Vascular-related diseases, including Blackfoot Disease and atherosclerosis, are prominent clinical findings among populations residing in arseniasis areas. While oxidative stress provided a general but nonspecific mechanistic base for arsenic-induced endothelial cell damage in vitro, more specific mechanisms are needed to explain the highly targeted vascular lesions induced by arsenic in vivo. Based on our previous studies, we hypothesized that arsenic exerted its action on blood vessels via the neurogenic inflammation process involving release of a neuropeptide (substance P) and activation of endothelial Neurokinin 1 (NK-1) receptor in vivo. Indeed, our present study demonstrated a significantly higher substance P levels in arsenic-treated tissues when compared to saline-treated controls indicating a rapid release of substance P under the influence of arsenic. Furthermore, the arsenic-induced vascular leakage could be significantly reduced when the neurogenic inflammation process was interrupted (via either disruption on the release of substance P, interference on the action of substance P, or blockage of endothelial NK-1 receptor) showing that the neurogenic inflammation process was indeed involved. Histamine release was found to play a significant role in arsenic-induced vascular permeability change. Our present study affirmed a de novo concept that a pathophysiological mechanism involving the neurogenic release of substance P and activation of endothelial NK-1 receptor underlies the arsenic-induced vascular injury and dysfunction in vivo. This pathophysiological process constituted a two-tiered biological interaction between the nervous system and vascular system and therefore was not readily unveiled by traditional in vitro studies in the past. Our present finding unveiled an important de novo concept on arsenic vascular toxicity in vivo.

Key Words: arsenic toxicity; vascular injury; substance P; neurogenic inflammation; vascular injury.

Because of its global existence and serious health impacts, arsenic remains as a top environmental concern in the United States as well as worldwide. Aside from cancer risk, epidemiological studies provided ample evidence that cardiovascular-related diseases including atherosclerosis, stroke, and ischemic heart disease were significantly increased among residents in arseniasis areas in the world (Chang et al., 2004; Rahman, 2002; Yu et al., 1984). Indeed, the well-known Blackfoot disease, a syndrome which involved degenerative collapse of the peripheral vasculatures and gangrenous conditions of the extremities, was one of the prominent clinical hallmarks in patients chronically exposed to high levels of arsenic via drink water in southeastern Taiwan in the 1960’s (Yu et al., 1984). Therefore, the action of arsenic on vascular system was actually quite specific and targeted.

Because of the close association of arsenic and vascular diseases or dysfunction, there were many investigations on the effects of arsenic on the endothelial cells in the past. However, most, if not all, of these studies were conducted with endothelial cell cultures in vitro. Free radicals production and oxidative stress generated by arsenic were reported to be associated with and could induce endothelial cell damages in vitro (Griffin et al., 2003; Gurr et al., 1998; Huang et al., 1993; Lynn et al., 2000; Wang et al., 2001). However, under in vivo conditions, not all chemicals which were known to produce free radicals or oxidative stress induced vascular changes as those observed in arsenic poisoning. Therefore, in addition to oxidative stress, other mechanistic process may also be involved in the production of the highly targeted vascular changes in arsenic poisoning.

One of the major influences on vascular permeability in vivo is via neurogenic control (Jancso et al., 1967). The term “neurogenic inflammation” is frequently used to refer to an induction of vascular permeability via neurogenic release of a neuropeptide, substance P, by the sensory C-fibers. Substance P is a potent ligand to the endothelial Neurokinin 1 (NK-1) receptor (also known as the substance P receptor). Increased activation of the NK-1 receptor enhances vascular permeability (leakage) (Holzer, 1998; Inoue et al., 1996; McDonald, 1988; Saria et al., 1983).
Disturbance in vascular permeability (vascular leakage) is a pathophysiological phenomenon which is frequently seen in early vascular damage and dysfunction. It has also been used as an indication of early vascular injury (Joris et al., 1990). We have previously developed an animal study model through which we could evaluate arsenic-induced vascular leakage (injury) in vivo both qualitatively and quantitatively (Chen et al., 2004b; Tsai et al., 2005). Using this in vivo study model, we had demonstrated that the vascular effects of mustard-oil (allyl isothiocyanate), a well known model-compound which was known to induce vascular leakage via neurogenic inflammation in vivo, was significantly enhanced in animals fed with sodium arsenite (Chen et al., 2004a). This study strongly suggested that neurogenic inflammation process might be involved in arsenic-induced vascular dysfunctions in vivo. The objective of our present investigation was to provide further scientific evidence, via strategic experiments, to confirm our hypothesis that neurogenic inflammation (substance P release and endothelial NK-1 activation) is indeed involved in arsenic-induced vascular leakage in vivo.

MATERIALS AND METHODS

To confirm our hypothesis that neurogenic inflammation was indeed involved in the arsenic-induced vascular leakage, a significant accumulation, reflecting rapid release of substance P in the ears of animals treated with arsenite must be demonstrated. Furthermore, significant reductions in arsenic-induced vascular leakage must also be evident when the neurogenic inflammation process is interrupted (interference of substance P binding with NK-1 receptor, inhibition of the endothelial NK-1 receptor, or disruption of the substance P release). A series of strategic experiments were designed for such demonstrations (see below). Since histamine release by mast cells may also contribute to the increased vascular leakage induced by arsenic co-treated with H1 receptor antagonist was also conducted to clarify this concern.

Animals Used and Evaluation of Vascular Leakage In Vivo

Mice (ICR) were obtained from the National Animal Center (Nan-Kang, Taipei, Taiwan) for this investigation. Young male mice with average body weight of 25 g were used (details see below). Arsenic used in this study consisted of sodium (meta) arsenite dissolved in 0.9% NaCl solution. A predetermined dose (385nmol) of arsenite, which was found to be effective in the induction of vascular leakage in the mouse ear (Tsai et al., 2005), was employed in all experiments in this investigation. The animal model that we have established for evaluation of vascular leakage in vivo (quantitative Evans Blue [EB] tracer technique) was used in the present study (Chen et al., 2004b; Tsai et al., 2005). Some details of these procedures, however, will still be presented below.

Measurement of Substance P in Mouse-Ear Tissue

If neurogenic inflammation was involved in arsenic-induced vascular dysfunction in vivo, arsenic would enhance the release of substance P with an elevation of this neuropeptide in the tissues. This experiment was conducted to evaluate such phenomenon.

In a group of anesthetized mice (N = 6), 5 µl of sodium arsenite (385nmol) was injected intradermally into the left dorsal ears. Right ears of mice were similarly injected with saline solution and served as controls. Mice were sacrificed at different time intervals (10, 30, and 60 min) after the injections. Ears were then removed and stored in liquid nitrogen. Isolation and measurement of tissue-substance P was performed in accordance to the method of Fehder et al. (1998).

Before homogenizing the frozen ears were submersed in 1 ml of 4% acetic acid and heated in a water bath at 100°C for 10 min. After the samples had cooled down, another 1 ml of 4% acetic acid was added. Ear samples were homogenized with Polytron at 25,000 rpm for 1 min. The homogenate was centrifuged at 825 × g (Beckman, Fullerton, CA, Allegra 64R) for 15 min. Supernatants were then centrifuged again at 10,000 × g for 30 min (Beckman, Optima TLX). Protein contents in the supernatants were quantified with Bradford method. Supernatants containing 700 µg of protein were loaded into Sep-Pak C18 columns (Waters, Milford, MA) and washed with 6 ml of 4% acetic acid. The protein contents were eluted from the columns with 3 ml of acetonitrile:water-trifluoroacetic acid (60:39:1). The elution was evaporated to dryness under vacuum. The dried eluates were then redissolved in 0.4 ml of EIA buffer included in substance P ELISA Kit (Cayman Chemical, Ann Arbor, MI) before analysis. Substance P contents in tissue extracts were analyzed using the enzyme-linked immunosorbent assay (ELISA) technique. The procedure of ELISA technique was performed according to the manufacturer’s protocol (Cayman Chemical).

Interruptions of the Neurogenic Inflammation Process

Experiment 1: interference of substance P binding with NK-1 by a substance P competitor, SP-(4-11). SP-(4-11) is a fragment of substance P [L-Pro4, D-Try7,9,10, Phe11], which consists only amino acids 4–11 of the substance P neuropeptide. This SP-(4-11) will competitively bind to the endothelial NK-1 receptor without functional activation of the receptor (Mizrahi et al., 1984). Thus, it can effectively block substance P from binding with NK-1, thus reducing the impact of substance P on NK-1.

Mice (N = 6) were anaesthetized with pentobarbital sodium (50 mg/kg, ip) and were given EB (30 mg/kg, iv; 100 µl) intravenously through the tail vein. SP-(4-11) was dissolved in aqueous acetic acid (50%) at concentration 5 × 10−4 M and subsequently diluted with saline to have final concentration 1 × 10−3 M. Mice were intravenously injected via the tail vein with 0.01 ml of SP-(4-11) (1pmol/g body weight). Control mice received saline injection. Three minutes later, 5 µl of sodium arsenite (10.0 mg/ml; 385nmol) was injected intradermally into the left dorsal ear skin (center of ear) and right ear received saline injection. After 60 min, mice were sacrificed under anesthesia via intracardial perfusion with 0.9% NaCl solution and each ear was excised whole and soaked in 1 ml of formamide at 25°C for 72 h. Quantitative evaluation of vascular leakage was performed in accordance to the method that we have previously established (Tsai et al., 2005). In brief, EB dye in the ear tissues was extracted by soaking the excised ear in 1.0 ml of formamide at 25°C for 72 h and filtered with a syringe filter (glass fiber). The eluted EB was measured with a spectrophotometer (620 nm). The result was expressed as microgram of dye per ear.

Experiment 2: Inhibition of NK-1 receptor with a NK-1 antagonist, RP67580. RP67580 has been found to be an effective inhibitor of functional NK-1 (substance P) receptor (Rupniak et al., 2003). It blocks NK-1 receptor and significantly reduces NK-1 activation induced by substance P.

In our present study, a group of mice (N = 6) were subcutaneously injected with NK-1 receptor antagonist RP67580 (dissolved in dimethyl sulfoxide; 0.6 mg/kg; Tocris Cookson Inc. Ellisville, MO) on the back of the mice. Control mice were injected with same volume of vehicle. Twenty minutes later, anesthetized mice were given EB intravenously followed by intradermal injection of sodium arsenite or saline (ear) as described above. Mice were sacrificed 60 min after arsenite or saline injection. Vascular leakage evaluation (EB measurement) was performed in accordance to the method as shown above. Arsenic-induced vascular leakages with or without NK-1 antagonist RP67580 treatment were compared.

Experiment 3: disruption of substance P–releasing sensory C-fibers. Neurogenic substance P was released by sensory C-fibers. Such release was found to be effectively reduced when the sensory C-fibers were disrupted with capsaicin during early developmental age (postnatal days 7–10) (Gamse et al., 1980; Jancko et al., 1967; Lembeck and Holzer, 1979; Lundberg and Saria, 1982).

In our experiment, a group of mice (N = 6) were pretreated with capsaicin on neonatal day 7 according to the method of Gamse et al. (1980). The dorsal
skin around mice neck was injected by a single subcutaneous injection of capsaicin (50 mg/kg). Control mice were injected with corresponding volume (0.1 ml) of capsaicin-vehicle (1:1.8 ethanol/Tween 80/NaCl 0.9% v/v). After 28 days of capsaicin pretreatment, arsenic exposure and quantitative EB procedures were performed as described above. Vascular (EB) leakage with or without capsaicin pretreatment was measured and compared in accordance to the quantitative procedures that we had previously established (Tsai et al., 2005).

Vascular Labeling with India Ink Technique

While the EB technique could provide a visual assessment in tissues (“blue-ear” effect) and quantitative measurement of vascular leakage induced by arsenic, it could not actually label or demonstrate specific vascular involvement of the leakage histologically under the microscope. India ink tracer technique, however, could provide such demonstration. This technique was employed in the present investigation to provide the needed visual demonstration on comparative vascular leakage induced by arsenic with or without NK-1 inhibition.

India ink (Waldeck, Germany, Billerica, MA), filtered at 5.0 μm (Millipore), was diluted with saline (1:10) and injected intravenously via the tail veins of the anesthetized animals at a dose of 1 ml/kg. In a separate group of mice (N = 6), mice were treated with NK-1 blocker RP67580 (0.6 mg/kg) as above. Control mice (N = 6) were injected with same volume of vehicle. Twenty minutes later, mice of both group received intradermal injection of either sodium arsenite or saline as above. Sixty minutes later, mice were sacrificed via intracardial perfusion with saline. Then the ears were excised and ear skins were peeled off from the cartilage carefully and immersed in formalin fixative for 12 h. Ear skin was dehydrated with ethanol, cleared with xylene, mounted with Permount (Fisher Scientific, NJ) and examined under the microscope. Conditions of vascular leakage in different treatment conditions were photographed and compared.

Evaluation on Histamine Involvement

In separate group, mice (N = 6) were anesthetized and given EB intravenously as above. Three minutes later, a 10 μl solution of pyrilamine maleate salt (99nmol) and histamine dihydrochloride (270nmol) was coinjected intradermally into the left dorsal ears. The right ears of these mice received histamine injection only. For comparison, in another group of mice (N = 4), a 10 μl solution of pyrilamine maleate (99nmol) and sodium arsenite (385nmol) was coinjected intradermally into the left dorsal ears and the right ears received arsenic injection only. Sixty minutes later, mice were sacrificed and the vascular leakages were evaluated via EB extractions and measurements as described above.

Statistical Analysis

Values are presented as means ± SD. The analysis of variance (ANOVA) test was used to compare the means of different groups of data. If the ANOVA test indicated an overall significant difference (p < 0.05), the pairwise multiple comparison test (Tukey) was used to determine the significance of a difference in the mean between any two groups. Differences were considered significant when p < 0.05.

RESULTS

Substance P Level in Mouse-Ears Treated with Arsenic

The tissue levels of substance P in mouse ears treated either with saline (control) or with arsenic were analyzed and compared. Our study showed that the tissue levels of substance P in mouse ears were consistently and significantly higher in the arsenic-treated animals than in controls (Fig. 1). This increase in tissue levels of substance P remained 130% of control even 60 min after arsenic exposure. Our data firmly indicated that there was an increased release of substance P into the tissues in the presence of arsenic.

Arsenic-Induced Vascular Leakage with or without Interfering with the Neurogenic Inflammation Process

Neurogenic inflammation is the result of interaction between substance P and NK-1 receptor and thus is heavily depended on the status of substance P (production, release, and binding) and NK-1 (activation). If arsenic-induced vascular dysfunction was related to neurogenic inflammation, interfering with this process (modulation on the substance P and NK-1 status) would reduce the vascular leakage induced by arsenic.

Our present investigation clearly demonstrated that introduction of a substance P fragment, SP-(4-11), which competed with substance P for NK-1 receptor binding, significantly reduced the arsenic-induced vascular leakage (Fig. 2). Similar reduction was also observed when a NK-1 receptor blocker or inhibitor (RP67580) was introduced (Fig. 3). These two studies strongly affirmed our hypothesis that interaction between substance P and NK-1 receptor played an important role in arsenic-induced vascular permeability change in vivo. This concept was further supported by our finding that destruction of the substance P–releasing sensory C-fibers by capsaicin also significantly reduced the vascular response (leakage) to arsenic (Fig. 4). These three experiments provided compelling evidences demonstrating that neurogenic inflammation was indeed
the pathophysiological process underlying the arsenic-induced vascular injury and dysfunction in vivo leading to an enhanced vascular leakage.

**Visualization of Arsenic-Induced Vascular Leakage with or without NK-1 Receptor Inhibition**

India ink tracer technique allows vascular leakage to be visually demonstrable under the microscope. In our present study, we have applied this technique in one group of our animals just to provide a visual demonstration in support of the quantitative data that we have obtained from our EB studies. Vascular leakage could be observed microscopically as dark or black particles along side of the blood vessels. Prominent dark particles were seen in arsenic-treated ears indicating a significant vascular leakage (Fig. 5A). A significant reduction in the dark particles along side of the blood vessels (decrease in arsenic-induced vascular leakage) in animals cotreated with arsenic and the NK-1 inhibitor RP67580 was observed (Fig. 5C). This result provided the needed support and visual confirmation of our EB study as demonstrated in Figure 3. No significant vascular leakage was observed in tissues treated with either saline or RP67580 alone (Fig. 5B, D).

**Role of Histamine in Arsenic-Induced Vascular Permeability Change**

Histamine is a well-known substance released by mast cells and is a potent factor in the induction of vascular permeability (leakage). In our present study we found that pyrilamine maleate, a known histamine receptor antagonist, significantly reduced histamine-induced vascular leakage but not arsenic-induced vascular leakage (Fig. 6). This finding demonstrated that although histamine could stimulate or enhance vascular permeability in many situations, it did not play a pivoting role in arsenic-induced vascular permeability changes.

**DISCUSSION**

Arsenic is a globally concerning environmental toxic metal inducing pathological changes in a number of tissues and organs (Chappell et al., 1997; Chen et al., 1992; Chen and...
Wang, 1990). Epidemiological studies indicated that, aside from cancer, cardiovascular-related diseases, including high blood pressure, arteriosclerosis, heart attack, stroke, and diabetes were significantly elevated in the arsenic exposed populations (Chang et al., 2004; Rahman, 2002; Yu et al., 1984), strongly suggesting a close interrelationship between vasculopathy and arsenic exposure. Blackfoot Disease was a unique clinical syndrome found among arsenic-affected individuals in Taiwan in the 1960’s. This disease was characterized by degenerative vascular collapse with gangrenous changes of the extremities of the patients (Yu et al., 1984). Thus, the vascular system was commonly believed to be a targeted system for arsenic toxicity.

While many research in the past provided data indicating that free radical generation and oxidative stress played an important role in inducing endothelial cell damages in vitro (Griffin et al., 2003; Gurr et al., 1998; Huang et al., 1993; Lynn et al., 2000; Wang et al., 2001), this generalized “oxidative stress” based mechanism failed to fully address the specific injury targeting onto the vascular system by arsenic in vivo. Furthermore, many other potent oxidative stress–inducing agents, including other toxic metals such as mercury, lead, or cadmium, were not known to produce similar or specific vascular injuries in patients as those observed in arsenic poisoning. Therefore, aside from free radical generation and oxidative stress, other mechanistic process may be involved in arsenic-induced vascular injury.

Our previous studies (Chen et al., 2004a,b; Tsai et al., 2005) demonstrated that arsenic indeed induced vascular injury and leakage which could be visualized and quantified with the EB tracer technique (the “blue-ear” effect). Furthermore, we demonstrated a synergistic enhancement of arsenic-induced vascular injury in vivo. While many research in the past provided data indicating that free radical generation and oxidative stress played an important role in inducing endothelial cell damages in vitro (Griffin et al., 2003; Gurr et al., 1998; Huang et al., 1993; Lynn et al., 2000; Wang et al., 2001), this generalized “oxidative stress” based mechanism failed to fully address the specific injury targeting onto the vascular system by arsenic in vivo. Furthermore, many other potent oxidative stress–inducing agents, including other toxic metals such as mercury, lead, or cadmium, were not known to produce similar or specific vascular injuries in patients as those observed in arsenic poisoning. Therefore, aside from free radical generation and oxidative stress, other mechanistic process may be involved in arsenic-induced vascular injury.

Our previous studies (Chen et al., 2004a,b; Tsai et al., 2005) demonstrated that arsenic indeed induced vascular injury and leakage which could be visualized and quantified with the EB tracer technique (the “blue-ear” effect). Furthermore, we demonstrated a synergistic enhancement of arsenic-induced vascular
leakage by mustard oil which was a well-known chemical compound with the ability to induce vascular injury and leakage via the neurogenic inflammation process (Chen et al., 2004a). Thus, involvement of the neurogenic inflammation in arsenic-induced vascular permeability change was suspected.

Neurogenic inflammation is a pathophysiological process characterized by an increased vascular plasma leakage. This process primarily involves the release of neuropeptides, such as substance P, from stimulated sensory nerve fibers (C-fibers) (Harrison and Geppetti, 2001; Holzer, 1998; Janco et al., 1967; Lundberg and Saria, 1982; McDonald, 1988). This released neuropeptide (substance P) will in turn bind and activate the endothelial NK-1 receptor leading to an enhancement of vascular permeability change (leakage) (Baluk et al., 1997; Gamse et al., 1980; Harrison and Geppetti, 2001; Inoue et al., 1996; Lembeck and Holzer, 1979; McDonald, 1988). We hypothesize that arsenic induces vascular leakage in vivo via the neurogenic inflammation process. In our present investigation, we have implemented a series of experiments in attempt to challenge and affirm this hypothesis. Our result clearly demonstrated that tissue content of substance P was significantly elevated after arsenic exposure indicating that there was an increased release of substance P under the influence of arsenic. Although there was a trend of general reduction in tissue-substance P with time, the level of tissue-substance P in arsenic-treated skin remained significantly elevated (130% of control) even at 60 min after exposure showing that the arsenic effect may be quite persistent.

Moreover, all our experiments designed to intervene with the neurogenic inflammation process (interference of substance P binding to NK-1 receptor, inhibition of the NK-1 receptor, and disruption of the substance P–releasing nerve fibers) all resulted in significant reductions of arsenic-induced vascular leakage. These reductions were demonstrable both quantitatively via quantitative EB assay technique and visually via the India ink tracer technique. Histamine blocker (pyrilamine), however, failed to influence the arsenic-induced leakage significantly. We believe that we have provided clear and strong evidences that the pathophysiological process of neurogenic inflammation is indeed involved in arsenic-induced vascular leakage in vivo. It is important to note that none of the methods for intervention or disruption of the neurogenic inflammation process yielded complete elimination of vascular leakage induced by arsenic. The mechanism for arsenic vasculotoxicity is complex and is likely to be multifaceted. Other factors, including oxidative stress, as demonstrated by in vitro studies (Griffin et al., 2003; Gurr et al., 1998; Huang et al., 1993; Lynn et al., 2000; Wang et al., 2001), should still be considered as an important contributing factor. However, our study did demonstrate that histamine, although an important factor in the induction of vascular permeability change in many pathological conditions, was not one of the major contributing factors in arsenic-induced vascular permeability changes (leakage).

CONCLUSION

In sum, our present study provided a de novo concept on a mechanism for arsenic-induced vascular toxicity in vivo. This mechanistic path is a pathophysiologically based process involving a two-tiered interaction between the nervous system (sensory C-fibers) and the vascular system (endothelial cells). Therefore, it could only be explored with intact animals and remained undetected by traditional in vitro studies in the past. With the unique animal model that we have developed for studying arsenic-induced vascular changes (Chen et al., 2004b; Tsai et al., 2005), this important phenomenon and concept could then be tested, demonstrated, and confirmed. While we have unveiled a new mechanistic process involved in arsenic-induce vascular changes in vivo, this mechanism by no means is the only mechanism for arsenic vasculopathy. Arsenic toxicity is complex and the mechanism may be multifaceted. Other contributory factors such as oxidative stress and direct endothelial membrane injuries by arsenic probably exist. Nevertheless, we believe that our discovery has uncovered an important and probably a major mechanism for arsenic-induced vascular toxicity in vivo. While the precise molecular mechanism for arsenic in inducing the neurogenic inflammation process is still unclear and deserves further investigation, we believe that our discovery has important scientific significance and public health values. It is also our hope that our present report will inspire other research in therapeutic intervention on the neurogenic inflammation process induced by arsenic. Such intervention may be potentially beneficial in the reduction of vascular-related diseases for populations exposed to arsenic.

REFERENCES


