 23% significant ($P=0.02$) increase in adhesion in the border zone as compared to the non-infarcted myocardium. However, anti-ICAM-1 coated model drug carriers injected 48 h, 1 week, and 2 weeks post-MI did not show a significant increase in adhesion in the border zone as compared to the non-infarcted myocardium. Anti-E-selectin coated model drug carriers injected 4, 6, or 24 h post-MI did not show a significant increase in adhesion in the border zone as compared to non-infarcted myocardium in the heart.

Discussion

Selectively targeting pro-angiogenic compounds in the infarcted myocardium represents an innovative approach for rebuilding damaged tissue. Our findings indicate that anti-P-selectin coated model drug carriers and liposomes can be preferentially targeted to infarcted regions in the myocardium post-MI. Furthermore, our data indicate that

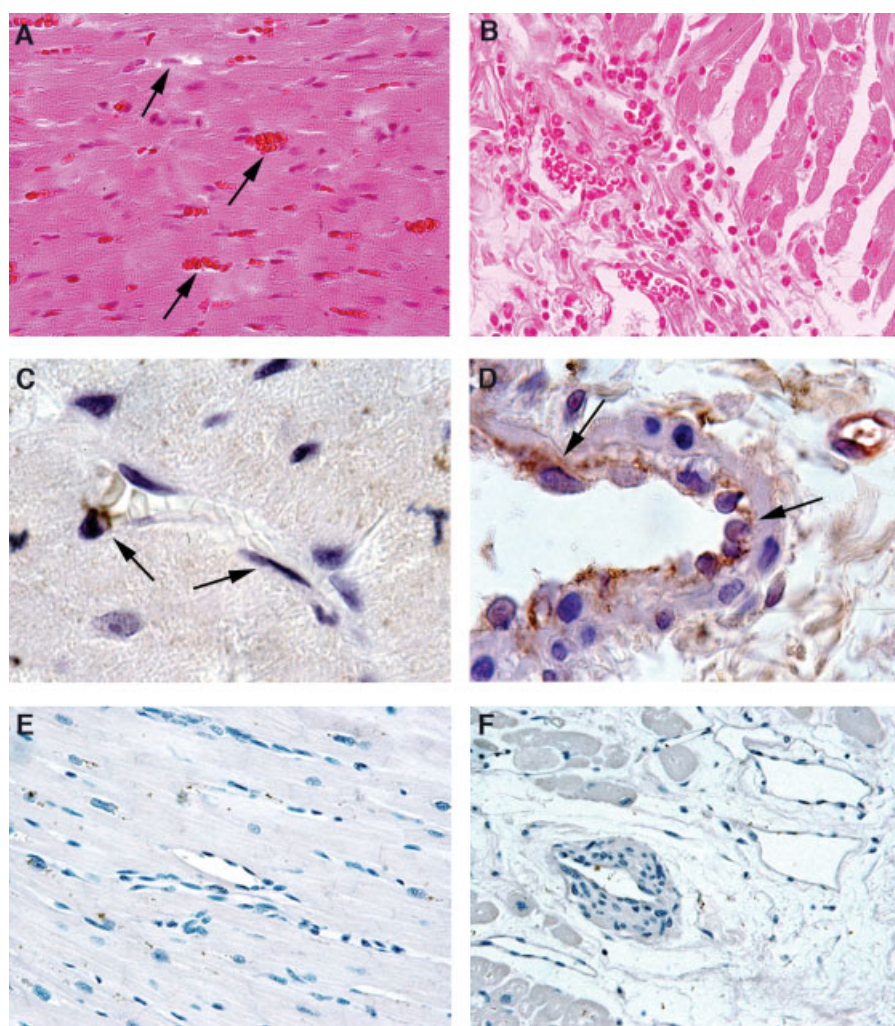


Figure 3. Low magnification view of a normal area of the heart shows numerous cylindrical myocardial cells with cytoplasmic striations and an abundant micro-capillary network (arrows) (**Panel A**, H&E staining). An area of infarction (4 h post-MI) demonstrates prominent loss of myocardial cells, which show fading cytoplasm and picnotic nuclei, and areas of coagulative necrosis. Fewer but more prominent blood vessels as well as inflammatory infiltration can be observed in the necrotic areas (**Panel B**, H&E staining). Immunohistochemistry for P-selectin shows weak reactivity in few capillary endothelial cells (**Panel C**, arrow), compared with a robust immunoreactivity in prominent endothelial cells in the areas of infarction 4 h post-MI (**Panel D**, arrow). Isotype matched negative controls for the immunohistochemistry in the normal areas (**Panel E**), and the infarction areas (**Panel F**), are shown. Original magnification for Panels A and B is 200 \times , Panels C to F is 1,000 \times . $n = 2$ animals.

the drug of interest (e.g., VEGF) may be targeted to infarcted tissue using anti-P-selectin ILs 0–24 h post-MI. No increase in adhesion of anti-P-selectin (or IgG) coated model drug carriers was observed in the non-infarcted myocardium.

Model drug carriers used in this study are easy to produce and provide a valuable tool for testing the feasibility of various vascular targeting schemes (Sakhalkar et al., 2003). However, these model drug carriers are not clinically relevant (Yuan et al., 2005). Utilizing the liposomal drug carriers, adapted from our work in the field of targeted drug delivery to tumors (Nallamotheu et al., 2006; Pattillo et al., 2005), the upregulation of P-selectin due to inflammation and hypoxia can be used to deliver various compounds (e.g., pro-angiogenic agents such as VEGF) to diseased tissue. We observed a significant accumulation of clinically relevant

anti-P-selectin coated ILs in the infarct region within the first 24 h post-MI. These findings could have significant clinical implications in that delivering a 100–200% increased dose of a drug to the diseased area with no increase in the dose to normal tissue could yield a large benefit to the patient without an increase in side effects of the drug.

It is in the border zone in which the inflamed tissue still receives blood flow providing an excellent avenue for selectively targeting drugs to the diseased myocardium. In the model drug carrier experiments it was possible to identify only the border zone of the infarct region for analysis. Due to the difficulty in isolating only the border zone in the IL experiments, the entire infarct region of the left ventricle wall was isolated and tested separately from the non-infarcted myocardium. A significant portion of the

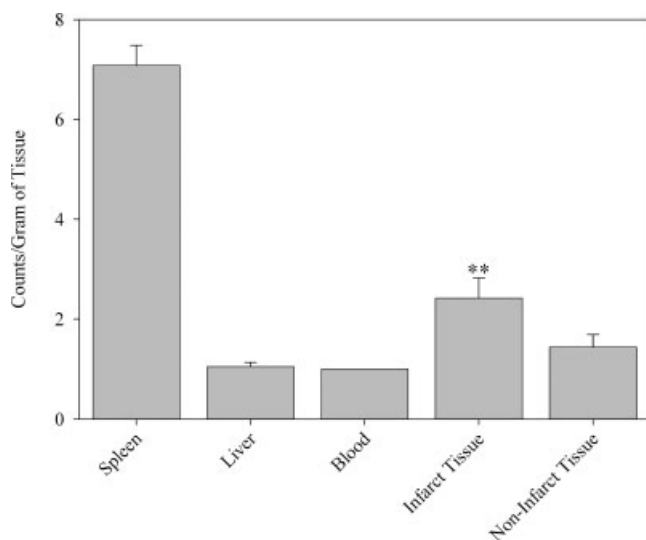


Figure 4. Radiolabeled anti-P-selectin immunoliposomes injected immediately post-MI and allowed to circulate for 24 h before sacrifice showed a preferential accumulation in the infarcted myocardium. Each measurement was normalized for number of radiolabeled immunoliposomes injected by dividing the number of counts per tissue by the animal weight and the liposome concentration in the blood. Mean ± SEM; $n=4$ animals. ** $P<0.01$ indicates significant differences in anti-P-selectin adhesion in infarcted heart tissue compared to non-infarcted heart tissue.

infarcted region is devoid of any blood flow making it impossible for liposomes to reach the majority of the necrotic tissue (which was still included in mass that was measured as the infarcted region). Therefore, it is reasonable

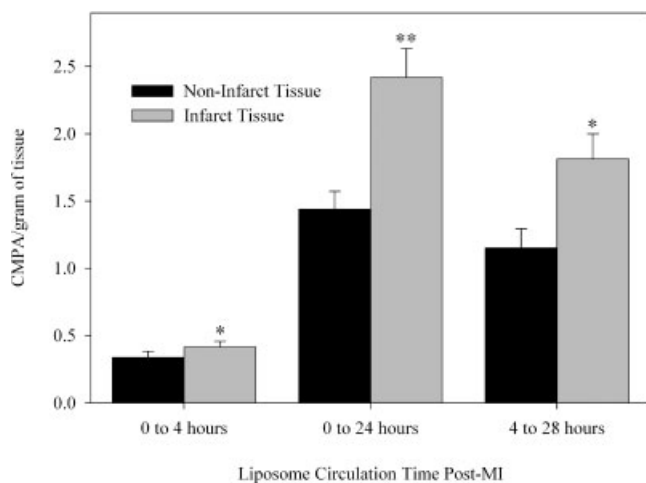


Figure 5. Anti-P-selectin coated immunoliposomes (IL) were found to preferentially accumulate in the infarcted myocardium at all the time points tested. Measurements were taken at various time points: 0–4 h (injected immediately post-MI, measured 4 h post-MI; $n=3$ animals), 0–24 h (injected immediately post-MI, measured 24 h post-MI; $n=7$ animals), 4–28 h (injected 4 h post-MI, measured 28 h post-MI; $n=7$ animals). Mean ± SEM. ** $P<0.01$, * $P<0.05$ indicates significant differences in anti-P-selectin adhesion in infarcted tissue compared to non-infarcted tissue at the same time point.

to assume that the number of liposomes targeted to the actual border zone (border zone is a relatively small fraction of the entire infarct region) is even higher than the values measured in our experiments.

The development of a minimally invasive treatment for regenerating lost vasculature after a MI would be very beneficial in our attempt at long-term treatment of heart disease. This treatment could provide a neovasculature for other treatments (e.g., stem cells) which are limited by the lost blood flow after the onset of an MI. We are currently investigating the possibility of using the approach outlined in this study to selectively deliver pro-angiogenic compounds (e.g., VEGF, bFGF) to infarct tissue in an attempt to rebuild the vasculature that is necessary to support regenerating myocardium (Weber et al., 2003).

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