CHAPTER V

DISCUSSION

Arsenite is a human carcinogen, but the diverse acute physiological effects caused by arsenite are likely due to the activation of various signaling pathways rather than genotoxicity. We used immunofluorescence microscopy and laser scanning confocal microscopy to study the effects of sodium arsenite on the actin cytoskeleton, focal adhesion vinculin, and mitochondrial localization in mouse fibroblasts. At 5 μM, sodium arsenite caused lamellipodia (Rac activation) but 25 μM of sodium arsenite caused a severe loss of F-actin and vinculin and most cells detached from the substrate. Signaling by tyrosine phosphorylation plays an important role in regulation of the actin cytoskeleton and focal adhesion formation because co-treatment with the tyrosine kinase inhibitor genistein can block these toxic effects induced by sodium arsenite. Furthermore, we also found that epidermal growth factor receptor (EGFR) inhibitor 4,5-dianilophthalimide, phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, RNA synthesis inhibitor actinomycin D, protein synthesis inhibitor cycloheximdie and MAP kinase inhibitor apigenin completely blocked these toxic effects induced by sodium arsenite. At 25 μM sodium arsenite did not cause apoptosis in sodium arsenite-exposed mouse fibroblasts that was detected by *in situ* cell death detection kit. Apoptosis did not play an important role in these toxic effects induced by sodium arsenite. Sodium arsenite also caused cervical cancer cell morphology changes related to the reorganization of the actin cytoskeleton and focal adhesion vinculin.

The Rho GTPases (Rho, Rac and Cdc42) which are the downstream effectors of the protein tyrosine kinase signaling pathway can lead to actin reorganization and activation of mitogen signal transduction such as SAPK/JNK and p38 MAP kinase, and thus lead to long-term changes in gene expression. Zhang *et al.* (1995) also reported that p21-activated kinase (PAK) is a potential mediator of Rac/Cdc42 signaling. Rac and Cdc42 appears to regulate a protein kinase initiated at the level of PAK and leading to activation of SAPK/JNK and p38 MAP kinase. From immunoblot assay, the protein tyrosine kinase inhibitor genistein, EGFR inhibitor 4,5-dianilinophthalimide, PI3K

inhibitor wortmannin, Src-family tyrosine kinas inhibitor PP1, as well as RNA synthesis inhibitor actinomycin D and protein synthesis inhibitor cycloheximide can not block the SAPK/JNK, phospho-SAPK/JNK, p38 MAP kinase, and phospho-p38 MAP kinase and PAK expression induced by sodium arsenite. The Rac or Cdc42/PAK/SAPK/JNK and p38 MAP kinase signaling pathway may not play an important role in the actin cytoskeleton and vinculin disruption induced by sodium arsenite. Rho proteins have been proposed to participate as molecular switches in the control of PI3K (Zhang *et al.*, 1993), phosphatidylinositol-4-phosphate-5-kinase (Chong *et al.*, 1994), phospholipase D (Malcolm *et al.*, 1994), myosin phosphatase (Kimura *et al.*, 1996) and smooth muscle contraction (Hirata *et al.*, 1992), cell-cell contact (Tominaga *et al.*, 1993) and endocytosis (Schmalzing *et al.*, 1995). Furthermore, Rho subtype proteins may play a role in transcriptional activation (Olson *et al.*, 1995; Hill *et al.*, 1995), and in the transformation of cells induced by the oncogene product Ras (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). The ADP-ribosylation of Rho proteins at asparagine-41 leads to the inhibition of the interaction of Rho with its effectors. Alternatively, ADP-ribosylation may affect the activation of the Rho protein by guanine nucleotide exchange factors. This pattern is found in the *Clostridium botulinum* ADP-ribosyltransferase C3 reaction with Rho protein. Furthermore, C3 protein also inhibits LPA-stimulated stress fiber assembly and focal adhesion clustering. Lynn *et al.* (1998) reported that arsenite treatment may generate nitric oxide to damage DNA which then stimulates poly(ADP-ribosylation) because arsenite induced DNA strand breaks and NAD depletion in bovine aortic endothelial cells, and these could also be suppressed by S-methyl-L-thiocitrulline, the inhibitor of nitric oxide synthase.

The tyrosine phosphorylation of MLCK is linked to EGFR and Src. Actin filament inhibitor (cytochalasin D) or MLCK inhibitor can also cause round-shape cells (retracted form) (Suzuki and Takahashi, 2001; Kamm and Stull, 2001). The RhoA/Rho kinase signaling pathway, downstream effector of EGFR and GPCR, which regulates the actin cytoskeleton and focal adhesion formation may play a major role in these toxic effects induced by arsenite. The filamentous actin disruption induced by arsenite which was shown to be mediated by EGFR and PI3K may be working through Rho GTPases especially in the RhoA/Rho kinase/myosin light chain kinase (MLCK) system.

Rho kinase (ROCK), an effector molecule of RhoA, phosphorylates the myosin binding subunit (MBS) of myosin phosphatase and inhibits the phosphatase activity. This inhibition increases phosphorylation of myosin light chain (MLC) of myosin II, which is suggested to induce RhoA-mediated assembly of stress fibers and focal adhesions. Rho is also known to directly phosphorylate MLC in vitro; however, the physiological significance of this MLC kinase (MLCK) activity is unknown. Totsukawa *et al.* (2000) found that MLC phosphorylation was both necessary and sufficient for the assembly of stress fibers and focal adhesions in 3T3 fibroblasts. The assembly of stress fibers in the center of cells required Rho kinase activity in addition to the inhibition of myosin phosphatase, suggesting that Rho kinase not only inhibits myosin phosphatase but also phosphorylates MLC directly in the center of cells. At the cell periphery, on the other hand, MLCK but not Rho kinase appeared to be the kinase responsible for phosphorylating MLC. They suggested that Rho kinase and MLCK played distinct roles in spatial regulation of MLC phosphorylation. Katoh *et al.* (2001) also showed that there were at least two different stress fiber systems in the cell. The central stress fiber system was dependent more on the activity of Rho kinase than on that of MLCK, while the peripheral stress fiber system depended on MLCK.

It is widely accepted that actin filaments and the conventional double-headed myosin interact to generate force for many types of nonmuscle cell motility, and this interaction occurs when the MLC is phosphorylated by MLCK together with calmodulin and Ca^{2+}. However, recent studies indicate that Rho kinase is also involved in regulating the smooth muscle and nonmuscle cell contractility. More rapid and extensive stress fiber contraction was induced by MLCK than was by Rho kinase. When the activity of Rho kinase but not MLCK was inhibited, cells not only lost their stress fibers and focal adhesions but also appeared to lose cytoplasmic tension (Katoh *et al.*, 2001).

Besides being activated by ligand binding, the EGFR can be transactivated by a growing number of different pathways, including GPCRs, cytokine receptors, ion channels, integrin and other RTKs (Kalmes *et al.*, 2001). Simeonova *et al.* (2002) also reported that arsenite-induced EGFR phosphorylation was independent of autocrine EGF and did not involve the major autophosphorylation site, Tyr 1173. The EGFR

transactivation induced by arsenite and its relationship to the Rho A/Rho kinase signaling pathway are being investigated.

It is becoming clear that the Arp2/3 complex, a complex of seven proteins including the actin-related proteins Arp2 and Arp3, regulates the assembly of new actin filament networks at the leading edges of cells. Proteins of the WASP (Wiskott-Aldrich Syndrome Protein) family bind directly to the Arp2/3 complex and stimulate its ability to promote the nucleation of new actin filaments. Upstream of WASP-family proteins, receptor tyrosine kinases, the Rho-family GTPase Cdc42, and likely G protein-coupled receptors, receive and transmit the signals leading to WASP-Arp2/3 complex-mediated actin nucleation (Machesky and Insall, 1999).

The role of Arp2/3 complex in lamellipodia concerns how it becomes localized and activated at sites of new actin polymerization. A family of candidates has now been found, including WASP, its more widely expressed homologue N-WASP and a related protein group, the Scars. These proteins bind directly to the Arp2/3 complex and regulate its behavior in cells. This connection between a family of signaling proteins and the Arp2/3 complex suggests a pathway through which multiple signaling cascades could activate actin plymerization. WASP and N-WASP bind to receptor tyrosine kinases such as the PDGF and EGF receptors, via adapter molecules such as Nck and Grb2. WASP and N-WASP also bind to Cdc42 and are therefore implicated in actin cytoskeleton reorganization downstream of Cdc42. The regulation of actin filament network formation through Arp2/3 complex and WASP is also controlled by PI3K in cell polarity and chemotaxis (Machesky and Insall, 1999).

Dröge (2001) reported that there are various examples of growth factors, cytokines, or other ligands that trigger reactive oxygen species (ROS) production in nonphagocytic cells through their corresponding membrane receptors. Such ROS production can mediate a positive feedback effect on signal transduction from these receptors since intracellular signaling is often enhanced by ROS or by a pro-oxidative shift of the intracellular thiol/disulfide redox state. For example, the role of ROS has been demonstrated for nerve growth factor (NGF) signaling in neuronal cells, for epidermal growth factor (EGF) signaling in human epidermoid carcinoma cells, and for PDGF. Stimulation by any of these growth factors results in a transient increase in intracellular

ROS through the signaling protein Rac1. Elimination of hydrogen peroxide by catalase was shown to inhibit EGF- and NGF-induced tyrosine phosphorylation of various cellular proteins, including phosphorylation of the growth factor receptor itself. In the case of the EGF receptor, induction of ROS production was reported to require the kinase activity of the receptor but not the phosphorylation of the four autophosphorylation sites at the COOH terminus of the EGF receptor (Bae *et al.*, 1997).

In view of the many growth factors, cytokines, or other ligands that trigger endogenous production of ROS, there is a strong possibility that the redox dependency of the signal transduction process may facilitate synergistic interactions between different types of membrane receptors. This physiologically advantageous cooperatively between different receptors may have been a major driving force in the phylogenetic evolution of the rather complex mechanism of redox regulation. The enhancing effect of ROS on tyrosine phosphorylation and catalytic activation, as exemplified by the EGF receptor, applies in a similar manner to various other protein components of intracellular signaling pathways.

IL-6 is a pleiotropic cytokine that is produced by many different cell types and the main sources are macrophages, fibroblasts, and endothelial cells. IL-6 plays a role in a wide range of responses, such as immune responses, acute-phase reactions, and hematopoiesis. The major steps in IL-6-type cytokine signaling have been elucidated. It was found that these cytokines signal via the activation of Janus kinases (Jaks) and transcription factors of the STAT family (Heinrich *et al.*, 1998). In this study, we investigated the function of IL-6 production in cells exposed to sodium arsenite by *in vivo* study. We found that plasma IL-6 levels were decreased in sodium arsenite-exposed rats in a dose-dependent manner and this toxic effect was blocked by apigenin. Chakravortty and Nanda Kumar (2000) reported that the dynamic and orchestrated organization of the cytoskeletal filaments and actin assembly in response to LPS may be a prime requirement for the LPS induced increase in percentage of "S" phase cells and IL-6 synthesis. This suggests that sodium arsenite may disrupt the PI3K-MAP kinase signaling pathway that relates to the reorganization of actin cytoskeleton and actin assembly because this effect can be completely blocked by apigenin.

Proinflammatory cytokines such as IL-1, TNF, IL-6, and IL-8 are produced by leukocytes in response to bacteria or bacterial components. A great deal has been learned during the past few years about the synthesis and release of proinflammatory cytokines by leukocytes; however, relatively little is known about the intracellular events that lead to leukocyte proinflammatory cytokine gene transcription (Chen *et al.*, 2002).

It is proposed that arsenite may act through tyrosine kinase and through Rho A/Rho kinase/MLCK signaling pathways related to actin cytoskeleton. We presented evidence that apigenin can be used as an antidote for arsenite.

Proposed Mechanisms of Sodium Arsenite on Cell Signaling and Actin Cytoskeleton