Differential effects of arsenic on cutaneous and systemic immunity: focusing on CD4+ cell apoptosis in patients with arsenic-induced Bowen’s disease

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Bowen’s disease (BD), a carcinoma in situ of the skin, has been identified as an early lesion in arsenic carcinogenesis. Patients with arsenic-induced Bowen’s disease (As-BD) showed both cutaneous and systemic immune dysfunctions. We set out to evaluate the interactions between keratinocytes and lymphocytes in the context of As-BD carcinogenesis. Our results showed that As-BD lesions demonstrated a significant dermal CD4+ cell, an essential regulator of proper tumor immunity, undergoing apoptosis. In addition, it was found that the As-BD patients have lower percentage of peripheral CD4+ cells as compared with control subjects. However, the CD4+ cells from As-BD patients were less susceptible to arsenic-induced apoptosis, due to reduced tumor necrosis factor receptor 1 expression. Interestingly, arsenic was found to induce Fas expression on CD4+ cells and increase the soluble Fas ligand (sFasL) production from keratinocytes. This sFasL-containing keratinocyte supernatant was able to induce comparable CD4+ cell apoptosis for both patients and controls. Using immunofluorescent staining, increased FasL was observed in keratinocytes of As-BD lesions and Fas was expressed among infiltrating CD4+ cells. Our findings suggested that systemically, the percentage of CD4+ cells was decreased in the peripheral blood of As-BD patients. These residual CD4+ cells were less susceptible to arsenic-induced apoptosis. However, once infiltrated into the As-BD lesions, the selective CD4+ cell apoptosis might be mediated by FasL from keratinocytes. This additional tumor-immune phenomenon present in the cutaneous environment provides a reasonable explanation for frequent occurrence of arsenic cancers in the skin.

Materials and methods

Human subjects

Patients from arsenic endemic areas in the southwest coast of Taiwan with biopsy-proven As-BD were included in this study. Twelve tissue specimens of As-BD, non-arsenical BD and psoriasis vulgaris were retrieved from pathology archive of Kaohsiung Medical University Hospital. Blood specimens were obtained from 12 gender-matched As-BD patients (63.4 ± 12.2 years old) and controls (60.0 ± 10.3 years old) with age within 5 years proximity. All experiments performed in this study were approved by Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-960119).

Immunohistochemical and immunofluorescent staining

Paraffin-embedded 5 μm sections were obtained from As-BD, non-arsenical BD, psoriasis vulgaris patients and normal skin. Following deparaffinization and rehydration, the slides were autoclaved in 10 mM citrate buffer for 20 min for antigen retrieval.

For immunohistochemical staining (21), endogenous peroxidase was blocked with 3% H2O2 for 5 min. Antibody against FasL (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated for 3 h at room temperature. The slides were washed with phosphate-buffered saline (PBS) and incubated with biotinylated link antibody (DAKO, Glostrup, Denmark) for 30 min at room temperature. Diaminobenzidine served as the chromogenic detection reagent. Counterstain was performed by Mayer’s hematoxylin staining for 1 min. Immunofluorescent slides were mounted with Entellan (Merck, Darmstadt, Germany) for microscopic observation. For immunofluorescent staining (22), the sections were stained with phycoerythrin (PE)-conjugated antibodies against CD4 or CD8 (BD Pharmingen, San Diego, CA) at room temperature. The slides were washed 3 times with PBS for 5 min. Antigen retrieval was performed by antigen retrieval solution (DAKO, Glostrup, Denmark) at 70°C for 30 min, followed by 3% H2O2 for 5 min. The sections were then blocked with 1% BSA in PBS for 30 min at room temperature and incubated with PE-conjugated antibodies (anti-CD4 and anti-CD8) for 1 h at room temperature. The final slides were mounted with fluorescent mounting medium (DAKO, Glostrup, Denmark), and the images were obtained using a confocal laser scanning microscope (Zeiss LSM710, Germany).

Abbreviations: AP-1, activator protein 1; As-BD, arsenic-induced Bowen’s disease; BD, Bowen’s disease; FITC, fluorescein isothiocyanate; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; sFasL, soluble Fas ligand; TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRADD, tumor necrosis factor receptor-associated death domain.
San Diego, CA). After CD4 or CD8 staining, the section slides were washed with PBS and stained with fluorescein isothiocyanate (FITC)-fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reagent (Roche, Penzberg, Germany) according to the manufacturer’s instructions. These double-stained sections were then mounted and observed under a fluorescence microscope or confocal laser scanning microscope (LSM Fluoview 500, Olympus, Tokyo, Japan). The percentage of apoptotic cells was averaged by counting 100 cells under $\times 200$ fields.

Cell culture and arsenic treatment

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood after centrifugation at 2000 r.p.m. for 20 min over a Ficol-Hypaque cushion (specific gravity 1.077) (23). Freshly obtained PBMCs (1.0 $\times$ 10$^6$ cells/ml) were suspended in RPMI-1640 medium (Invitrogen–Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen–Gibco). Keratinocyte culture was based on the method by Liu et al. (24) with modifications. We isolated the keratinocytes from normal human foreskin. Freshly obtained foreskin specimens were washed with PBS and cut into 5 $\times$ 5 mm pieces. These tissue pieces were then put into a 10 U/ml dispase solution (Invitrogen–Gibco) and incubated for 30 min at 37°C. After incubation, the epidermal layer of human keratinocytes was lifted from the dermis and placed into a 0.05% trypsin–ethylenediaminetetraacetic acid solution (Invitrogen–Gibco) for 15 min at 37°C. A 60 min reaction at 37°C of recombinant epidermal growth factor. Isolated keratinocytes were cultured at 37°C in a humidified incubator with 5% CO$_2$ atmosphere. The third passage of keratinocytes was used in our experiments.

DNA ladder testing for apoptosis

The DNA ladder testing was assessed by agarose gel electrophoresis according to the method by Enari et al. (25) with a slight modification. Cells (2 $\times$ 10$^6$ cells) were collected and suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM ethylenediaminetetraacetic acid, 1.2% sodium dodecyl sulfate, 150 mM NaCl and 0.2 mg/ml proteinase K), followed by incubation at 37°C over night. Cellular DNA was isolated by phenol extraction. The DNA samples were carefully loaded into the wells of a 2% agarose gel. Electrophoresis was carried out in Tris-acetate-EDTA buffer at 50 V for 1 h and the DNA was visualized by ethidium bromide staining.

Measurement of TNF-$\alpha$ and soluble Fas ligand by enzyme-linked immunosorbent assay

After 48 h of arsenic treatment, cell-free supernatant of cultured PBMCs and keratinocytes were obtained for TNF-$\alpha$ and soluble Fas ligand (sFasL) measurement, respectively, using commercially available enzyme-linked immunosorbent assay kits (Quantikine, R&D System, Minneapolis, MN) according to the manufacturer’s instructions. TNF-$\alpha$ and sFasL concentrations were calculated based on linear regression standard curves in which $R^2$ were $>0.99$. The detection ranges were 1.6–1000 pg/ml and 2.7–1000 pg/ml for TNF-$\alpha$ and sFasL, enzyme-linked immunosorbent assay kits, respectively.

Flow cytometric analysis of apoptosis and TNF-R1 expression in PBMC subpopulations

Apoptotic PBMC subpopulations (CD4$^+$ or CD8$^+$) were detected by flow cytometry (26). Cells were stained with PE-conjugated antibodies against CD4 or CD8 (BD Pharmingen) and fixed by 4% paraformaldehyde. A commercially available TUNEL kit (Roche) was then used to detect apoptotic cells. After washing twice with PBS, the CD4- or CD8-stained cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Permeabilized cells were washed with PBS and then incubated with 50 $\mu$l of TUNEL reaction mixture (contains terminal deoxynucleotidyl transferase and label solution provided by the kit). Label solution without terminal deoxynucleotidyl transferase was used as negative control. A 60 min reaction at 37°C was required to label the DNA strand breaks with FITC. The percentages of TUNEL-positive cells among CD4 or CD8 subpopulations were analyzed by flow cytometry. The discriminator on the cytometer was set by FITC fluorescence of the negative control, fluorescence higher than negative control was taken as TUNEL positive.

The flow cytometric method was also used for determination of TNF-R1 expression on CD4$^+$ cells. PBMCs were collected and costained with monoclonal anti-TNF-R1 antibody conjugated with PE (Caltag, Burlingame, CA) and CD4-FITC. The double-stained PBMCs were washed with PBS, fixed with 4% paraformaldehyde (in PBS) and analyzed by flow cytometry. The
discriminator on the cytometer was set at 16 on FITC fluorescence of isotype control and 11 on PE fluorescence of isotype control. Fluorescence higher than isotype control was taken as positive in the present study.

Western blotting analysis of apoptosis-associated proteins

For western blotting of tumor necrosis factor receptor-associated death domain (TRADD) caspase-8 and caspase-3 (27), total cellular protein extract from PBMCs was obtained by lysing the cells in ice-cold lysis buffer (50 mM tris, 5 mM ethylenediaminetetraacetic acid, 0.1% triton X-100, 150 mM NaCl and mixed cocktail protease inhibitors). After 12 000 r.p.m. centrifugation, the supernatants were collected and the protein concentrations were measured by protein quantification kit (Bio-Rad, Hercules, CA). Samples containing 40 μg protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (using 12.5% acrylamide gels) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked in 2% skim milk/PBS with 0.5% Tween-20 for 1h at room temperature. Antibodies against human TRADD, activated caspase-8 and activated caspase-3 (Santa Cruz Biotechnology) were used at a dilution of 1:500. The chemiluminescence substrate kit (Pierce, Rockford, IL) was used to visualize the presence of specific proteins in the polyvinylidene difluoride membrane.

Blocking tests

For FasL blocking (28), PBMCs (1 × 10⁶ cells/ml) were incubated with 1 μg/ml of anti-FasL neutralization antibody (Clone NOK-1; BD PharMingen). These cells were then cultured for 48 h at 37°C without removing the neutralization antibody. Treated cells were double stained with CD4 and TUNEL for apoptotic cell detection by flow cytometry.

Statistical analysis

All values were presented as mean ± SD. Non-parametric statistical tests were used in this study. The comparisons between two groups were assessed by the Mann–Whitney test, and the comparisons among multiple groups were assessed by Kruskal–Wallis test followed by Dunn’s post-test as appropriate. A P-value of <0.05 was considered as statistically significant.

Results

Apoptotic CD4⁺ cells were observed in As-BD lesion

Cell apoptosis was observed in the epidermis (dyskeratotic cells) and dermis (infiltration cells) of As-BD lesions (Figure 1a). The dermal apoptotic cells were positively stained with CD4⁺ (Figure 1b), but not CD8⁺ cells in serial sections (Figure 1c). This CD4⁺ specific apoptosis was mostly observed in As-BD specimens, but less in non-arsenical BD and psoriasis vulgaris. As-BD showed 87.3 ± 7.8% TUNEL copositive rate in total CD4⁺ cells, non-arsenical BD showed 12.7 ± 8.6% and in psoriasis vulgaris, the copositive rate was 5.3 ± 3.2%. The CD4/TUNEL copositive rates in non-arsenical BD and psoriasis vulgaris were significantly less than that in As-BD (see supplementary Figure S1 available at Carcinogenesis Online).

Peripheral CD4⁺ cells from As-BD patients were less susceptible to arsenic-induced apoptosis

We isolated peripheral PBMCs from healthy donors (controls) and As-BD patients. The percentages of peripheral CD4⁺ cell population, but not CD8⁺ cell population, were significantly less in As-BD patients (22.7%) as compared with controls (40.5%) (Figure 2a). Arsenic-induced apoptosis was then studied. Apoptotic DNA laddering was observed in control PBMCs with 5 μM arsenic treatment,
but not in patients’ PBMCs after the same dosage of arsenic treatment (Figure 2b). We further found that 5 μM of arsenic induced significant CD4+ cell apoptosis (Figure 2c), but not CD8+ cell apoptosis (Figure 2d), for both control and As-BD PBMCs. Unexpectedly, the percentage of apoptotic CD4+ cells in the As-BD group (7.2%) was significantly lower than control group (13.2%) (Figure 2c).

**PBMCs from As-BD patients showed significantly less TNF-α release and TNF-R1 expression compared with normal controls**

To explore the mechanisms involved in CD4 apoptosis, the TNF-R1-related pathway was studied. Arsenic (1 and 5 μM) stimulated TNF-α release from both the control and the patients’ PBMCs. However, the levels of arsenic-induced TNF-α release from the patients’ PBMCs were significantly lower than that from the control PBMCs (Figure 3a). Furthermore, 5 μM of arsenic significantly enhanced the TNF-R1 expression on control CD4+ cells, but only marginally on patients’ CD4+ cells (Figure 3b). Corroborating with these results, the expression of TRADD protein was enhanced by 5 μM arsenic treatment in the PBMCs from both the normal donors and As-BD patients. The expressions of activated caspase-8 and caspase-3, the TNF-R1 downstream apoptosis proteins, were also enhanced by 5 μM arsenic treatment. However, the arsenic-induced levels of caspase-8 and caspase-3 were less in As-BD patients as compared with normal controls (Figure 3c). Therefore, arsenic was able to induce CD4+ cell apoptosis, but the CD4+ cells from As-BD patients showed less susceptibility to arsenic-induced apoptosis as compared with controls.

**Fig. 3.** A significant decrease in TNF-α release and TNF-R1 expression in arsenic-treated PBMCs form patients as compared with normal controls. (a) After 48 h exposure to 0, 1 and 5 μM of arsenic, the supernatants (per 10^6 cells) of PBMCs were collected and the concentrations of TNF-α were measured by enzyme-linked immunosorbent assay (mean ± SD, n = 12). *P < 0.05, arsenic-treated group versus non-treated group by Kruskal–Wallis test. (b) Arsenic-treated PBMCs were double stained with TNF-R1-PE (y-axis) and CD4-FITC (x-axis). The percentages of TNF-R1+ cells in CD4+ cells from controls and patients were analyzed by flow cytometry. (c) Total cell lysates were obtained from 0, 1 and 5 μM arsenic-treated PBMCs. The expressions of TRADD, caspase-8 and caspase-3 were detected by western blotting (n = 6).
sFasL enhanced CD4⁺ cells from As-BD patients to arsenic-induced apoptosis. (a) Fas and FasL expression on CD4⁺ cells from normal controls. (b) Fas and FasL expression on CD4⁺ cells from As-BD patients. (c) Cultured human keratinocytes were treated with 0.1, 1, 5 or 10 μM of arsenic. After 48 h incubation, the supernatants of keratinocytes were collected. The concentration of sFasL was detected by enzyme-linked immunosorbent assay (mean ± SD, n = 12). *P < 0.05, treated group versus non-treated group by Kruskal–Wallis test. (d) Control or As-BD patient’s PBMCs were cultured with 5 μM arsenic, 5 μM arsenic-treated keratinocyte supernatant (As-KC supernatant) or As-KC supernatant with anti-FasL neutralizing antibody. After 48 h treatment, the percentages of apoptotic CD4⁺ cells were measured by flow cytometry (mean ± SD, n = 12). *P < 0.05, As-KC supernatant-treated groups versus arsenic only or +anti-FasL groups by Mann–Whitney test.
sFasL from arsenic-treated keratinocytes enhanced arsenic-induced CD4+ cell apoptosis from both As-BD patients and normal controls

To explore the interactions between CD4+ cell apoptosis and keratinocytes, the effects of arsenic on cultured cells in terms of Fas/FasL axis was studied. Arsenic induced Fas, but not FasL, expression on peripheral CD4+ cells from normal controls (Figure 4a) and As-BD (Figure 4b). On the other hand, arsenic (1, 5 and 10 μM) was able to stimulate, in a dose-dependent manner, sFasL production from cultured keratinocytes (Figure 4c). Supernatant from 5 μM arsenic-treated keratinocyte (As-KC supernatant) significantly increased the percentage of apoptotic CD4+ cells from both controls and As-BD patients (Figure 4d). However, while As-BD CD4+ cells were less susceptible to apoptosis than control CD4+ cell under direct arsenic treatment, the CD4+ cells from As-BD patients showed similar susceptibility as control after As-KC supernatant treatment. Anti-FasL neutralizing antibody reduced CD4+ cell apoptosis induced by As-KC supernatant from normal controls and patients to the level similar to direct arsenic treatment (Figure 4d).

**Discussion**

As-BD patients are characterized by dysfunctional systemic and cutaneous immunity. In the present study, we demonstrated that patients...
with As-BD have lower percentage of CD4+ cells in the peripheral blood as compared with control subjects. In addition, the peripheral CD4+ cells from As-BD patients were found to be less susceptible to the arsenic-induced apoptosis as compared with control subjects. Mechanistically, we showed that the peripheral CD4+ cells from As-BD patients were less susceptible to TNF-α/TNF-R1 apoptotic signaling as demonstrated by significantly lower TNF-α concentration, TNF-R1 expression and lower levels of TNF-R1 downstream apoptotic protein (including TRADD, caspase-3 and caspase-8) expression after treatment with arsenic. Other possible explanations for arsenic tolerance of CD4+ cells include multidrug resistance protein expressions and immunosenescence under chronic arsenic exposure (29,30).

Paradoxically, in the skin of As-BD, the dermal CD4+ cells were remarkably vulnerable to apoptosis. These results indicated that in As-BD patients, there appeared to be a differential cutaneous and systemic immune dysfunction, particularly in regard to CD4+ cell apoptosis. It is probably that alternative apoptotic pathways are activated in CD4+ cells infiltrating As-BD lesions. As epidermal keratinocytes are in proximity to dermal lymphocytes, apoptosis of dermal lymphocytes may be mediated through keratinocytes. Arsenic treatment was found to induce Fas, but not FasL, expression on CD4+ cells. In addition, arsenic dose-dependently stimulated sFasL release from cultured keratinocytes. Appropriate blocking tests indicated that the sFasL from arsenic-treated keratinocytes significantly enhances arsenic-treated CD4+ cell apoptosis from As-BD patients. To examine the keratinocyte/CD4+ cell interaction in vivo, immunohistochemical staining of As-BD lesions was performed. Our results showed increased Fas expressions on dermal (infiltrating) CD4+ cells and enhanced FasL expression on epidermal (basal) keratinocytes in As-BD lesions, corroborating with the CD4+ cell apoptosis via Fas/ FasL seen in culture system. These results suggested that arsenic might induce selective CD4+ cell apoptosis via increased FasL expression from keratinocytes in As-BD.

Arsenic has been shown to activate the activator protein 1 (AP-1) transcription factor via blocking the Jun N-terminal kinase phosphatase activity (31). AP-1 is a critical factor in regulating FasL expression since the promoter region of human FasL gene contains AP-1-binding site (32,33). We have previously reported that arsenic-induced AP-1 activation is associated with increased FasL expression on human keratinocytes (34). Therefore, the increased FasL expression by keratinocytes in As-BD lesions might generate a proapoptotic microenvironment to the infiltrating lymphocytes. In other words, an ‘immune escape’ effect might be present in the As-BD lesions via Fas/ FasL pathway (35–37). Immune escape is considered to be a common event in cancers. Most immune privilege effects were found to be the result of altered gene expression or biological changes in cancer cells (38,39). Indeed, FasL-associated tumor-anti-immune effects have been reported in different tumors, including colon cancers (38), ovarian carcinoma (40), hepatoma (41), breast cancer and head and neck malignancies (39,42). In our present study, we unveiled that arsenic-induced FasL-associated tumor-anti-immune phenomenon is also present at As-BD lesions between keratinocytes and CD4+ cell. Despite the observation that the peripheral CD4+ cells were less susceptible to direct arsenic-induced apoptosis, this FasL-mediated signal from keratinocytes rendered the CD4+ cells around the As-BD lesions more vulnerable to apoptosis (Figure 6). Since CD4+ cells are the central immune regulator in cancer immunology (43), we believe that the aforementioned immune escape mechanism contributed to the multiple and recrudescent characteristics of As-BD.

Differential effects of arsenic on systemic (TNF-R1-mediated) and cutaneous (Fas-mediated) CD4+ cell death were explored in the present study. Both TNF-R1 and Fas are the death receptors transducing signals that promote cell death by apoptosis. However, there are differences between these two pathways. TNF-R1 regulates both cell activation and death, whereas Fas specifically provide death signals. Ligand-activated TNF-R1 recruits TRADD as a mediator for both cell survival and apoptosis. TRADD may recruit TNF receptor-associated protein-2 to promote cell survival, while TRADD may also recruit Fas-associated protein with death domain to enhance apoptosis. In contrast, Fas directly interacts with Fas-associated protein with death domain and subsequently results in apoptosis (44,45). The differential activation of these pathways may provide explanation to the differential effects of arsenic on systemic and cutaneous CD4+ cell death, as
well as the organ specificity of arsenic carcinogenesis. While only TNF-R1 signaling was activated systemically, both TNF-R1 and Fas well as the organ specificity of arsenic carcinogenesis. While only TNF-R1 and Fas signaling were specifically detected in As-BD lesions, but not in non-arsenical skin. From previous studies, it has been shown that skin is the main target organ for the development of cancer in patients with chronic arsenism. We propose that this additional tumor-anti-immune phenomenon present in the cutaneous environment may contribute to this observation.

In summary, the present study demonstrated a selective immune suppression in As-BD. It is possible that these phenomena are characteristic of BD itself, but not directly linked to arsenic exposure. However, we found that higher amount of apoptotic CD4+ cells was specifically detected in As-BD lesions, but not in non-arsenical BD or psoriasis vulgaris specimens, indicating the correlations between arsenic exposure and CD4+ cell dysfunction. Systemically, arsenic induced a selective immune suppression of CD4+ cell via TNF-α/TNF-R1 pathway, resulting in decreased CD4+ cell percentage in peripheral blood. Paradoxically, this direct effect was less prominent in As-BD patients. Decreased sensitivity to arsenic in regard to TNF-α/TNF-R1 expression after long-term arsenic exposure may explain this phenomenon. Organ specifically, arsenic might induce cutaneous immune escape by mediating selective CD4+ cell apoptosis via increased FasL expression from keratinocytes in As-BD, contributing to its clinical recalcitrant characteristics.

Since As-BD lesions represent early carcinogenic state in human skin, our results provide a scientific basis for understanding the immune escape responses induced by arsenic during the early stage of chemical carcinogenesis.

Supplementary material
Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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