



Review

Drug-induced vascular injury—a quest for biomarkers[☆]

Expert Working Group on Drug-Induced Vascular Injury¹

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1. Introduction

Drug-induced vascular injury in animals has been a topic of intense discussion and debate in the toxicology, clinical, and regulatory arena for the past 25 years. A lack of understanding of the basic mechanisms by which drugs cause vascular injury in animals, the absence of specific and sensitive biomarkers, and low cross-species safety multiples have become significant barriers in the development of many classes of therapeutic agents.

Vascular injury has been reported, with increasing frequency, as an adverse histological observation in preclinical toxicity studies that are conducted to support the safe introduction of new medicines in humans. Vascular lesions, primarily arterial, can be induced within hours of drug administration; their morphological and pharmacological reversibilities are not clear. Animals exhibit no clinical signs, and routine clinical pathology data are normal. While reported and postulated mechanisms are varied, vascular injury in animals is induced by either altered hemodynamic forces (shear and/or hoop stress), direct drug-induced toxicity, and/or immune-mediated injury of the endothelium and/or medial smooth muscle. Aside from histological methods, the detection, noninvasively, of acute drug-induced vascular injury in animals or humans is not currently possible due to the lack of specific and sensitive biomarkers of endothelial and/or vascular smooth muscle injury.

Historically (ca. 1980–1995), regulatory authorities and pharmaceutical companies have been able to manage the risk of drug-induced cardiovascular toxicity in animals as sufficient data emerged that appeared to correlate the occurrence of myocardial and vascular toxicity with decreases in blood pressure and reflex tachycardia. Founded on this principle, it became generally accepted that as long as therapeutic doses of candidate drugs in humans did not induce hypotension and reflex tachycardia, safety and regulatory concerns were lessened resulting in many products finding a clear path to the market place and/or approvable status (Table 1).

Hypotension and marked reflex tachycardia have been proven to cause myocardial necrosis. This tenet may not

apply to drugs that cause vascular lesions that are associated with myocardial lesions as with minoxidil and the phosphodiesterase (PDE) 3 inhibitors. Industry is now developing drugs that cause vascular injury in animals, but without changes in systemic blood pressure or heart rate, e.g., endothelin receptor antagonists, dopamine (DA1) agonists, adenosine agonists, second- and third-generation PDE 4 inhibitors, and others. It appears that the two events, myocardial and vascular toxicity, have different pathogenic mechanisms, the former related to myocardial ischemia and the latter unknown.

When toxicities in animals, e.g., drug-induced vascular injury, are reported and the therapeutic index and/or safety margins are either low or negative and there are no obvious associations with predictive biomarkers, it is incumbent upon industry to provide data to confirm that the new drug will be reasonably safe in humans. In the past, experience with many of the potent vasodilators (Minoxidil, PDE III inhibitors, hydralazine) led scientists to conclude that cardiovascular lesions observed in animals were associated with dramatic changes in hemodynamics leading to marked reflex tachycardia, which resulted in the induction of

Table 1
Approved or approvable drugs that cause arterial toxicity in animals

Drug	Mechanism	Preclinical cardiovascular effects
Milrinone	PDE 3 inhibitor	Decreased MABP/reflex tachycardia
Fenoldopam	DA1 agonist	Decreased MABP
Theophylline	PDE 3 inhibitor/adenosine antagonist	Decreased MABP/reflex tachycardia
Minoxidil	Potassium channel opener	Decreased MABP/reflex tachycardia
Adenosine	A1 agonist	Vasodilator
Hydralazine	?	Decreased MABP/reflex tachycardia
Bosentan	Endothelin receptor antagonist	No significant change in MABP or HR
Cilomilast	PDE 4 inhibitor	No significant change in MABP or HR
Nicorandil	Potassium channel opener/nitrate	Decreased MABP/reflex tachycardia

cardiovascular lesions (Joseph et al., 1996a, 1996b, 1997; Mesfin et al., 1987, 1989, 1995). Drug doses that did not cause marked reflex tachycardia did not cause lesions. These kinds of drugs progressed to clinical studies where patient safety was monitored by carefully evaluating blood pressure and heart rate and avoiding doses that caused marked decreases in systemic vascular resistance, hypotension, and reflex tachycardia.

Regulatory pressure on industry to identify reliable biomarkers has created very high hurdles for drugs that cause vascular injury. Concerns have been heightened by the known association between chronic vascular injury and inflammation (atherosclerosis) and the increased incidence of cardiovascular morbidity in humans (Pearson et al., 2003). The lack of “safety margins” and the lack of biomarkers has hindered development of life saving therapies in asthma, stroke, cerebral hemorrhage, pulmonary hypertension, chronic obstructive pulmonary disease, and others. However, as noted above, several drugs that cause vascular injury without hypotension and reflex tachycardia have been approved, without evidence of any known increased clinical risk. Although the messages between the regulatory authorities and industry are somewhat confusing, it is clear that industry must strive to develop methods to monitor for endothelial and/or smooth muscle compromise in normal animals and humans to clear the path for effective and efficient drug development in the future.

Adverse vascular toxicity, as described in animals, has not been reported in humans with the compounds listed in Table 1. Some compounds, like fenoldopam, bosentan, and cilomilast did not cause the classical picture of hypotension and reflex tachycardia in animal models, but nevertheless were approved or are approvable. In some of these cases, the animal to human safety multiples are very low or negative. While each drug is approved based on its own merit and safety and risk–benefit analysis, decision making must be based on consistent scientific principles, reinforcing the need for a review of the issues by the Expert Working Group.

The regulatory dilemma of vascular risk management will not be resolved quickly. In the interim, where unique physiology, pharmacology and metabolism, and adequate therapeutic indices and safety margins serve to segregate animal findings from the human, reasonable safety and risk determinations are possible. At this time, while it may not be possible to make generalizations and assumptions of clinical value for humans, decisions regarding clinical safety must continue to be based on weight of evidence and experienced and sound scientific clinical judgment on a case-by-case basis.

To address the gap in managing the risk of drugs that induce vascular injury, a Vascular Injury Expert Working Group (EWG) was assembled. Membership was confirmed in January 2001 and the EWG on Vascular Injury had its first organizational meeting in May 2001. The EWG was charged with the following: (1) evaluate the current state of

knowledge of drug-induced vascular injury in animals; (2) identify opportunities for discovery and validation of predictive biomarkers of acute vascular injury that could be used in preclinical drug safety programs and eventually in Phase I/II clinical programs. This report summarizes findings of the EWG to date, including:

1. current status of the field
2. current thinking on mechanisms of drug-induced vascular injury
3. opportunities for practical biomarkers and high-potential research areas
4. emerging technologies that hold promise in the search for new markers
5. opportunities for collaborative work between public and private partners

1.1. Background

1.1.1. Preclinical perspective

Arterial and venular injury is a relatively uncommon hazard identified during preclinical toxicity testing; however, it is commonly observed in over a dozen different pharmacological classes of drugs, including some that are approved products. The lesions of interest can be induced within hours in selected vascular beds in rats, dogs, pigs, monkeys, and/or mice. Depending on the induction protocol, lesions are usually reversible, although the literature is conflicting on this point. In rat and dog, vascular beds prone to drug-induced vascular injury are also susceptible to development of spontaneous vascular disease.

It is recognized that drugs that induce vascular lesions in animals present a safety assessment dilemma to toxicologists, physicians, and regulators wishing to assess safety of new medicines for humans. This dilemma is confounded by gaps in our knowledge regarding pathogenesis of injury, as well as limited knowledge regarding comparative physiology, pharmacology, and metabolism in various species, and, importantly, the absence of validated preclinical methods for monitoring vascular integrity noninvasively. Contrary to past thinking, preclinical experience with new and novel pharmaceuticals suggests that vascular injury is not associated with systemic changes in blood pressure and heart rate, rendering these parameters of little predictive clinical value. Variation in species responsiveness to vasoactive and nonvasoactive agents and marked differences in reactivity of selected vascular beds, taken together with contributions from numerous reactive cell types (e.g., endothelium, vascular smooth muscle, and inflammatory/immune cells) all add complexity to defining mechanism(s) and identifying robust biomarkers that are sensitive and specific.

Historically, toxicology studies in normal animals have not predicted the occurrence of the drug-induced hypersensitivity vasculitides, which are by far the most common drug-induced adverse vascular responses observed in

humans. In general, drug-induced hypersensitivity angitis of humans is not recognized in animal toxicology studies. Conversely, the common drug-induced vascular lesions recognized in animals are not known to occur in humans; it is possible, however, that this could be related to our lack of knowledge and methods for monitoring in humans. Given the extensive clinical experience with various drugs that cause vascular toxicity in animals, representing several different pharmacological classes, along with biopsy and autopsy data (Sobota, 1989; Sobota et al., 1980), this seems an unlikely possibility. Vascular wall biology is complex and heterogeneous within a species, making comparisons across species challenging and perhaps impossible. It is this complexity that creates the need for biomarker discovery, validation, and implementation.

Lack of consistency with histological terminology across laboratories in characterizing the key components of vascular damage has been problematic, as has the lack of precision, on occasion, in defining the vascular system, in contrast to its host tissue, as the correct target organ. The use of the words “drug-induced vasculitis, arteritis, or phlebitis” has created communication issues with clinicians because these diagnoses convey a picture of hypersensitivity angitis and this is clearly not the issue preclinically.

1.1.2. Clinical perspective

Pathologists define vascular injury in a broad, generic manner when structural changes occur in the vascular wall, which deviate significantly from normal and are visualized microscopically using accepted standards of science (Langford, 2003). In humans, the vast majority of drug-related vascular injury is pathogenetically inflammatory in nature, it affects small arterioles and venules, and generally an immune-mediated process is highly suspect until proven otherwise (Hoffman and Weyand, 2002; Langford, 2003; Sanguenza and Requena, 2003; Simon and Rogers, 2001). While the skin is frequently the target issue with hypersensitivity angitis, it is estimated that a small minority of these patients have internal organ involvement based on biopsy and autopsy data. Drug-induced vasculitis, in general, is reported to occur in <1% of patients as an adverse event during postmarketing surveillance (Federal Register, 1998; Physicians Desk Reference, 2003). To date, there are no examples of drug-induced vascular injury or vasculitis resulting in early withdrawal of a drug from the marketplace (Federal Register, 1998); perhaps with the exception of the sympathomimetic amine, phenylpropanolamine (PPA) (Horwitz et al., 2000). Drugs that cause altered vasomotion (constriction, dilatation), when administered acutely or over long periods of time, may be factors in lesion induction and remodeling that take place in chronic vascular injury. In addition, vessels already compromised by spontaneous disease, e.g., atherosclerosis or rheumatoid vasculitis, could possibly have increased susceptibility to drugs that specifically injure the vascular wall. It is well known that vasopressors (sympathomimetics), like PPA,

dopamine, and the ergot alkaloids can cause vascular lesions in humans that result in clinical disease. It is also known that these pressor agents cause vascular lesions that are morphologically distinct from lesions caused by the drugs listed in Table 1 (Kerns et al., 1989a).

Biomarkers of vascular injury in humans are usually not specific and are of limited sensitivity. In general, these markers are epiphenomena and include C-reactive protein, sedimentation rate, immunoglobulins, anti-neutrophil cytoplasmic antibodies (ANCA), and immune-complexes. Although lacking specificity, they are useful to the clinician in diagnosis and disease monitoring. Biopsies of skin lesions, skeletal muscle, superficial peripheral nerve, and kidney, when indicated, may be extremely valuable. Currently, the diagnosis of drug-induced hypersensitivity angitis is usually established on a total weight of evidence approach rather than presence of any particular biomarker. Morphological diagnoses remain the major means of identifying both human and experimental lesions.

2. Mechanisms

2.1. Introduction

Limited understanding of the mechanisms by which drugs cause vascular injury has been a significant barrier to progress in the development of reliable biomarkers of vascular injury. This facet is further confounded by studies suggesting multiple and distinct mechanisms operating in these syndromes. This is not entirely surprising because a wide variety of drugs, with varied pharmacological effects, could potentially act on different cells but trigger a common downstream event leading to vascular injury. A review of the literature indicates that at least three major mechanistic pathways may contribute to drug-induced endothelial and smooth muscle compromise, resulting in vascular injury in animals. These mechanisms can be broadly classified into the following categories.

Primary:

- Biomechanical injury following changes in shear and/or hoop stress.
- Toxicity following direct pharmacological and/or chemical compromise.
- Injury via immunological and inflammatory mechanisms.

Secondary:

- Injury secondary to marked drug-induced inflammation in the host tissue “bystander effect”.

Such mechanisms are not mutually exclusive and in fact there could be significant overlap between these mechanisms.

2.1.1. Biomechanical injury

The relevance and uncertain extrapolation of drug-induced arterial toxicity in rats, dogs, and other species to humans continues to be an obstacle to drug discovery and development scientists. In addition, the lack of reliable animal and human biomarkers for vascular injury and predictive animal models is an ongoing concern to regulatory scientists. The issue of arterial toxicity particularly in the dog is further complicated by the occurrence of spontaneous coronary arterial lesions that are reported to mimic drug-induced vasculopathy (Detweiler, 1989). It therefore becomes paramount for scientists to identify clear and unequivocal markers suitable for assessment and monitoring of potential drug-related vascular injury in animals and humans. Historically, in the dog, vascular toxicity has been associated with profound hemodynamic changes in mean arterial pressure (MAP) and heart rate (HR), which have been used as surrogate markers of potential vascular toxicity in man at therapeutic doses (Dogterom et al., 1992; Mesfin et al., 1987, 1989, 1995). However, recent experiences with a novel class of vasoactive agents, endothelin receptor antagonists (ETRAs), and others, suggest that profound hemodynamic changes (MAP and HR) are not a prerequisite for development of coronary arterial lesions in the dog (Albassam et al., 2001; Loudon and Morgan, 2001). For this class of molecules, hemodynamic monitoring as a method to assess the potential hazard to humans is not possible, and extrapolation of the potential risk to the human population can only be made based on canine responses and the therapeutic index in the dog (Albassam et al., 2001; Dogterom et al., 1992; Loudon and Morgan, 2001; Mesfin et al., 1987, 1989, 1995). The composite of protein and message expression profiles for ETa and ETb receptors indicate a disproportionate distribution of ETb receptors within the dog right coronary artery and this along with functional consequences predispose the dog to endothelin- and ETRA-induced lesions (Loudon et al., 2000); similar data do not exist for the rat and human.

A number of structurally diverse pharmacological agents can induce arterial lesions in rats and dogs, and the mesenteric and coronary arteries, respectively, are primarily affected. The reasons for this tissue-specific selectivity are not clear but may be related to increased sensitivity in these respective vascular beds because spontaneous arterial lesions develop at these sites in both species (Boor et al., 1995; Detweiler, 1989; Hartman, 1987; Joseph et al., 1997; Kerns et al., 2001). The beagle dog, used routinely in toxicology studies, is considered to be extremely sensitive to the development of drug-induced coronary arterial lesions. For example, adenosine agonists and endothelin receptor antagonists (ETRA) will induce coronary arterial lesions in dogs and monkeys (Albassam et al., 1998, 1999, 2001). However, in the dog, lesions occur after a very short duration (hours) of treatment at lower systemic exposure compared to the monkey. The mechanisms for this selective

species response are ill-defined but may be related to localized changes in blood flow (Humphrey and Zins, 1984) as demonstrated with adenosine (Albassam et al., 2001; Bacchus et al., 1982; Berne, 1980; Berne et al., 1983), an endogenous substance released from the heart or the adenosine agonists (Macallum et al., 1991; Metz et al., 1991; Steffen et al., 1986), and several structurally diverse vasoactive pharmacological agents (Dogterom et al., 1992; Loudon and Morgan, 2001). In the rat, there is indirect evidence linking increases in localized mesenteric blood flow and development of drug-induced mesenteric arterial lesions (Kerns et al., 1989a, 1989b). Therefore, exploration of the contributions of localized changes in blood flow (BF) and subsequently changes in shear and hoop stress, to drug-induced arterial injury, is worthy of investigation in both animals and humans.

Flow-mediated mesenteric arterial lesions in the rat. Within the mesenteric arteries, physiological activation of specific dopaminergic receptor subtypes (DA1) by fenoldopam leads to marked vasodilation and mesenteric arterial lesions (Ikegami et al., 2001, 2002; Kerns et al., 1989b). To test the vasodilation hypothesis, fenoldopam was administered with DA1 receptor antagonist and this treatment regimen prevented induction of hemorrhagic lesions in rats (Kerns et al., 1989b). Other studies have also reported that co-administration of the vasoconstrictor methoxamine attenuated the fenoldopam induced splanchnic arterial lesions (Kerns, unpublished data). The vasodilation hypothesis was also tested using SK&F 95654, a potent phosphodiesterase inhibitor (PDEIII) that causes marked reduction in mean arterial pressure (~40%) and induction of mesenteric arterial lesions. In this model, co-administration of arginine vasopressin, a vasoconstrictor, reversed the hypotensive effect in a dose-dependent manner and prevented induction of mesenteric arterial lesions (Joseph, 2000).

When administered at doses that induce mesenteric arterial lesions, SK&F 95654 and Minoxidil, a potent K⁺ channel opener, produced long-lasting increases in mesenteric blood flow for 5–7 h post dosing (Joseph, 2000). These data indicate that prolonged vasodilation by certain agents will contribute to vascular injury.

Flow-mediated coronary arterial lesion in dogs. Several vasoactive agents that lower blood pressure and increase heart rate induce regional, right coronary arterial damage (Dogterom et al., 1992; Loudon and Morgan, 2001). The basis for this selective coronary arterial lesion in dogs may be related to localized increases in coronary blood flow because under normal physiological conditions, localized control and regulation of coronary blood flow is mediated by endogenous adenosine in response to increased oxygen demand (Albassam et al., 2001; Bacchus et al., 1982; Berne, 1980; Berne et al., 1983). It is therefore not surprising that pharmacologic mimicry of adenosine (i.e., adenosine agonists) by compounds such as CI 914, CI 947, and *N*-

(2,2-Diphenylethyl) adenosine (DPEA) all induce coronary arterial lesions in dogs and increase coronary arterial blood flow (Albassam et al., 1998; Macallum et al., 1991; Metz et al., 1991; Steffen et al., 1986). Therefore, it is now well accepted that administration of adenosine (A1) agonists as a pharmacological class are associated with coronary arterial lesions in dogs. A "class" effect for coronary arterial lesions has also been ascribed to K^+ channel openers (Mesfin et al., 1987) and ETAs (Albassam et al., 2001; Louden and Morgan, 2001) because these agents cause profound increases in regional blood flow.

Several reports from studies in dogs indicate that extreme increases in regional coronary blood flow precede coronary arterial damage. For example, minoxidil, a long-lasting vasodilator, when given to dogs at cardiotoxic doses induced a 6–10-fold increase in regional cardiac blood flow and this sustained increase in flow resulted in damage to the coronary vasculature (Humphrey and Zins, 1984; Mesfin et al., 1989). Other structurally and diverse pharmacologic agents such as SB 209670 (Louden et al., 2000), hydralazine (Chelly et al., 1986), SK&F 94836 (Gristwood et al., 1988), and milrinone (Liang et al., 1987), which are vasoactive, have been reported to increase CBF and induce coronary arterial lesions in dogs.

Other studies in dogs have shown medial hemorrhage and necrosis of extramural coronary arteries of the right atria that were associated with minor but sustained increases in heart rate (10–30 beats/min) and slight decreases in mean arterial pressure (10–15 mm Hg) after infusion of an endothelin receptor antagonist for 5 days (Louden et al., 1998). These changes were further shown to be associated with sixfold increases in regional blood flows and a disproportionate distribution of ET_B receptors in the right coronary arteries of dogs (Louden et al., 2000).

Blood flow and biomechanical injury. It has been postulated that mesenteric and coronary arterial lesions develop because of marked vasodilation, increased blood flow, decreased shear (endothelial cells), and increased hoop stress (vessel wall). Hoop stress or tension is the product of transmural pressure and the vessel radius divided by wall thickness. Decreased shear stress leads to leukocyte adhesion, interendothelial breaks, gradual breakdown of vessel wall integrity, breaks in the internal elastic lamina, and hemorrhage (Joseph, 2000; Mesfin et al., 1989). It has also been speculated that vasodilation changes normal laminar flow to turbulent flow that imitates changed shear stress and this leads to a cascade of effects in the vessel wall culminating in hemorrhage and necrosis. It is not clear to what extent similar changes occur in humans given pharmacologically active doses of certain vasodilators. It is also not clear that such changes in vessel wall tension in the absence of concurrent receptor-mediated cellular alterations would result in the observed vessel injury.

2.1.2. Direct pharmacological and/or chemical toxicity

Pharmacological. The target cell of drug-induced vascular toxicity is presumed to be endothelial and/or smooth muscle cell. Development of vascular lesions has been linked to the pharmacologic effect of the drug because the pharmacologic target is often located on these cells. For example, ETAs as a class cause arterial lesions in dogs because the drug target, endothelin receptors, are located on endothelial and smooth muscle cells (Cines et al., 1998). ET receptors on EC and SMC regulate vascular tone through opposing vasoconstrictor and vasodilator effects (Cines et al., 1998; Clozel et al., 1992). Therefore, an upset of this delicate balance resulting in loss of vasoconstriction or vasodilation might lead to vascular damage. For example, it was shown that the disproportionate distribution of vasoconstrictive receptors, which when antagonized within the right atrium and right coronary arteries predisposes these sites to pharmacological vasodilation, results in sixfold increases in regional blood flow, but minimal changes in heart rate and blood pressure (Louden et al., 2000). A similar observation has been reported in the rat for DA_1 receptors that are the target for fenoldopam (Kerns et al., 1989a). Other pharmacological targets associated with vascular injury that are located on EC and SMC cells include adenosine receptors, K^+ channels, and the cGMP-inhibitable isoenzyme of phosphodiesterase (Joseph et al., 1996a, 1996b). Vascular toxicity then may result from pharmacological interaction with the molecular target that initiates a series of interactive cascades among cellular and noncellular components leading to eventual damage. Contributing to toxicity may be the sustained alterations by these agents on EC components normally responsible for targeting interactions with the immune system.

Chemical. Allylamine and β -Amino-propionitrile (β -APN) are chemical substances that are direct acting vascular toxicants (Boor et al., 1995). Allylamine causes medial hypertrophy and subintimal proliferation and β -APN causes aneurysms of the aorta and coronary and mesenteric vasculature in man (Boor et al., 1995). Based on the different morphological presentations and mechanisms of vascular injury by these agents, pathogenesis is very likely different from the drug-induced lesions of concern in this report.

2.1.3. Immunological and/or inflammatory mechanisms

Although the precise mechanisms by which adverse immunological or inflammatory mechanisms mediate injury to endothelial cells (EC) and smooth muscle cells (SMC) is not clear, there is evidence that drugs and biologics can activate the immune system, which in turn can cause EC and SMC injury. Moreover, drugs may modulate the EC directly in such a way as to trigger an inflammatory response. While the immune effectors mediating acute drug-induced vascular injury have not been extensively identified, several types of vasculitis as seen in chronic autoimmune diseases and

several infections have been well characterized. Thus, the latter may serve as a model to address the possible mechanisms involved in acute and chronic phases of drug-induced vascular injury. Such disease-related vascular injuries are discussed below to provide additional clues on how drugs may induce vascular injury.

Neutrophils/eosinophils. Neutrophils and eosinophils have been widely associated with vascular injury in humans. Chemotactic agents including complement components, factors from fibrinolysis, activate them as well as initiate kinin systems further causing neutrophil stimulation. Similarly chemokines produced by endothelial cells and by other leukocytes activate neutrophils. Chemotactic stimuli facilitate their binding to the endothelial cells. Neutrophils carry a variety of granules containing hydrolases, myeloperoxidase, proteinases, and muramidase. In certain forms of vascular injury, antibodies against myeloperoxidase have been reported. Immune complexes can bind Fc receptors on neutrophils and activate them to release enzymes that could cause endothelial cell injury. Cytokines at the inflammatory site may contribute significantly to the generation of reactive oxygen species (ROS), particularly by PMNs. The vascular endothelium is a major target of oxidant stress, playing a critical role in the pathophysiology of several vascular diseases and disorders. Specifically, oxidant stress increases vascular endothelial permeability and promotes leukocyte adhesion, which trigger alterations in endothelial signal transduction and redox-regulated transcription factors (Lum and Roebuck, 2001). Histopathological analysis of drug-induced vascular injury in human skin has shown two distinct patterns (Calabrese and Duna, 1996). The first type consists mainly of neutrophils and eosinophils and the second, predominantly mononuclear cells.

Mast cells. Mast cells have been well characterized for their role in induction of allergies. Although their precise role in endothelial cell injury is not clear, several recent studies suggest that they may play a key role in endothelial cell functions and injury. For example, mouse or human mast cells can produce and secrete vascular permeability factor/vascular endothelial cell growth factor (VPF/VEGF) that can potentially enhance vascular permeability and induce proliferation of vascular endothelial cells (Boesiger et al., 1998). Mast cells play a critical role in regulating the expression of EC adhesion molecules, ICAM-1 and VCAM-1, and thereby enhance leukocyte-mediated inflammatory response (Meng et al., 1995). In mercuric chloride-induced vasculitis in rats, mast cells were implicated as a trigger in the inflammatory process (Kiely et al., 1997). Enhanced local degranulation of mast cells has been reported (Zhang et al., 2002a, 2002b) in SK&F 95654-induced mesenteric vascular injury in rats.

Endothelial cells. Sustained endothelial cell activation has been implicated as an important event in the induction of

vasculitis and vascular leak syndrome. Endothelial cell activation is considered as an immunological event (Blann, 2000). Activated endothelial cells can function as antigen-presenting cells and participate in T-cell-mediated immune reactions (Pober and Cotran, 1991). Activated endothelial cells up-regulate the expression of major histocompatibility complex (MHC)-encoded molecules (Pober and Cotran, 1991). MHC class I antigens on the activated endothelial cells interact with CD8 T lymphocytes, while endothelial MHC class II antigens interact with CD4 T lymphocytes in cell-mediated immune responses (Pober and Cotran, 1991). Furthermore, activated endothelial cells express many immunologically relevant surface molecules, such as adhesion molecules of the immunoglobulin gene superfamily. In SK&F 95654-induced mesenteric injury in rats, evidence for early (hours) endothelial cell activation has been reported (Joseph et al., 1996a, 1996b; Zhang et al., 2002a, 2002b). Moreover, increased numbers of circulating endothelial cells have been detected during vascular injury such as during myocardial infarction and endotoxemia (Gerrity et al., 1976; Mutin et al., 1999). Thus, circulating endothelial cells and markers of endothelial injury may serve as biomarkers for vascular injury.

Adhesion molecules. Several endothelial cell adhesion molecules belonging to the Ig superfamily play a critical role in the interaction between EC and leukocytes. These include ICAM-1, ICAM-2, VCAM-1, and MAcCAM. These molecules are either expressed or inducible in EC. In addition, integrins present on leukocytes are involved in adhesion to EC. Also, E-selectin expressed on EC binds to the carbohydrate ligands on leukocytes. Adhesion molecules play a critical role in the recruitment of leukocytes in many forms of vascular injury. Increased expression of ICAM-1 has been reported in vasculitic lesions involving nerve and muscle as well as in vessels of SLE patients (Majewski et al., 1991; Panegyres et al., 1992). Also, in patients with diffuse vasculitis, the skin vessels exhibited increased expression of E-selectin and ICAM-1 (Leung, 1991). The levels of soluble ICAM-1 have been shown to be higher in individuals who develop atherosclerosis there by suggesting that sICAM-1 may serve as a biomarker for such vascular lesions. ICAM-1 immunostaining on endothelial cells is enhanced in the mesentery of rats exposed to SK&F 95654 (Zhang et al., 2002a, 2002b).

Autoantibodies. Several drugs can cause vascular injury in humans associated with anti-neutrophil cytoplasmic autoantibodies (ANCA) (Merkel, 2001). These patients produce Abs primarily against myeloperoxidase or proteinase 3 (Cambridge et al., 1994; Lotti et al., 1998). Although it is not clear that these antibodies are directly pathogenic, the levels of ANCA may serve as a marker for disease activity and response to drug withdrawal (Merkel, 2001). There is strong evidence to suggest that hydralazine and propyl thiouracil can cause ANCA-

positive drug-induced vascular injury (Merkel, 2001). In addition to ANCA, anti-EC Abs (AECA) have also been detected in a variety of vascular diseases. AECA may upregulate expression of adhesion molecules such as E-selectin, ICAM-1, as well as cytokines and chemokines by EC, which in turn may recruit leukocytes (Praprotnik et al., 2000).

T cells. Rats treated with mercuric chloride develop necrotizing vasculitis that occurs in two phases: an early and a late phase. In the latter phase, T cells seem to play an important role inasmuch as treatment with monoclonal antibodies to inhibit T cell function completely prevents the development of late vasculitis (Kiely et al., 1997). Furthermore, infiltration of T cells into vascular lesions and elevated soluble IL-2 receptors in the serum have been reported in Kawasaki disease (Uchiyama and Kato, 1999). In Takayasu arteritis, which exhibits vasculitis that involves the aorta and pulmonary arteries, the infiltrating cells consist of cytotoxic T cells and NK cells. They injure the vascular cells by producing perforin (Seko, 2000). Vascular injury caused by cytolytic T cells and NK cells has been well documented with the use of IL-2 and other cytokine therapies including GM-CSF, IL-1, and IL-4 (Vial and Descotes, 1995). IL-2 was shown to activate T cells to express high levels of CD44, which interacts with EC and induces EC injury by producing Fas ligand and perforin (Rafi et al., 1998a, 1998b; Rafi-Janajreh et al., 1999). Activated cytolytic T cells and NK cells express Fas ligand, which in turn can induce significant levels of apoptosis in EC (Janin et al., 2002). In giant cell arteritis, the vascular injury appears to depend on T cells that produce IFN- γ , which activates macrophages (Weyand and Goronzy, 2000). In a patient with acute drug-induced cutaneous vasculitis, a 100-fold increase in plasma concentrations of IFN- γ was noted, thereby suggesting that IFN- γ may up-regulate adhesion molecule expression on EC and activation of T cells (Shiohara et al., 1992).

Macrophages. Activated macrophages are known to participate in the process of instability and rupture of atherosclerotic plaque (Weyand and Goronzy, 2002). In giant cell arteritis, the mechanisms of injury have been mostly attributed to the effector macrophages. Macrophages specialize in oxidative damage with lipid peroxidation attacking smooth muscle cells and matrix components. These macrophages also produce reactive oxygen intermediates, which, in combination with nitrogen intermediates, cause protein nitration of endothelial cells (Weyand and Goronzy, 2002). In Kawasaki disease, activation of cytokines produced by macrophages elicit proinflammatory and prothrombotic responses in endothelial cells (Igarashi et al., 2001). In this disease, macrophage colony stimulating factor has been shown to play a critical role in the pathogenesis and can be used as an indicator for the risks of valvulitis and coronary arteritis (Igarashi et al., 2001).

2.1.4. Vascular injury secondary to a “bystander effect”

In the evaluation and diagnosis of vascular injury, it is critical to ensure that the vascular tissue under evaluation is indeed a target organ and not affected “innocently” by adjacent and severe inflammation in the host tissue. There are compounds in development that have, as their major toxicity, drug-induced neutrophilic tissue inflammation in multiple tissues (Kerns, unpublished observations). In these cases, it is expected that occasional arteries in selected tissues may be compromised, secondarily. In these cases, affected small muscular arteries and arterioles have marked infiltration of the wall with neutrophils and fibrin-like material. A singular diagnosis of arteritis in this instance would not be appropriate, as it does not communicate what is really happening in the host tissue. Importantly, there are many acute phase inflammation biomarkers and also neutrophil counts to monitor in animals and humans to predict the onset of these unexpected drug-induced pro-inflammatory syndromes.

2.2. Research opportunities

1. Identification and development of mechanistically based novel biomarkers of drug-induced vascular injury that targets both the vascular cells and immune cells.
2. Direct measurement of early and sustained alterations in regional blood flow using radioactive or color-coated fluorescent microspheres (Glenny et al., 1993).
3. Measurement of coronary arterial diameter by echocardiography (Stephan-Guedner and Inomata, 2000).
4. Evaluation of endothelial nitric oxide synthetase (eNOS) because it is elevated by increased shear stress. It has been suggested that increased shear elevates eNOS, which is co localized with caveolae, and this coupling transducer signals into EC as well as SMC cells (Arnal et al., 1999) in the vessel wall.
5. Develop methodologies (in vitro followed by in vivo studies) for direct measurement of shear stress or biochemical assays to determine eNOS or caveolin-1 concentrations that may be elevated because of increased shear.
6. Electron microscopy to evaluate the status of EC and SMC caveolae because disruption of this cellular organelle is associated with vascular pathology in caveolin-1 gene disruption in mice (Drab et al., 2001).
7. Immunohistochemistry, Western blotting, laser capture dissection, and microarray technology to evaluate gene and protein changes in target cells i.e., EC and SMC.
8. For agents that are potentially direct vascular toxicants employing cytotoxicity assays with cultured EC and SMC at relevant concentrations that may be achieved in vivo.
9. Identification of the mechanisms by which immune cells are activated directly by drugs, which, in turn, may contribute to EC injury. This involves identification of the nature of effector cells (e.g., neutrophils,

lymphocytes, monocytes, macrophages, basophils, eosinophils, and mast cells) adhesion molecules that are upregulated on leukocytes, which facilitate the leukocyte-EC interactions, and the effector molecules produced by leukocytes involved in EC injury.

10. In instances where drugs modulate the ECs in a way that would secondarily trigger an immune attack, it is important to identify the nature of molecular and phenotypic changes occurring in EC that trigger inflammation and immune attack.
11. The studies on the role of immune components in EC injury can be facilitated by the use of pharmacologically immunocompromised, genetically immunodeficient animals, transgenic animal models deficient in specific immune components (knock-out), and transgenic animal models where the original strain has a pathologic defect that can be corrected by supplying the normal functional gene (knock-in).
12. Laser capture to isolate EC or inflammatory cells and DNA microarray analysis may help in identifying specific pathways of immune cell and EC activation.
13. The mechanism of immune cell-mediated EC injury such as necrosis or apoptosis will be useful in identifying better biomarkers and to develop strategies to prevent EC injury.
14. Role of anti-neutrophil cytoplasmic autoantibodies (ANCA) in drug-induced vascular injury.
15. Analysis of genetic risk factors, which may play a role in immune-mediated EC injury.
16. Quantitative analysis of gene induction in living animals.

2.3. Gaps

1. Correlate blood flow changes in the dog using the various methods and different pharmacological agents to clearly establish what magnitude of blood flow increase causes vascular lesions.
2. Technology is evolving for measuring blood flow in rat. A reproducible and validated model is not available and so the increased blood flow hypothesis in the rat has not been thoroughly tested with multiple drugs.
3. Measurement of shear stress and biomechanical effects has not been attempted in rats or dogs.
4. No available data on the biochemical (signal transduction pathway) effects of these drugs on EC or smooth muscle cells in vivo or in vitro.
5. Lack of hard scientific data that provides an understanding of the biomechanical forces interplay in the vascular wall during vasodilation that leads to subsequent injury.
6. Shear stress and/or exaggerated pharmacology and vasodilation may cause apoptosis of EC and SMC in the vessel wall. Develop methods to test.
7. Understanding and ability to separate direct vs. indirect effects.

8. Understanding the nature of leukocyte–EC cell interactions during drug-induced vascular injury.
9. To identify biomarkers of inflammation and injury as they relate to drug-induced vascular compromise.

2.4. Summary

It is clear that a more complete understanding of the mechanisms of drug-induced vascular injury will accelerate development of solutions to managing risk in clinical studies. Directed approaches to understand mechanisms will provide a link between structural damage and derangement of specific vascular functions. Unraveling mechanism (s) of toxicity will ultimately lead to identification of relevant biomarkers. Investigative studies will link deranged vascular alterations to cellular, biochemical, and molecular events. The ultimate product of this effort then would be identification of mechanistically linked, relevant, and bridging biomarkers of vascular toxicity for both preclinical and clinical application. Employment of new technologies such as proteomics, metabonomics, and transcriptomics should be utilized as appropriate in these scientific investigations. Use of specific gene-targeted transgenic, knock-out, and knock-in animals should provide useful information on the precise role of immune cells in drug-induced vascular injury. It is also clear that a balance in the interaction between leukocytes and endothelial cells is critical for immune system and vascular homeostasis. In contrast, perturbations in this balance can contribute to vascular and immune system anomalies. Thus, understanding the effect of drugs on EC, SMC, and circulating leukocytes would likely lead to development of better models to study vascular injury and further help identify biological markers that can be used to evaluate and predict drug-induced vascular injury.

3. Biomarkers (Tables 2–10)

3.1. Introduction: why vascular injury biomarkers?

Data from animal toxicology studies are generally needed from drug developers to demonstrate for regulatory agencies a safe strategy for initiating human clinical trials. When drug-induced toxicities, such as vascular injury, occur in animals, reasonable assurances for safety are needed from animal studies when appropriate clinical monitoring is not feasible. Among the many goals for performing animal toxicology studies, the key objectives include (1) an identification of all dose-limiting and associated toxicities and their reversibility and identification of the dose–exposure and dose–response relationships seen up to maximally tolerated doses, (2) an identification of a safe starting dose for human trials, and (3) a definition of an appropriately safe patient monitoring strategy for clinical trials. Data from animal studies are best applied to define the relationship between dose, drug exposure, therapeutic effect,

and dose-limiting toxicity to evaluate appropriateness for clinical trials. In such animal studies involving extensive histopathological evaluations, however, the use of serum or plasma biomarkers is often limited, in routine analyses, as described by a working group of clinical pathologists in 1996 (Weingand et al., 1996). This working group has recommended routine application of a core set of clinical endpoints for routine toxicity screening. These endpoints, together with hematology data, serve as reporters of diminished renal function (e.g., BUN, creatinine), altered hepatic integrity (e.g., ALT, AST), altered general homeostasis (e.g., electrolytes, pH), or tissue response to injury (e.g., alterations in circulating cell populations). These same clinical endpoints have been used for routine monitoring for well over 25 years despite great strides in biological research over this same time frame. The integrity of most tissues is either not being monitored or only monitored on a limited basis and clearly vascular injury is not covered with the recommended panel (Weingand et al., 1996).

New chemical entities from several pharmacologic classes have been developed that present with histopathological evidence of vascular injury that is clearly associated with drug administration in a dose- and time-dependent manner. Many of the earlier studies that have been published in the peer-reviewed literature describing such findings were seen with agents at doses that produced dramatic effects on vasomotor tone, blood pressure, and heart rate. It was felt with a reasonable level of assurance that if higher doses were avoided and cardiovascular parameters were monitored in dose escalation clinical trials, vascular injury could be avoided in the clinic. However, many of the agents under development more recently have been associated with histopathological evidence of vascular injury in animal toxicology studies, without any easily measurable effects on vascular tone, blood pressure, or heart rate. In addition, in some cases, the therapeutic index [defined as the ratio between the plasma exposure achieved at the highest dose at which no significant toxicity is observed (NOAEL) compared to the plasma exposure achieved at the lowest dose achieving a therapeutic effect in the same species] is not broad. The lack of a practical clinical monitoring strategy often results in the sponsor and regulator sorting through speculative discussions surrounding relevance of animal findings to humans, and the inaccuracies of any defined exposure–response relationships during the proposed ensuing clinical phases of development. There is growing concern and recognition of the role of vascular injury and vascular inflammation as a risk for cardiovascular disease, myocardial infarction, and stroke (Patel et al., 2001). There are also experiences indicating that toxicities can sometimes be species-specific and irrelevant to humans. Nevertheless, there is a need and desire for the identification and validation of mechanism-based easily accessible biomarkers that can be measured in animal toxicology studies and integrated with histopathological findings. These new biomarkers should identify the early onset of vascular compromise and be

robust enough to use to monitor progression or reversibility of injury across pharmacological classes and across species, including humans.

Tables 2–10 list in detail potential biomarkers based on recognition of a common association of vascular injury with endothelial and smooth muscle cell injury, endothelial cell activation, and a subsequent inflammatory response in and around affected vessels.

3.2. Scope of the problem and path forward

The pathogenesis of the human vasculitides is poorly understood and a definitive classification of the vascular syndrome can be difficult. The underlying mechanisms, the earliest initiating events, the role and interplay of specific immune cell components involved, the vessels targeted, the progression of early disease to later forms, the availability of diagnostic biomarkers are all important areas under active investigation. Currently, recognized biomarkers for human vasculitides are of limited value, tend to be focused on immune manifestations of the disease, and may reflect late stages of disease. For example, anti-neutrophil cytoplasmic antibodies to neutrophil myeloperoxidase (pANCA) and to neutrophil proteinase 3 (cANCA) are late-appearing and are not present in all forms of clinical vasculitides. However, pANCA and other markers are largely untested in animal toxicology studies and their predictive value in vascular injury in animals is unproven. In atherosclerosis, an arterial disease with a prominent inflammatory component, several prospective clinical studies have shown that plasma levels of myeloperoxidase (Brennan et al., 2003) and high-sensitivity C-reactive protein (hsCRP), an acute phase reactant, is a strong predictor of future myocardial infarction and stroke in otherwise healthy men and women (Ridker, 2001, 2002) although its value has been recently debated in the literature (Danesh et al., 2004). Measurement of hsCRP in humans may soon become routine as a screen for risk of cardiovascular events. For the many examples of drug-induced vascular injury that have been noted in animal models, there may be pathogenetic differences among the models resulting in a blurring of boundaries across mechanisms. Future research needs to carefully characterize the early histopathology and to link this data with altered biomarkers, to understand pathogenesis, to understand the role, if any, of immunological components in the initiation and progression of disease. For pragmatic solutions, the identification of practical and easily accessible diagnostic interspecies biomarkers is of prime importance. Biomarkers useful to drug development would ideally be useful for defining specific pathogenic mechanisms; be easily accessible; be sensitive and specific to appear with the earliest manifestations of vascular injury, define the site and type of vessel affected, track with disease severity, and regress as disease mitigates; and be measurable with reagents that identify regions of proteins conserved across species including humans. It is also recognized that biomarkers

Table 2
Potential markers of endothelial injury

Biomarker (references)	Reagent availability	Key points
sE-selectin (CD62E) (Becker-Andre et al., 1992; Fries et al., 1993; Weller et al., 1992)	Rat-N, Dog-N, Human-Y	<ul style="list-style-type: none"> expressed selectively on endothelial cells following activation by TNF, IL-1 and LPS ligands include: sialyl-Lewis (sCD15), P-selectin glycoprotein ligand-1, cutaneous lymphocyte antigen involved in leukocyte tethering and rolling velocity reduction during the initial stages of inflammation and following endothelial activation shed from the endothelium and circulates as sCD62E in several inflammatory diseases that affect the endothelium, including diabetes, atherosclerosis, smoking etc in healthy humans, the levels of circulating sCD62E are very low potential marker with high sensitivity and high specificity in healthy volunteers and high sensitivity and moderate to low specificity in selected patient populations maximal transcript level of E-selectin at 3–6 h in a murine model high degree of structural and functional homology of E-selectin among human, mouse, and rabbit significant increase of soluble levels of E-selectin in patients with a variety of vasculitides (\approx 1–3-fold increase above control subjects)
Circulating endothelial cells (CEC) (Hadlovec, 1978; Dignat-Gorge and Sampol, 2000)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> may require multiple markers markers may include a pan-endothelial functional marker such as uptake of acetylated lipids (DiI-Ac-LDL), and a variety of endothelial surface markers such as CD31, vWF, CD146, Tie 2, and flk-1 tail vein blood-drawing may give high background in rats have been shown to increase after varied causes of vascular damage in both humans and rats. causes in humans include coronary angioplasty, sickle cell anemia, rickettsial vasculitis, cytomegalovirus infection, thrombotic thrombocytopenic purpura, and acute coronary syndromes increased CEC in rats has been associated with administration of endotoxin, streptokinase, adrenaline, and 5-hydroxytryptamine
vWF (Cid et al., 1996; Newsholme et al., 2000; Reidy et al., 1989; Zhang et al., 2002a, 2002b)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> activated endothelial cells release vWF into the plasma and subendothelial matrix through exocytosis of the cytoplasmic Weibel–Palade bodies exocytosis of vWF is associated with the mobilization of P-selectin from Weibel–Palade bodies to the surface of endothelial cells elevation after varied causes of vascular damage in humans and after drug-induced vascular injury in rats with a short T1/2 (newsholme) possible sequential blood drawing artifacts vWF elevated in smokers high levels of vWF in patients with hypersensitivity vasculitis in endotoxin-treated or balloon-injured rats, the immunostaining content of intracellular vWF was increased to threefold, indicating that endothelial cell injury leads to increased vWF levels up-regulated vWF expression on the endothelial cells of the mesenteric artery in rats treated with SKF95654 high-molecular-weight procoagulant product of endothelium is a constituent of platelets (alpha granules) and mRNA of vWF is found in platelets; vWF can also be obtained from isolated platelets in vitro circulating vWF may be a specific marker of endothelial damage and contribution from platelets is minimal platelet vWF after discharge remains bound to platelet surface clinical evidence of increased circulating vWF-inflammatory and atherosclerotic vascular diseases, after surgical procedures—bypass surgery, infarction with ischemic heart disease, thromboembolic disease, rheumatoid arthritis, increased with acute phase reactants; sensitive marker—perhaps too sensitive 18 h half life
vWF Pre-protein (Vischer et al., 1998)	Rat-N, Dog-N, Human-Y	<ul style="list-style-type: none"> sensitive marker acute and progressive injury; not found in normal plasma, rapid turnover and short half-life, releases as a 1:1 ratio with mature protein no extensive human experience, baboon only animal species with data.

Table 2 (continued)

Biomarker (references)	Reagent availability	Key points
Endothelial microparticles (EMPs) (Ferrer et al., 1995; Jimenez et al., 2001)	Rat-?, Dog-N, Human-Y	<ul style="list-style-type: none"> • a marker of endothelial cell activation in thrombotic thrombocytopenic purpura • the number of EMPs can be measured by monoclonal antibodies CD31 and CD51 using flow cytometry • elevated EMPs in the plasma correlate to increased expression of ICAM-1 and VCAM-1 • CD31 (also called PECAM-1) is expressed on endothelial cells, platelets, and megakaryocytes. Plays a role in the interaction of the endothelium and platelets • CD51/CD61 (also called $\alpha 2\beta 3$ integrin) is an endothelial cell adhesion molecule, which is mainly expressed on endothelial cells but is also expressed on leukocytes and macrophages. Plays a role in the interaction of endothelial cells and extracellular matrix
Thrombomodulin (Boehme et al., 2000; Schwartz and Karmochkine, 1998)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • important regulator of activated thrombin-TM converts thrombin from a procoagulant to an anticoagulant • constitutive membrane protein—therefore probably needs to be cleaved into soluble forms to be detected in circulation • increased circulating concentrations of an antigenic component has been reported with thrombocytopenic purpura, disseminated intravascular coagulation, atherosclerosis, and inflammatory connective tissues diseases • in vitro studies suggest that TM is in endothelial cell tissue culture supernatants; suggesting comes from damaged endothelial cells. Other cell sources—synovial cells, rheumatoid patients, platelets, megakaryocytes, mesothelial cells, PMNs; unlike vWF, TM concentrations appear independent of inflammatory cytokines IL-1 and TNF.; unknown if assay works in humans—utility in rats
TPA (Cockwell et al., 1997; Jurd et al., 1996)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • anticoagulant • cytokine-activated endothelial cells decrease tPA anticoagulant activity and increase plasminogen activating inhibitor-1 (PAI-1) procoagulant activity • increased levels of soluble tPA in patients with vasculitis
Tissue factor (Cotran, 1987; Bach et al., 1994; Jurd et al., 1996)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • procoagulant, not expressed in normal endothelial cells • cytokine-activated endothelial cells increase TF procoagulant activity • marker of endothelial cell activation • increased levels of soluble TF in patients with vasculitis

whose performance attributes may fall short of these ideals may nevertheless have some value in specific contexts. Because pathogenic mechanisms may differ across pharma-

logical classes and possibly across species within a class and because the presentation of vascular lesions may vary by vessel and organ affected, panels of biomarkers may be necessary to help detect all forms of vascular injury and distinguish mechanisms.

Table 3
Potential markers of smooth muscle injury

Biomarker	Reagent availability	Key points
Caveolin (Arnal et al., 1999; Drab et al., 2001; Feron et al., 2001)	Rat-?, Dog-Y, Human-Y	<ul style="list-style-type: none"> • caveolin-1, plasmalemmal invaginated vesicle, key role in cell signaling pathway • expressed on EC, SMC and fibroblasts; loss impairs nitric oxide and calcium signaling causing aberrations in endothelium-dependent relaxation, contractility, and maintenance of myogenic tone
Smooth muscle alpha actin	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • smooth muscle alpha actin expressed in vascular smooth muscle • antibody available that cross reacts across mammals • unknown whether increases in circulation after vascular injury

Table 4
Inflammatory markers

Biomarker	Reagent availability	Key points
Neutrophils	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • neutrophils and monocytes are oftentimes seen in areas of vascular damage • unclear whether inflammatory cells are the cause or result of vascular damage
Mast cells and basophils (Zhang et al., 2002a, 2002b)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • mast cells are often times associated with mesenteric vascular injuries in SD, SHR, and WKY rats; the possible release of inflammatory mediators and cytokines from mast cell degranulation is suggested as a pathogenetic mechanism

Table 5
Acute phase proteins and cytokines

Biomarker	Reagent availability	Key points
C-reactive protein (CRP) (Gentry, 1999)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> c-reactive protein is relatively conserved in its response among species (rat, dog, and man). CRP is believed to bind bacterial and fungal cell walls, resulting in opsonized particles and serving as a complement cascade attractant; CRP is elevated in animals (rats and dogs) with vascular lesions
Serum amyloid A (Gentry, 1999)	Rat-N, Dog-Y, Human-Y	<ul style="list-style-type: none"> serum amyloid A appears to respond as an acute phase protein in man and dog, but may not be an appropriate marker in rat function of serum amyloid A is not completely understood but may play a role in lipid metabolism during inflammation
Haptoglobin (Zhang et al., 2002a, 2002b)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> haptoglobin is a mucoprotein that traps hemoglobin from lysed red blood cells; it has been reported to be elevated in inflammatory disorders haptoglobin has been found to be elevated in dogs with vascular lesions
Fibrinogen (Castell et al., 1988; Geiger et al., 1988)	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> acute phase protein related to the release IL-6 fibrinogen is increased to 4.8-fold 4 h after the administration of IL-6 in male Wistar rats fibrinogen is increased to 3.8-fold after the administration of IL-6 in human hepatocytes
α 2-macroglobulin (Gentry, 1999)	Rat-Y, Dog-N, Human-N	<ul style="list-style-type: none"> this particular protein may be a respondent specifically in the rat and is not relevant for dogs and humans
IL-1	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> some data demonstrate that this marker is elevated in rats with vascular lesions interleukin-1 (IL-1) is an important mediator of the immune system, playing key role during infection, inflammation, cell differentiation, and apoptosis may act as an activator of cardiovascular cells inasmuch as cells of the vessel wall and the heart can produce IL-1 IL-1 can be detected in the serum using ELISA
IL-6 (HogenEsch et al., 1995)	Rat-Y, Dog-N, Human-Y	<ul style="list-style-type: none"> IL-6 is primarily produced by macrophages, TH2 cells, and bone marrow stromal cells reports suggesting that in some types of vasculitis, IL-6 may play a critical role induces synthesis of acute phase proteins in the liver IL-6 can be detected in the serum using ELISA
TNF α	Rat-Y, Dog-N, Human-Y	<ul style="list-style-type: none"> TNFα is a major mediator of apoptosis as well as inflammation and immunity TNFα is the prototypic proinflammatory cytokine and endothelial cells are the principal cellular targets of its actions found in the serum and can be detected using ELISA
Chemokines (Cockwell et al., 1997; Tesar et al., 1998; Wong et al., 1997)	Rat-Y, Dog-Y/N, Human-Y	<p><i>IL-8</i></p> <ul style="list-style-type: none"> marker of endothelial cell activation activator of neutrophils (formerly known neutrophil activating protein-1) and a potent neutrophil chemoattractant plays a role in the transendothelial migration of neutrophils, particularly in post-capillary venules enhances the interaction of neutrophils with ICAM-1 increase in soluble levels of IL-8 in patients with vasculitis <p><i>MCP-1, MIP-1, RANTES</i></p> <ul style="list-style-type: none"> MCP-1, MIP-1, and RANTES are synthesized and secreted by activated endothelial cells MCP-1 is an activator of monocytes and a monocyte chemoattractant, thereby playing a role in the regulation of monocyte extravasation MIP-1 and RANTES are selectively chemoattractant for T cells significant increase in MCP-1, MIP-1a, and RANTES gene expression levels in patients with vasculitis various assays exist for chemokines and cytokines, including those that are protein-based as well as mRNA-based. Usefulness of these markers in vascular injury has not been established

Careful analyses of genomic and proteomic alterations at sites of tissue injury could elucidate pathogenic mechanisms and target potential biomarkers. Genomic, proteomic, and metabonomic technologies are expected to assist with questions of interspecies response differences, pathogenesis, and by identifying additional biomarkers. Such approaches are both hypothesis-generating and hypothesis-directed. Investigations of altered protein and gene expression in

circulating immune cells could serve a practical role in identifying accessible and monitorable biomarkers. Investigations of serum and urine components using proteomic and metabonomic technologies are already beginning to yield useful results. Expansion of such approaches is expected. In addition to broad-based biomarker discovery approaches, the measurement of alterations in specific endpoints is detailed based on an evaluation of the

Table 6
Adhesion molecules

Biomarker	Reagent availability	Key points
sCD44-Hyaluronic Acid (Griffioen et al., 1997; Lesley et al., 1993; Rafi-Janajreh et al., 1999)	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> • activation of T cells by HA or stimulation of monocytes with inflammatory cytokines induces CD44 to bind to hyaluronic acid (HA), an adhesion event implicated in leukocyte-leukocyte and leukocyte-endothelial cell interactions • CD44 expressed on activated T cells and NK cells is involved in endothelial cell injury and vascular leak • activated endothelial cells also express CD44 • may be unique isoforms expression by endothelial cells; thus, detection of soluble CD44 and HA in blood may constitute a useful biomarker for vascular injury
MAdCAM	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> • expression of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in acute and chronic inflammation • MAdCAM-1 is an endothelial cell adhesion molecule, which is specifically expressed on the high endothelial venules in lymphoid tissues • L-selectin and $\alpha 4\beta 7$ integrin are receptors of MAdCAM-1 • involvement of L-selectin-mediated or $\beta 7$ integrin lymphocyte rolling • in rat, $\alpha 4$ integrins on the surface of neutrophils interact with MAdCAM-1 on the endothelium, suggesting that rat neutrophils may use $\alpha 4$ integrins to mediate selective recruitment of neutrophils to sites of inflammation in vivo • rat neutrophils express $\alpha 4$ and $\beta 1$ integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). • unique to Peyer's patch endothelial cells • L-selectin-mediated lymphocyte rolling on MAdCAM-1
ICAM-1(Cotran, 1987; Coll-Vinent et al., 1997; Gonzalez-Amaro and Sanchez-Madrid, 1999; Zhang et al., 2002a, 2002b)	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> • ICAM-1 serves as a marker of endothelial cell activation • ICAM-1 on the activated endothelial cells as a ligand interacts with Mac-1 (CD11b/CD18), a leukocyte integrin receptor • ICAM-1 plays a role in margination and extravasation of lymphocytes at sites of inflammation or a localized immune response • increased levels of soluble ICAM-1 in patients with vasculitis • up-regulated ICAM-1 expression on the endothelial cells of the mesenteric artery in rat treated with SKF 95654
VCAM-1 (Ara et al., 2001; Gearing and Newman, 1993; Gonzalez-Amaro and Sanchez-Madrid, 1999)	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> • a marker of endothelial cell activation • VCAM-1 on the activated endothelial cells as a ligand interacts with VLA-4 (CD49d/CD29), leukocyte $\alpha 4\beta 1$ integrin • VCAM-1 plays a role in the firm attachment and subsequent transendothelial migration of leukocytes • increased levels of soluble VCAM-1 in patients with vasculitis

published literature (Table 2). These endpoints are hypothesized to be associated with drug-induced vascular injury and are furthermore hypothesized to add value to these investigations in preclinical safety studies. To further investigate the value of these potential biomarkers, collaborative investigations are envisioned with the design of common preclinical protocols, utilization of technology experts contributing common assay reagents, and strategies to support the analytical and biological assay validation. Ultimately, this work needs to lead to clinical assays that are practical and that can be validated.

4. New technologies

4.1. Toxicogenomics

4.1.1. Background

Toxicogenomics is an emerging discipline that uses genomic tools including gene expression profiling technol-

ogies to address problems of toxicological significance. Several recent publications on toxicogenomics have been published and can be reviewed for a more detailed discussion of general principles (Castle et al., 2002; Pennie et al., 2001; Ulrich and Friend, 2002). Toxicogenomics can contribute to a mechanistic understanding of vascular injury at the molecular and cellular levels, and can lead to the development of gene-based screens to improve selection of compounds for drug development, as well as potentially identify more sensitive and specific biomarkers. To date, no reports specifically describing the use of toxicogenomics techniques for vascular injury in preclinical animal models have been published. However, there have been numerous publications from the field of cardiovascular disease demonstrating that arterial tissue can be characterized using expression profiling approaches, and therefore the mechanistic understanding of vascular injury should be a realistic goal (Henriksen and Kotelevtsev, 2002; Ye et al., 2002).

There are at least two ways that toxicogenomic approaches can facilitate the identification of vascular injury

Table 7
Chemical analytes

Biomarker	Reagent availability	Key points
Histamine	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> histamine is the main vasoactive mediator released from mast cells and basophils in dogs and humans
Serotonin	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> serotonin (5HT) is the main vasoactive mediator released from mast cells and basophils in rats and other rodents
PGs and LTs	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> RIAs and ELISAs exist for a variety of prostaglandin (PGs) and leukotrienes (LTs) and their major serum and urinary metabolites these are nonspecific indicators of inflammatory disease; are of unproven use in vascular injury
Chymase	Rat-?, Dog-?, Human-?	<ul style="list-style-type: none"> potential marker, but no data to date
Tryptase	Rat-?, Dog-?, Human-?	<ul style="list-style-type: none"> potential marker, but no data to date

biomarkers. First, by monitoring genes that are differentially expressed in blood vessels isolated from animals treated with compounds that induce vascular injury, it might be possible to narrow the search for candidate biomarkers by specifically focusing on genes that encode cell-surface or secreted proteins. These genes encode potential circulating biomarkers that could be further characterized directly in plasma or serum using immunoassays or other diagnostic methods. Second, by profiling circulating leukocytes, which

can be easily obtained from whole blood, might also identify surrogate biomarkers. [Alcorta et al. \(2002\)](#) have reported that gene expression changes in circulating leukocytes from patients with a variety of renal diseases, including small vessel vasculitis (ANCA positive disease), can be clustered according to disease type. The latter approach will be particularly useful for characterizing vascular injury in animals and humans.

4.1.2. Platform(s)

There are numerous technologies and platforms that can be used for genome-wide expression profiling. While there are several ways to categorize genomic approaches, most relevant for the study of vascular injury are so-called “closed” and “open” profiling systems. Closed systems typically use a microarray-type format and are based on predefined sets of genes. Such genes could be selected to address a specific hypothesis (e.g. oxidative-stress array), to characterize certain tissues (e.g. kidney array), diseases (e.g. breast cancer array), or profile an entire genome (e.g. rat genome array). Microarrays are generally based on cDNA clones, PCR-amplified cDNA fragments, or chemically synthesized oligonucleotides that are “spotted” or affixed to a solid support such as a glass slide or a nylon membrane. These arrays can be obtained from a variety of commercial vendors or constructed in house using commercially available or homemade equipment. Another type of array—the GeneChip from Affymetrix—is based on oligonucleotides that are synthesized directly on a glass surface. Regardless of the platform used, experiments typically

Table 8
Apoptosis markers

Biomarker	Reagent availability	Key points
Fas ligand (Janin et al., 2000 ; Rafi et al., 1998a, 1998b)	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> FasL is a 40-kDa type 2 transmembrane protein belonging to the tumor necrosis factor (TNF) family ligation of Fas, a cell-surface protein, transduces an apoptotic signal leading to cell death FasL is expressed mainly on activated leukocytes but recent studies suggest that it may also be expressed on endothelial cells; membrane-bound FasL is rapidly cleaved into a soluble form that can be detected in the serum (1) Fas ligand has been implicated in endothelial cell injury (2,3). Currently, there are no tests in the market to detect soluble FasL in the serum growth of FasL-bearing tumor cells in syngeneic murine host induces apoptosis and toxicity in Fas(+) organs
Caspases	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> there are a series of caspases that are activated during apoptosis that can be detected using cells undergoing apoptosis this assay would require having sufficient numbers of recovered apoptotic cells to perform the biochemical assays
Annexin V	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> Annexin V is a useful marker for human and rodent apoptosis Annexin V binds to cell membranes that bleb during the apoptotic process exposing phosphatidyl serine residues Annexin V techniques have not proven useful in dog studies
TUNEL	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> the TUNEL assay can be performed using both flow cytometry and immunohistochemistry it relies upon DNA repair enzymes that will incorporate biotinylated UTP into DNA

Table 9

Immunological/functional markers

Biomarker	Reagent availability	Key points
Autoantibody (Ara et al., 2001; Gross et al., 1993; Mayet et al., 1993, 1994)	Rat-Y, Dog-Y/N, Human-Y	<i>Myeloperoxidase (MPO) and Proteinase 3 (PR3)</i> <ul style="list-style-type: none"> • autoantibodies directed against cytoplasmic antigens of neutrophils (ANCA), especially those with specificity for MPO and PR3, are valuable markers for vasculitides • ANCA are specific granule proteins of granulocytes and monocytes • cytoplasmic (classic) cANCA is induced by antibodies directed against proteinase 3 (PR3; PR-ANCA) while the perinuclear pANCA is induced by antibodies against myeloperoxidase (MPO; MPO-ANCA) • positive correlation between MPO-ANCA and PR3-ANCA levels and increased sE-selectin, sP-selectin, sICAM-1, and sVCAM-1 was reported in patients with ANCA-associated vasculitis using the quantitative anti-MPO-ELISA and anti-PR3-ELISA • cytokines TNF-α and IL-β increase PR3 expression in the cytoplasm of endothelial cells. A possible direct pathogenic effect of anti-PR3 antibodies, through ANCA-endothelial interaction, has been suggested in vasculitides • cytotoxic effects of antibodies to PR3 on human vascular endothelial cells a PR3 antibody-mediated mechanisms of endothelial injury
Regional blood flow by imaging	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • vascular flow and pressure changes may or may not be predictive of vascular injury—the techniques needed to measure flow in animals are invasive and as such are not true biomarkers • in humans, there are new imaging techniques that may be useful to measure flow changes in regional vasculature

involve isolating RNA from control and treated tissues or cell lines, labeling the RNA, and hybridizing it to the array. Depending on the specific platform, absolute or differential expression levels of the genes represented on the arrays are then calculated and used for statistical analysis. Because mechanisms of vascular injury are not known, it is preferred to use the genome-wide microarrays that are generally available for rat, mouse, and human. Although these closed systems are evolving as the genomes become better defined, genes critical to the pathogenesis of vascular injury could be overlooked.

In contrast to the “closed” systems, “open” expression-profiling systems do not begin with a preselected set of

genes. Instead, the differences between two populations of RNA are determined directly, generally using some combination of RT-PCR, gel electrophoresis, and DNA sequencing. Examples include SAGE (Serial Analysis of Gene Expression), differential display, and subtractive hybridization. Kits for many of these techniques are commercially available, and several biotechnology companies use proprietary versions to build expression databases. One advantage of open methods for characterizing vascular injury is that, depending on the platform’s sensitivity, most of the genes directly involved in the lesion should be identified using these techniques; this would include some genes that have not been previously identified and therefore would not be

Table 10

Potential of omics

Biomarker	Reagent availability	Key points
Proteomics	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • techniques such as Power Blotting, 2D gel electrophoresis, and 2D HPLC combined with SELDI, LC/MS/MS, and MALDI techniques may be useful to define profiles of proteins that may be associated with vascular injury • this work is more hypothesis-generating than hypothesis-driven and is at an early stage of development • the hope is that novel protein markers may be discovered through global scanning or proteins
Genomics	Rat-Y, Dog-Y/N, Human-Y	<ul style="list-style-type: none"> • techniques such as gene array may be useful to define profiles of genes that may be associated with vascular injury • this work is more hypothesis-generating than hypothesis-driven and is at an early stage of development • the hope is that novel gene markers may be discovered through global scanning and that quantitative PCR technique can be developed to monitor gene biomarkers
Metabonomics	Rat-Y, Dog-Y, Human-Y	<ul style="list-style-type: none"> • initial studies suggest that metabonomics NMR profiling and computer techniques may be useful to identify animals with vascular injury • however, these techniques rely on nonspecific changes in kidneys and other organs that may occur far downstream from the toxicologic events and often do not allow for mechanistic interpretation

represented on microarrays. Another advantage is that they may be better suited for characterizing vascular injury in species whose genomes have not been extensively sequenced, such as the dog. Limitations of the open methods include their technical difficulty, time-consuming nature, and expense.

4.1.3. Knowledge gaps/limitations

There are several technical issues that must be considered when working with blood vessels. Certainly one of the most significant is that most blood vessels are small, making it difficult to obtain sufficient quantities of RNA for most profiling methods. This problem is of course more acute in rodents than dogs. The development of increasingly sensitive amplification techniques offers promise that starting RNA amounts will not be a limitation in the future. Moreover, like most tissues, blood vessels are complex tissues comprised of multiple cell types and can be found in close association with surrounding connective tissues such as fat, pancreas, or lymph nodes. When interpreting expression data from vascular tissue, this complexity can make it difficult to distinguish the contributions of endothelial cells from vascular smooth muscle cells (VSMCs), infiltrating leukocytes, and surrounding tissues. One possibility is to perform *in situ* hybridization or immunohistochemistry to localize the cellular source of specific transcripts of interest, assuming the necessary antibody and cDNA reagents are available or can be generated. Another is to use laser capture microdissection (LCM) to isolate “pure” populations of endothelial cells, VSMCs, or other targeted cell populations (Stagliano *et al.*, 2001). Inherent to the lack of mechanistic understanding of vascular injury, good *in vitro* models are not available, limiting the ability to generate higher-throughput gene-based *in vitro* screens for vascular injury-inducing potential.

As with all large-scale expression profiling experiments, data analysis and interpretation remains a key challenge. Lists of genes that are up- and down-regulated in tissues from animals with vascular injury will easily be generated, but how can we separate cause from effect? This task will be facilitated by solid experimental designs that include time courses (i.e., take samples at early time points to capture potential initiating events), dose-response (i.e., include a low dose that does not cause toxicity to help separate pharmacologically mediated changes in gene expression from toxicological ones), and careful choice of positive and negative controls (i.e., generating expression data from animals treated with compounds that cause inflammation but not vascular injury will facilitate the search for more specific biomarkers). Furthermore, bioinformatic approaches to link differentially expressed genes to altered metabolic and signaling pathways and well-designed and focused follow-up studies are critical to confirm new hypothesis that global gene expression approaches might generate.

Ultimately, the success of identifying biomarkers for vascular injury and understanding mechanisms of vascular

injury may require application of multiple technologies, including genomics, proteomics, metabonomics, flow cytometry, imaging, etc. This creates the additional challenge of combining disparate data from these various approaches and integrating them to allow cross-platform querying and extraction of biological knowledge to gain a more holistic understanding of vascular injury.

4.2. Proteomics

4.2.1. Background

Proteomics technologies are broadly characterized as tools designed to examine the expression of proteins in a given set of samples. Types of information derived from proteomics technologies include protein identity, quantity, interactions, structure, and posttranslational modification. The nature of the proteome presents a series of challenges, most importantly protein heterogeneity, that limit the ability of any single technology to completely assay and characterize it. Proteins can be large or small, globular or compact, hydrophobic or hydrophilic, basic or acidic. Posttranslational modifications such as glycosylation, lipidation, or phosphorylation can have dramatic effects on how the proteins behave in biochemical assays. A second challenge of the proteome is the extraordinary wide dynamic range of protein expression, which is estimated to be one billion fold (Corthals *et al.*, 2000). This presents an extraordinary challenge to any detection method. A third challenge of the proteome is the absence of a method of direct amplification. While the polymerase chain reaction can amplify DNA and RNA, no such technique exists for proteins (although there are promising approaches in development). These challenges together create a large opportunity for technology development, as well as niches for the myriad of technologies that have already been developed. For the researcher studying vascular injury, these technologies can provide powerful tools to discover biomarkers or to understand fundamental mechanisms. To date, there have been no published reports detailing proteomic-derived biomarkers for vascular injury; however, several laboratories are currently pursuing the approach and recent progress has been presented in the field of cardiac toxicology (Petricoin *et al.*, 2004).

4.2.2. Platforms (strengths/weaknesses)

This document cannot, of course, list all proteomic technologies, which are constantly being developed and refined. Instead, it will focus on several well-established techniques that are broadly aimed at expression profiling (although some have additional applications such as studying posttranslational modification). Consequently, a discussion of high-throughput crystallography, as an example, will not be presented here. Several themes will develop. First, all proteomics technologies consist of some permutation of separation followed by detection. Second, no single proteomics technology in its current state can possibly hope

to characterize the entire proteome. It is therefore the recommendation that each researcher apply multiple approaches to the study of the proteome.

Classically, two-dimensional gel electrophoresis (2-D PAGE) has been used to assay the proteome (Aebersold and Goodlett, 2001). Protein samples are first separated based on *pI* using an isoelectric focusing gradient. These proteins are then further separated in a second dimension based on their migration in an electric field, which is generally a function of their hydrodynamic volume, which roughly correlates to their molecular weight. Detection can then be performed using any of a myriad of agents, including silver staining and fluorescent dyes. Individual spots on the gels can be excised, digested with trypsin, and tryptic fragment fingerprint matched against a database. Where indicated, tandem mass spectrometry can be performed and matched against the theoretical tandem mass spectra of peptide sequences to provide (in most situations) definitive identification. Its drawbacks include poor visualization of low-abundance proteins, generally poor visualization of extremely basic or acidic proteins, poor visualization of hydrophobic proteins, and low throughput (Gygi et al., 2000). Matching of 2D gels from different samples is time-consuming, although computer algorithms that aid this process are improving. Thus, this technique is useful for the researcher who wishes to perform differential profiling and has abundant sample volume but limited numbers of samples. It is not useful for the researcher with limited sample volume or many samples. This may limit the statistical validity of some of the conclusions derived from 2D gel studies. Recent advances in software used to align 2D gels, integrated platforms, and independent 2D gel service providers can overcome many of these limitations.

Surface-enhanced laser desorption ionization and time-of-flight mass spectrometry (SELDI-TOF MS) is a proteomics technique with a broad range of applications, including protein expression profiling (Fung et al., 2001). Separation is performed using a combination of traditional elution chromatography (usually on spin columns) followed by retentate chromatography. Retentate chromatography is performed by incubating samples (or fractions of samples) on arrays containing chromatographic substrate on its surface. Traditional chromatographic steps of binding and washing are performed, but what is detected by laser desorption TOF MS are the proteins that have been retained on the arrays. This technique can be used for differential profiling, particularly when samples are scarce (only 20 μ l of serum is required) as well as when a relatively large number of samples need to be profiled. In addition to differential profiling, this technology can be used to study protein-protein interactions, posttranslational modifications, and epitope mapping. Therefore, it may be quite useful in studying mechanisms of vascular injury in addition to identifying novel biomarkers. Its principal drawback is the molecular identification of signatures, which generally requires additional biochemical analytical techniques.

A relatively novel technique, ICAT, for differential expression profiling requiring labeling of samples has recently been described and commercialized. In this method, the proteins in two samples to be compared are labeled separately on the side chains of their reduced cysteinyl residues using one of two isotopically different, but chemically identical sulfhydryl-reactive ICAT reagents (one being an isotopically "light" reagent, d(0), the other being a "heavy" reagent containing eight deuterium atoms on its carbon backbone, d(8)). The labeled protein mixtures are combined and enzymatically digested (e.g. with trypsin), and the labeled peptides are isolated by affinity chromatography, using the affinity tag (biotin group) that is part of the ICAT reagents. The selected peptides are separated by liquid chromatography and analyzed by tandem mass spectrometry (Gygi et al., 1999). This technique assumes quantitative and equivalent labeling and recovery of analytes and labels only proteins containing cysteines. In addition, it is not well suited to the study of proteins that have been modified posttranslationally. It may be a good technique for differential profiling of a limited number of samples, but is not high throughput.

Multidimensional liquid chromatography followed by tandem mass spectrometry is another technique for identification proteins contained in a complex sample (Griffin and Aebersold, 2001). Samples for this procedure are generally globally digested, and then liquid chromatography using orthogonal separations principles is performed serially, and the individual peptides are then sequenced by tandem mass spectrometry. While useful in providing information of proteins contained within a sample, quantitation information is minimal and the procedure is notoriously low-throughput.

Finally, a brief discussion of protein arrays in their many forms is warranted (Walter et al., 2002). There has been much interest in developing arrays on which hundreds to thousands of proteins or antibodies are immobilized, and then incubated with a sample and binding information for each of these analytes obtained simultaneously. This has, however, been difficult to achieve in practice, in large part because identifying reagents (i.e., purification of the thousands of proteins or antibodies) is difficult, immobilization strategies that retain protein function are difficult to achieve, and various protein-protein interaction reactions often have differing binding requirements (e.g. pH, salt, divalent cations, etc). Specific types of protein arrays include tissue arrays and multianalyte western blots. In the future, it can be expected that many types of arrays will be developed, most with specific types of applications rather than global protein expression profiling.

These descriptions are not intended to be comprehensive, but rather provide insight into the major proteomic tools available to the researcher studying vascular injury. Some of these techniques are readily transferable to the individual researcher's laboratory; others are best done in

collaboration with experts versed in the use of the techniques. In addition, the vascular injury researcher will find many opportunities to participate in the improvement of existing techniques and the development of novel ones. Beyond the applications of protein expression profiling detailed here, the study of protein–protein interactions to delineate protein–interaction maps can be quite useful in studying the mechanisms underlying vascular injury. In addition, the study of posttranslational modifications is a core application of many of these proteomics technologies and can therefore be useful in studying how different types of posttranslational modification determine the function of the proteins involved in the mechanisms of vascular injury.

4.3. Metabonomics

4.3.1. Background and principles

Toxicants, by definition, disrupt the normal composition and flux of endogenous biochemicals in, or through, key intermediary cellular metabolic pathways. These disruptions alter, either directly or indirectly, the blood that percolates through the target tissues. This altered blood can then produce changes in urinary content, either directly or indirectly, producing characteristic biomolecular traces. The diagnostic utility of any single trace biomolecule is limited due to the number of variables affecting it along its route to the urine and by the commonality of biochemical processes disrupted by toxicants. However, if a significant number of trace molecules are monitored, the overall pattern, or “fingerprint” produced may be more consistent and predictive than any one marker. Metabonomics combines the techniques of high-resolution nuclear magnetic resonance (NMR) spectroscopy and pattern recognition technology to rapidly generate these diagnostic metabolic fingerprints (Holmes et al., 2000; Nicholson et al., 1999, 2002; Robertson et al., 2000).

4.3.2. Platforms

Although, in principle, many analytical techniques could be used for metabonomic evaluations, high-field NMR provides the distinct advantage of being able to simultaneously detect thousands of molecules in a bodily fluid with little sample preparation. This ability allows an unbiased assessment of metabolic response (Lindon et al., 1999, 2000). Proton (^1H) NMR spectroscopy is capable of detecting individual atoms in soluble proton-containing molecules with a molecular weight of approximately 20 kDa or less. Standard flow probes used with 600-MHz spectrometer are capable of detecting thousands of resonances in the urine from molecules at concentrations in the mid micromolar range or higher with a data acquisition time of a few minutes. Incorporation of cryoprobe technology will lower this limit significantly. The other half of the technology, pattern recognition, is considerably more diverse in the availability of platforms

(El-Deredy, 1997; Holmes and Shockcor, 2000). However, principal component analysis (PCA) has been the most frequently used technique for small-scale focused metabonomic studies (Robertson et al., 2000). An exciting area of ongoing research is the linking of metabonomics technology with proteomic and toxicogenomic platforms allowing potential biomarker assessment from gene to protein to phenotype.

4.3.3. Application of technology to drug-induced vascular injury

The application of metabonomic technology to drug-induced vascular injury is a relatively recent development. To date, most work has focused on PDE4-induced arterial lesions in the rat. Principal component analysis produced a clear urine spectral pattern separation between 8 of 11 rats with vascular lesions and 36 of 37 rats without lesions in samples collected after 3 or 4 days of treatment with CI-1018, a PDE4 inhibitor (Robertson et al., 2001). Furthermore, in rats immunosuppressed with dexamethasone, the urine spectral pattern was still evident, suggesting the spectral changes were not simply an indirect index of inflammation (Slim et al., 2003). The specificity of the spectral changes produced in rats with vascular lesions has been demonstrated with renal lesions in SHR rats that could be differentiated from spectral changes associated with vascular lesions produced by the same compound in WKY rats (Zhang et al., 2002a, 2002b). Work with rolipram (nonselective PDE4 inhibitor) demonstrated that the spectral changes precede vascular lesions in both time and dose (Robertson et al., 2001).

4.3.4. Advantages/disadvantages

Using urine as a sample matrix, a distinct advantage of the technology is that comprehensive toxicological information can be gained from a single animal including onset, peak, and regression of toxicity without concerns over sample numbers or timing. Results can be readily correlated with concurrent clinical and clinical pathology assessments if necessary. Because sample collection is noninvasive, analyses are easily piggybacked onto other studies with minimal impact. Sample size is usually not an issue in rats as they produce 10–20 ml of urine/day. Sample preparation is minimal requiring only addition of antibacterial agent, dilution in deuterated buffer, and centrifugation to eliminate particulates—all of which are amenable to robotic processing in a microtiter plate format. The analyses themselves can be conducted relatively rapidly (200–300/day) and have a relatively low sample cost/analysis. Because NMR is nonselective, once a sample is analyzed, the spectra can be queried as needed to obtain different biological information without the necessity of rerunning the analyses. The primary disadvantages of the technology are the high initial capital costs of obtaining an instrument and the necessity for collecting samples in metabolism cages with cold collec-

tion capability as bacterial contamination of samples essentially eliminates the utility of the sample. Other disadvantages with the technology are not appreciably different than those associated with proteomic and genomic approaches. Because NMR is nonselective, any change in animal physiology or toxicity (other than the one of interest) may affect the urine NMR spectra, making it difficult to associate a pattern specifically with the lesion of interest. Additionally, it is often difficult to mechanistically associate bimolecular changes in urine to observed lesions. NMR is not particularly sensitive (relative to something like mass spectrometry) but it is not subject to efficiency bias and the utilization of longer acquisition times or the use of cryoprobe technology can reduce the sensitivity limitation. In the future, metabonomics will likely embrace both NMR and mass spectrometry technologies in a complimentary fashion. Given the wealth of data that can be generated and the uncertainty as to what it all means, bioinformatics limitations and information overload plague this technology as they do the other "omics" approaches.

4.3.5. Knowledge gaps

Metabonomics is a newer technology than proteomic and toxicogenomic approaches (at least to the toxicologist anyway). With that newness comes a certain amount of "black box" fear for those not familiar with NMR. However, NMR, as a platform, is much older and more stable than platforms for other technologies, which should help reduce those fears accordingly. The multivariate statistical methods are much less user friendly and represent a real knowledge gap between the users and those assessing the significance of the generated data. Within the vascular injury application, the primary knowledge gap is the lack of a clear mechanistic relevance of the biomolecular changes driving the pattern separation. It seems clear that the urinary changes are not simply a reflection of inflammation, but it is difficult to understand how minimal to moderate pathologic changes in one vascular bed (e.g. the mesentery) could drive micro to millimolar urinary changes in molecules involved in intermediate metabolism. It seems likely that other factors may be involved, some of which, when elucidated, may lead us to a better understanding of the etiology of this troublesome lesion in rats that may then lead to a more clear evaluation of the clinical significance of these changes.

4.4. Flow cytometry

4.4.1. Background and principles

There are numerous circulating cells present in the bloodstream of both clinical and preclinical species. These cells are exposed to the pharmacologic agent of interest, and the possibility exists that during vascular toxicity, cells within the vessel wall may dislodge and enter the circulation.

Information, both quantitative and functional, can be obtained by analyzing these circulating or dislodged cells. There is considerable heterogeneity between arterial, venous, and capillary vascular endothelium, and even organ-specific endothelial phenotypic alterations have been identified (Cines et al., 1998). This may allow discrimination of site-specific vascular injury, once appropriate markers have been identified. Flow cytometry has wide application in the study of circulating cells, which can be collected by minimally invasive means. It permits rapid simultaneous acquisition of multiple parameters, which are valuable for analyzing diverse functional or quantitative changes in individual cells (Shapiro, 1995). The advent of eight- or nine-color sorters and/or analyzers permitted multicolor fluorescence analyses, and the continuing development and commercialization of a wide range of fluorescent probes have made flow cytometry a unique technology to meet these needs. These capabilities coupled with sequential gating strategies permit analyses of extremely large numbers of cells leading to subsequent identification of even rare cells (Gossett et al., 1999).

4.4.2. Platforms

Flow cytometry allows for rapid measurement of numerous characteristics on individual cells/particles moving single file in a fluid stream. Forward angle light scatter can distinguish cell/particle size whereas side angle light scatter can determine internal complexity. Furthermore, light emitted from fluorescently labeled probes or antibodies allows identification of a wide variety of cell surface, cytoplasmic and nuclear structures. Light scatter and fluorescent emission are then detected by photodetectors. Flow cytometers use two basic platforms, bench top analyzers and sorters. These are available from a variety of manufacturers. Both platforms can be used for high-speed laser cell interrogation and analysis. The biggest difference is that a sorter can be used to select cells of interest into a receptacle for further analysis or culture. Typically, sorters are much more accommodating in design of lasers and optics compared to bench top analyzers, providing more flexibility in study design, and numbers of parameters that can be examined simultaneously.

4.4.3. Application of technology to drug-induced vascular injury

The application of flow cytometry to the study of drug-induced vascular injury has only begun recently. Flow cytometric analyses in relation to drug-induced vascular injury would be applicable in several areas: (1) alterations of normally circulating cells such as white blood cells, (2) cells released from the vascular wall into circulation after vascular damage, (3) assessment of oxidative stress in circulating cells. Single cell suspensions of circulating leukocytes can be monitored as sentinels of drug-induced effects as it lends itself amenable to rapid analyses of thousands of cells (Gossett et al., 1999). There are numerous commercially available antibodies to cell surface proteins on

leukocytes in most species, allowing for immunophenotyping and assessment of activation markers. It is currently unknown how drug-induced vascular damage impacts these measurements. There are small numbers of circulating endothelial cells present in normal individuals, and the numbers have been shown to increase after various insults resulting in vascular damage (Dignat-Gorge and Sampol, 2000). To date, most of these studies have utilized antibody linked magnetic beads to concentrate circulating endothelial cells for enumeration, and the use of flow cytometric quantitation is untested. There is *in vivo* evidence for oxidative stress in vascular endothelium and leukocytes of spontaneously hypertensive rats as compared to normotensive rats (Suzuki et al., 1995). Hypertension is well known to result in vascular damage and repair. This assay relies on the use of hydroethidine that is a reduced nonfluorescent precursor of the fluorescent molecule ethidium bromide. Hydroethidine, in the presence of superoxide radical, converts to ethidium bromide that can bind DNA and then fluoresces brightly. This assay could be applied to models of drug-induced vascular damage to assess either leukocytes or circulating endothelial cells.

4.4.4. Advantages/disadvantages

Advantages. The equipment is widely available in both clinical and preclinical laboratory settings. There is an ever-increasing number of commercially available antibodies and fluorochromes applicable to flow cytometry. Additionally, antibodies can be made to most antigens providing increased utility to flow cytometric analyses in toxicology. Flow cytometric examination of cells can be done at rates of tens of thousands of cells per second, and can frequently be done in an automated fashion.

Disadvantages. The capital costs for equipment, especially for sorters, are quite high. Qualified personnel are needed to run and interpret multiparameter data. Antibodies and fluorochromes are key to fully utilizing the potential of flow cytometry in assessment of vascular damage. Despite the availability of antibodies for endothelial cells, because of the heterogeneity of endothelial cells, truly pan-endothelial antigens likely do not exist. This could also be an advantage once the localization of antibodies is mapped to specific vascular endothelium, potentially allowing identification of vascular damage location. Making new antibodies to current antigens without antibodies or previously unidentified antigens takes both time and money. Many of the currently available antibodies do not cross-react across multiple species.

4.4.5. Knowledge gaps

It is currently unknown whether localized vascular damage, typical of the drug-induced vasculopathies, alters either normally circulating cells or dislodges vascular wall cellular components in a detectable fashion. Likewise, the

duration of increased or altered circulating cells after vascular insult is largely unknown, although it appears to return to normal as the symptoms subside (Grefte et al., 1993; Mutin et al., 1999; Sinzinger et al., 1988; Solovey et al., 1997). There is marked disagreement, in the literature, on the number of circulating endothelial cells present in normal individuals; these discrepancies may be related to differences in methods and/or antibodies utilized. It will be important to provide details regarding methodology for quantitation of cells when reporting on models of vascular damage. There is currently a need to more fully elucidate the phenotypic heterogeneity of the vascular endothelium in a localized manner, specifically in vascular beds where drug-induced vascular damage is common: coronary vascular bed in dogs, splanchnic vascular bed in rats. Localization of damage is currently only detected by postmortem examination. Reagents allowing this localization should be pursued by techniques such as injection of phage-display peptide libraries that detect specific surface molecules in the vascular endothelium (Pasqualini and Ruoslahti, 1996), or pursuing novel methods of culturing endothelial cells from specific vasculature (Dong et al., 1997; MacPhee et al., 1994).

4.5. Bioinformatics and multivariate statistics

4.5.1. Background

“Omics” dominates the list of new techniques available to the researcher studying almost any biological system. Rapid developments in technology platforms coupled with high-throughput techniques allow researchers to measure many analytes from many samples simultaneously, generating a staggering amount of data. This leads to the challenge of mining that data, archiving that data, and retrieving that data. The fields of bioinformatics and multivariate statistics, coupled with increasing computational power, present to the researcher solutions to their data-mining problems as well as opportunities for research and development of novel solutions to these problems. Because of this, computational biology should itself be thought of as a novel technology.

4.5.2. Platforms

The term bioinformatics in this discussion is reserved strictly for two tasks: first, to describe methods that mine primary and secondary sequences of nucleic acids and proteins as well as structural data for proteins to derive information regarding genes or proteins; and second, to archive and retrieve such data in a computationally efficient manner. Thus, bioinformatics encompasses strategies used to perform sequence alignment, to predict coding regions from genomic DNA, predict secondary structure of proteins, and so forth. It also encompasses databasing such information, such as is performed at the National Center for Biotechnology Information (NCBI) or SWISS-PROT. Another type of database is one of theoretical enzymatic digests of proteins contained in a protein database, which is

searched with an actual enzymatic digest of an unknown sample to obtain the identity of the protein. An outstanding review text is written by Baxevas and Ouellette (2001). Vascular injury researchers have at their disposal several tools, many of which are continually being improved. For example, better prediction of coding regions in genomic data leads to a more complete catalog of genes. This in turn leads to a more complete database of enzymatic digests for protein identification. A more complete understanding of protein function and structure allows researchers to catalog protein domains and sites of specific types of posttranslational modification, thus allowing a researcher to generate and test hypotheses regarding protein function more quickly. However, much remains to be done. For example, databases of theoretical translations do not account for real or theoretical posttranslational modifications. This can lead to incorrect protein identification or, more likely, the mistaken conclusion that one has found a heretofore unidentified protein. Algorithms that more accurately predict gene structure also remain to be developed. Bioinformatics can also be used to understand species differences in vascular injury (i.e., comparative genomics). For example, sequence alignment can reveal conserved domains that might dictate similar functions (and therefore similar responses to drugs) as well as nonconserved regions that might help predict divergent functions.

This discussion will not include the problem of visualization of genomics or proteomics data. A good review can be found in Chakravarti et al. (2002). Perhaps of more interest to the researcher employing these “omics” technologies is statistical analysis of data generated by these techniques. For example, if one were to use a 10-k cDNA microarray on 20 samples, there would be 200 000 data points. SELDI technology can easily generate data for 140 000 protein peaks for 40 samples. Particularly in the most common employment of these “omics” technologies, there is a profound asymmetry between sample number (few) vs. data points per sample (many). This presents a challenge for statisticians, in which the converse situation (many samples with fewer data points/sample) is preferred. Generally, the goal is to find the set of variables (genes or proteins) that distinguish classes (such as with vascular injury vs. without vascular injury) of samples from each other. Univariate analysis on a variable-by-variable basis is simple to perform, but in most complex biological conditions, it is unusual to find a single variable that is both sensitive and specific for a given condition. For example, many acute phase reactant proteins are increased in animal models of vascular injury, but these are generally not considered to be specific to vascular injury. Using multiple variables simultaneously is a common method to overcome the limitation of single markers. Multivariable analysis is therefore an important component of any study aimed at identifying biomarkers of vascular injury. Multivariate analysis can be conveniently divided into two broad classes, supervised and unsupervised. Supervised learning

requires that during the development of a classification model, information regarding outcome (class assignment) is available and is useful in developing a diagnostic. Unsupervised learning does not have such a requirement and is more powerful for data exploration. These classes of algorithms will be discussed in turn.

Supervised learning techniques are generally described as classification algorithms, in which data for each of the samples is supplied to the algorithm along with the class assignment for each sample (classes are the various groupings of samples such as drug-treated vs. untreated). The algorithm uses the class assignment to develop models that can be used to classify these samples (called the training set). In the ideal case, these models are then tested on different samples for which the identity is hidden (called the testing set). In general, the performance of the model on the test set is expected to be lower than that on the training set. The quality of the model is a function of the overall classification success rate as well as the decline in classification success rate from the training set to the test set of samples. It is not uncommon to develop a model that can classify the training set with high success but that does poorly on a test set—these models are generally described as being overfit. Examples of supervised learning techniques include support vector machines, neural nets, and decision trees. The vascular injury researcher is recommended to utilize algorithms that provide the underlying variables that contribute to the classification model, because these features can be concluded to be biomarkers for future workup.

Unsupervised learning techniques are generally described as clustering algorithms. No class assignments are presented to the algorithm, which attempts to distribute the samples as far apart as possible in n -dimensional space. Samples that are more similar to each other will be closer to each other in these models, and the distance between samples is therefore a measure of dissimilarity. Common unsupervised learning techniques include self-organizing maps, hierarchical and k -means clustering, and principal component analysis. These types of techniques are particularly useful in finding hidden classes. Because no a priori assumptions are made about the categorization of samples, the distribution of the samples may reveal heretofore-unknown subgroups. A drawback of unsupervised learning techniques is that deconvolution of these models may not reveal a defined, small subset of biomarkers. Instead, one often gets a long list of co-regulated genes or proteins. This can be particularly problematic for discovery of biomarkers that are to be used as a framework for mechanistic studies of vascular injury.

Statisticians are constantly developing refinements of these techniques as well as novel approaches. The challenge for the vascular injury researcher not versed in statistics will be to utilize the technique(s) that are most appropriate for the type of data being mined. Vascular injury researchers should seek out collaboration

with statisticians who are versed in the biology of genes and proteins, so that intelligent decisions regarding algorithm selection and parameters are made. Moreover, it should be recognized that proper experimental design is the first step toward optimal statistical analysis; poor experimental design can lead to the inability to make statistically sound conclusions or, worse, incorrect conclusions from the data. Finally, all data that are supplied to these types of algorithms must be intelligently preprocessed (e.g., calibrated and normalized) (Fung and Enderwick, 2002).

4.6. Conclusions

These exciting new technologies can be sources of new biomarkers as well as provide insight into the mechanisms of vascular injury. The combination of these technologies will provide a synergistic effect, both from the standpoint of validating results across the various platforms, as well as providing the opportunity for “meta” statistical analysis, i.e., the integration of information garnered from multiple techniques. It is recognized that the adoption of any one of these technologies is itself a resource heavy proposition, and to undertake more than one simultaneously creates even greater pressure on potentially limited resources. Therefore, it would be prudent for researchers across institutions to allocate resources and become centers of excellence in a specific subset of these technologies. The data can then be shared, archived, and mined. This will expedite the discovery and validation of biomarkers and enable a greater understanding of the mechanisms of vascular injury.

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