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The TNF-α 238A polymorphism is associated with susceptibility to persistent bone marrow dysplasia following chronic exposure to benzene

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Abstract

Chronic exposure to benzene can result in transient hematotoxicity (benzene poisoning, BP) or persistent bone marrow pathology including dysplasia and/or acute myeloid leukemia. We recently described a persistent bone marrow dysplasia with unique dysplastic and inflammatory features developing in individuals previously exposed to benzene (BID) [Irons RD, Lv L, Gross SA, Ye X, Bao L, Wang XQ, et al. Chronic exposure to benzene results in a unique form of dysplasia. Leuk Res 2005;29:1371–80]. In this study we investigated the association of single nucleotide polymorphisms (SNP) (−863 (C → A), −857 (C → T), −308 (G → A), −238 (G → A)) in the promoter region of the cytokine, tumor necrosis factor-alpha (TNF-α) on the development of BP, persistent BID and *de novo* myelodysplastic syndrome (MDS) in 394 individuals. Only the −238 (G → A) polymorphism was significantly associated with the development of BID (odds ratio (OR) = 7.4; 95% C.I. 1.23–44.7) and was specific for BID and not *de novo* MDS or BP. These findings are consistent with a role for inflammation in the development of BID and suggest that cell-specific alterations in TNF-α expression may promote clonal selection in the evolution of neoplastic hematopoietic disease.

Keywords: Benzene poisoning; TNF-α promoter gene polymorphisms; Leukemogenesis; Clonal selection; Genetic variation; Inflammation; Epidemiology; Toxins/drugs/xenobiotics

1. Introduction

Benzene is a widely known hematotoxic agent. However, the severity of hematotoxicity associated with chronic exposure to benzene varies widely. Individuals undergoing comparable exposure may demonstrate no significant hematologic abnormalities while others exhibit signs of benzene poisoning (BP) or even bone marrow failure. Some individ-

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uals with BP will undergo complete recovery after cessation of benzene exposure while others will go on to develop bone marrow dysplasia that persists for years after exposure has ended and/or develop acute myeloid leukemia (AML). We recently described a unique form of persistent bone marrow dysplasia developing in patients previously exposed to benzene (BID), which is characterized by severe inflammatory changes, including eosinophilic progenitor dysplasia and hematophagocytosis as well as dyserythropoiesis, dysgranulopoiesis and stromal degeneration [1]. In some individuals the dysplastic changes observed in BID are consistent with a defined subtype of myelodysplastic syndrome (MDS), predominantly refractory cytopenia with multiliineage dysplasia (RCMD). The pathology in BID also is frequently accompanied by lymphocyte activation, and prominent clonal proliferation of regulatory T lymphocyte subsets in the bone marrow of affected individuals. The underlying mechanisms that explain these inflammatory changes in the bone marrow microenvironment remain unknown. However, these findings appear to predate the development of clonal structural cytogenetic abnormalities frequently observed in MDS, and present as a unique diagnostic feature that distinguishes BID from the majority of cases of *de novo* MDS [1].

The cytokine, tumor necrosis factor-alpha (TNF-α), plays a pivotal role in inflammation, immunity, hematopoiesis and apoptosis [2,3]. Altered production of TNF-α has been implicated in the development and severity of several diseases including rheumatoid arthritis, psoriasis, congenital and acquired aplastic anemia [4,5], MDS [2,3], viral and bacterial infections and cancer [6]. Also, TNF-α recently has been implicated as playing an important role in mediating the regulatory activity and survival of CD34+ hematopoietic progenitor cells in HLA-mismatched bone marrow transplantation [7,8]. TNF-α production in human cells is influenced by genetic polymorphisms, several of which have been implicated in the development of disease [9–11]. However, neither the mechanisms whereby these polymorphisms influence TNF-α gene expression and transcription, nor their role in the pathogenesis of disease or hematopoietic stem cell biology is completely understood. The TNF-α gene is located within the highly polymorphic major histocompatibility complex (MHC) class III region on chromosome 6p21.3. It contains at least nine single nucleotide polymorphisms (SNP), including four located within the promoter region that have been extensively studied (occurring at positions relative to the transcription start site) −308 (G → A), −238 (G → A), −863 (C → A), −857 (C → T) [10]. The −308A haplotype is associated with increased production of TNF-α, is linked to erosive joint disease in rheumatoid arthritis and an increase in the severity of certain infections, such as malaria and leishmaniasis [11]. The −238A haplotype is not linked with increased TNF-α production but is associated with derepression of TNF-α expression in vitro in some, but not all cell lines studied [12]. The −238A haplotype also has been implicated in the severity of infections (*e.g.* hepatitis B, tuberculosis, malarial anemia) [13] and psoriasis [14]. The −863A allele is variously associated with increased TNF-α production as well as decreased serum TNF-α concentrations in healthy males [15] and also has been implicated in erosive joint disease in rheumatoid arthritis [16]. The −857T allele alternatively confers an increased risk of gastric ulcer and a decreased risk of lymphoma associated with *Helicobacter pylori* infection [17,18].

The association between inflammation and cancer development is well-established, and evidence has emerged to suggest that bone marrow failure in MDS may involve activation of immune cells that target antigens in the hematopoietic microenvironment accompanied by an increase in pro-inflammatory cytokines, including TNF-α [19–22]. Recent studies in our laboratory also demonstrated that the benzene metabolite, hydroquinone, sensitizes human bone marrow progenitor cells to TNF-α-induced apoptosis via a mechanism that involves inhibition of nuclear factor-κB (NF-κB) [23]. These observations, taken together with the prominent inflammatory changes described in BID, led us to investigate the potential role of TNF-α in the pathogenesis of benzene-induced hematotoxicity. We investigated the frequency of TNF-α genetic polymorphisms in individuals with current and previous chronic exposure to benzene and correlated them with evidence of hematotoxicity occurring during benzene exposure (BP) as well as the development of dysplasia in individuals previously but not currently exposed to benzene (BID).

2. Materials and methods

2.1. Subjects

Three hundred and ninety-four cases and controls, ≥18 years of age, were enrolled from two sources: (1) in-patients recruited from Shanghai hospitals, and (2) benzene-exposed workers recruited from factories involving the rubber, petrochemical, asbestos manufacturing and painting industries. Informed consent was obtained according to the Declaration of Helsinki, 2004 and the NIH Common Rule (45CFR46), and together with the protocol, were approved by the Combined Multiple Institutional Review Board of the University of Colorado at Denver and Health Sciences Center and the Internal Review Board at Fudan University in Shanghai, China.

Three different case groups were defined: (a) patients diagnosed with MDS ($N=95$), (b) workers diagnosed with bone marrow dysplasia persisting after previous chronic exposure to benzene (BID) ($N=23$) [1], and (c) workers diagnosed with benzene poisoning (BP) ($N=46$). Control groups included: (a) healthy workplace-based subjects ($N=141$) recruited from the same factories as BP and BID cases, and (b) hospital-based patients diagnosed with non-hematopoietic diseases ($N=89$). Information on age, gender, smoking and alcohol use was obtained through personal interviews when feasible, and subsequently accounted for

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in statistical analyses when indicated. Criteria used to diagnose BP included: (a) a total WBC count <4000 ($\times 10^6$ L^{-1}), or WBC count 4000–5000 ($\times 10^6$ L^{-1}) and a platelet count <80,000 ($\times 10^6$ L^{-1}), or an absolute neutrophil count <2000 ($\times 10^6$ L^{-1}), (b) employment in a factory with documented benzene exposure for at least 6 months and (c) exclusion of other causes for abnormal blood counts. Hospital-based cases of MDS and BID were diagnosed in our laboratory based on evaluation of peripheral blood and bone marrow using morphologic, immunohistochemical, flow cytometric, cytogenetic, and molecular genetic techniques as previously described [1,24]. Workplace controls were also required to have at least 6 months employment in a factory using benzene in order to maintain comparability with cases. Patients with a history or symptoms associated with the following conditions were excluded from both case and control groups: infectious disease, concomitant chronic disorders (*e.g.* renal failure, arteritis, autoimmune disease, diabetes or cancer) or hematological abnormalities due to nutritional deficiencies or other causes. In addition, subjects were excluded if they had been treated for tuberculosis or used any of the following medications within the previous 5 years: chloramphenicol, sulfonamides, meprobamate, phenatoin, colchicine, cyclophosphamide, propylthiouracil, tolbutamid, primaquine and Chinese traditional herbs (*e.g.* Bezoar, Angelica, arsenic or Thunder cloud vine).

2.2. Benzene exposure assessment

The exposure assessment employed several approaches according to how the subjects were recruited to the study and available work history details. Hospital based MDS subjects had a work history questionnaire administered by trained interviewers. Expert review of questionnaires suggested low to no benzene exposure potential and further evaluation was not warranted. Many of the BP and BID subjects and benzene exposed worker controls worked in the same facilities. Therefore, exposure assessment for these groups used the same approach. Occupational exposure to benzene was evaluated by review of factory industrial hygiene monitoring records, review of the Chinese literature on benzene exposed industries and jobs, Chinese regulatory authority benzene exposure databases and by current quantitative industrial hygiene surveys, including personal benzene exposure samples and breathing zone/area sample analyses. Based on current measurements, 210 subjects had recent full-shift time weighted air concentration exposures ranging from 0.26 mg/m^3 (0.08 parts per million (ppm)) to 353.5 mg/m^3 (110 ppm). The exposures summarized in Table 1 include retrospective as well as current exposure. The retrospective exposure estimates are based on current data, historic data, and consideration of ventilation, material composition and other exposure modifying changes in the factories. Expert judgment was used more extensively with available historic data to assess exposures for three of the BID subjects. A total of 193 subjects had ongoing exposure during our evaluation, while the majority of BID subjects were removed from exposure an average of 2.7 years prior to diagnosis and exposure evaluation. Since many of the BID and BP/worker control subjects worked in the same facilities, differences in the mean exposures between BID and BP/worker controls largely reflect changes in the working environment over the past 3–5 years. The varying exposure periods of time for all subjects ranged from 2 to 37 years.

Cumulative exposure and long term average exposure was usually treated as a continuous variable, but was also categorized. Categories of cumulative exposure (in mg/m^3 year) were: <175, 175 to <500, 500 to <1183, 1183 to <4330, and

Table 1
Distribution of demographic, lifestyle, exposure and polymorphisms among study groups

Characteristic	MDS, $N=95$	BID, $N=23$	BP, $N=46$	Worker controls, $N=141$	Hospital controls, $N=89$
Gender					
Male	51 (53.7%)	8 (35%)	24 (52%)	84 (60%)	53 (60%)
Female	44 (46.3%)	15 (65%)	22 (48%)	57 (40%)	36 (40%)
Smoking[a] (% ever smoked)	73 (81%)	17 (74%)	40 (87%)	91 (65%)	57 (64%)
Alcohol (% current use)	72 (81%)	21 (91%)	37 (80%)	94 (67%)	74 (83%)
Age	54.9 ± 17	45.5 ± 7.8	42.7 ± 6.3	43.4 ± 7.4	44.5 ± 18.7
(year, mean ± S.D.) (range)	19–87	34–59	29–53	25–59	17–90
Benzene: long term average exposure (mg/m^3) (mean, range) [mean ppm]	NA	977 (49–1436) [305]	395 (2.5–1675) [123]	207 (1.7–1280) [65]	NA
Benzene cumulative exposure (mg/m^3 year) (mean, range) [mean ppm year]	NA	14,300 (728–28,600) [4469]	5826 (10.8–44,400) [1821]	2870 (2.3–30,700) [897]	NA
Cumulative exposure category 3+ (%) (see text)	NA	100%	48%	43%	NA
Long-term average category 3+ (%) (see text)	NA	100%	65%	50%	NA
−238 N (% AG + AA)	2 (2.1%)	5 (21.7%)	3 (6.5%)	6 (4.3%)	5 (5.6%) (1 ND)
−308 N (% AG+AA)	10 (10.5%)	3 (13.0%)	10 (21.7%)	20 (14.2%)	14 (15.7%) (2 ND)
−857 N (% CT + TT)	ND	3 (13.0%) (3 ND)	6 (13.0%)	24 (17.0%) (9 ND)	20 (22.5%) (1 ND)
−863 N (% AC + AA)	ND	7 (30.4%) (3 ND)	6 (13.0%) (9 ND)	34 (24.1%)	25 (28.1%)

[a] MDS: five unknowns; BID: two unknowns; BP: two unknowns; ND: not determined

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4330+. For long term average, categories (in mg/m^3) were <10, 10 to <40, 40 to <100, 100 to <1000, and 1000+. Category cut-points exploited natural breaks in the distribution of exposure in control subjects. For example, for cumulative exposure, six control subjects had cumulative exposures between 1000 and 1182 mg/m^3 year, while the worker with the next highest exposure had a value of 1581 mg/m^3 year. Thus, the value of 1182 formed a natural boundary in the distribution of cumulative exposure.

2.3. Detection of polymorphic variants of the TNF-α gene

Genomic DNA was isolated from blood using A Qiagen QIAmp DNA mini Kit (Chatsworth, CA) according to the manufacturer's directions. Polymorphisms of the TNF-α gene were determined using Restriction Fragment Length Polymorphisms (RFLP). PCR primers used for amplification of the fragments with the −238 G→A variant were 5′AAACAGACCACAGACCTGGTC3′ and 5′CTCACACTCCCCATCCTCCCGGATC3′, the −308 G→A polymorphism, 5′GAGGCAATAGGTTTTGAGGGCCAT3′ and 5′GGGACACACAAGCATCAAG3′, the −857 C→T polymorphism, 5′GGCTCTGAGGAATGGGTTAC3′ and 5′CCTCTACATGGCCCTGTCTAC3′, and the −863 C→A polymorphism, 5′GGCTCTGAGGAATGGGTTAC3′ and 5′CTACATGGCCCTGTCTTCGTTACG3′.

Conditions for PCR were an initial denaturation at 94 °C for 3 min, and then 35 cycles of 94 °C for 30 s, 59 °C for 1 min and a final extension at 72 °C for 2 min. Restriction enzymes, *Bam*HI and *Nco*I, were used in digestions of PCR fragments containing the polymorphisms −238 G/A, A/A and −308 G/A, A/A, respectively, and *Taf*I was used in digestions of PCR fragments containing the polymorphisms −857 C/T, T/T and −863 C/A, A/A. The results were analyzed on 2% agarose gels.

2.4. Statistical analyses

Logistic regression models were the primary analytical tool. Cases and controls were defined as binary dependent variables. Separate regression models were run for the three different case types (*i.e.* MDS, BID and BP) versus controls. MDS cases were compared to hospital controls, BP cases were compared to workplace controls, while BID cases were compared to both worker and combined (worker plus hospital) control groups, given the source case populations. Independent predictor variables included the four TNF polymorphisms (−238A, −308A, −857T, and −863A), cumulative and/or career average benzene exposure and potential confounders (*i.e.* age, gender, alcohol use and smoking). Parsimonious models were sought therefore covariates were only retained in logistic models if they had a meaningful impact (*i.e.* an OR differing by ±50%) on the main effects of genotype and/or exposure. Pseudo-r^2 values were examined to assess the overall predictive value of logistic models; and models with relatively large pseudo-r^2 values were preferred. Odds ratios (OR) were derived from the logistic models, tested for statistical significance using the likelihood ratio test, and 95% confidence intervals were generated to assess the precision of the ORs. Hardy–Weinberg equilibrium (HWE) conditions were assessed for statistically significant results. The null hypothesis of HWE was not rejected, indicating genotypic frequencies are consistent with HWE. All analyses were performed using STATA 8.0 software.

3. Results

Distribution of demographic characteristics, exposure and TNF polymorphisms are shown in Table 1 for the five study subpopulations. The majority of worker controls, BP and BID cases were employed in the same factories and worked under the same conditions. Therefore, although mean benzene exposures differed, there was extensive overlap in the exposure frequencies for the groups. For example, 100% of BID cases, 65% of BP cases and 50% of worker controls all experienced cumulative benzene exposures in excess of 150 parts per million/year (ppm year).

Age ($p < 0.001$) and smoking ($p < 0.02$) were significant predictors for MDS compared to hospital controls. However, when age and smoking were retained in the model, neither the −238A ($p = 0.18$) nor the −308A ($p = 0.75$) polymorphisms were significant. When each polymorphism was tested separately (*i.e.* without age and smoking in the model) the same conclusion was reached: −238A ($p = 0.23$), −308A ($p = 0.27$). Very few MDS and hospital control patients were exposed to benzene, making quantitative exposure comparisons between these two groups uninformative (data not shown).

Cases of BID were compared to both worker controls and the combined population of worker and hospital controls. There were no significant differences in potential confounding variables: age, smoking, alcohol, and gender when BID cases were compared to the combined control group. However, age ($p = 0.01$), alcohol ($p = 0.03$) and gender ($p = 0.05$) differed between BID cases and worker controls. Both cumulative and lifetime average intensity of benzene exposure differed significantly between BID cases and worker controls ($p < 0.001$). The OR for each successive category of lifetime average concentration was 4.6 ($p < 0.001$) and for cumulative exposure was 4.9 ($p < 0.001$). When each of the four TNF-α polymorphisms was tested singly for BID cases against the combined control group, only the −238A polymorphism was significant: OR = 6.3 ($p = 0.001$). This persisted when controlling for benzene exposure and/or potential confounders. Results using the preferred model with greatest explanatory power for BID versus combined controls accounts for lifetime intensity of exposure, age, gender, and alcohol use (OR for −238A = 7.40, $p < 0.029$, pseudo-$r^2 = 0.40$) (Table 2). For

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Table 2
Benzene-induced dysplasia (BID) and benzene poisoning (BP) risks by TNF genotype for preferred[a] logistic regression models

Case type	Controls	Genotype	OR	95% CI
BID	Worker	−238	7.41	1.23–44.8
BID	Worker	−308	1.03	0.29–3.66
BID	Worker	−857	0.79	0.22–2.93
BID	Worker	−863	1.55	0.57–4.21
BID	All	−238	7.40	1.23–44.7
BID	All	−308	0.95	0.27–3.32
BID	All	−857	0.70	0.20–2.51
BID	All	−863	1.48	0.56–3.88
BP	Worker	−238	1.47	0.38–5.66
BP	Worker	−308	1.52	0.68–3.40
BP	Worker	−857	0.56	0.22–1.42
BP	Worker	−863	0.37	0.15–0.93

[a] Preferred model has greatest explanatory power, see Section 2.

the three other TNF polymorphisms, models with or without confounders produced very similar OR's which were not elevated or statistically significant.

When cases of BID were compared to worker controls only, the OR for the −238A polymorphism alone (OR = 7.2, $p = 0.001$) was similar to that generated when using all controls. Again, no other polymorphism was statistically associated with BID, and the model with greatest explanatory power for the BID versus worker control contrast possessed the same covariates as the model with combined controls, and very similar results for each TNF polymorphism (Table 2).

For BP cases, there were no significant differences compared to worker controls for age, gender, and alcohol use. More BP cases smoked compared to controls (OR = 4.8, 95% CI 1.7–13.3). As expected, both cumulative and average career benzene exposure differed significantly among BP cases versus controls ($p = 0.03$ and 0.01, respectively). The OR's for successive categories of cumulative and average career benzene exposure were 1.16 and 1.29, respectively.

Odds ratios for the −238A, −308A, and −857T polymorphisms were not significantly different among BP cases when compared to worker controls (Table 2). Adjusting for benzene exposure did not alter this observation. There was a reduced OR of 0.41 for the −863A polymorphism which was marginally significant ($p = 0.06$) without adjustment, and statistically significant with adjustment for either career average (OR = 0.30, $p = 0.02$) or cumulative exposure (OR = 0.36, $p = 0.04$) to benzene, as well as adjustment for smoking (OR = 0.37, $p = 0.03$). However, each of these models was compromised in terms of the variance explained (pseudo-r^2 values were 0.06 or less).

4. Discussion

A growing body of evidence has established that chronic inflammation plays an important role in the pathogenesis of a number of human malignancies, primarily those of epidermal origin as well as certain lymphoid neoplasms [25–28]. Nevertheless, the precise mechanisms that explain the association between chronic inflammation and neoplastic progression remain unclear. Immune-mediated inhibition of hematopoiesis is thought to play a causal role in the pathogenesis of aplastic anemia [29]. However, chronic inflammation as a predisposing factor in the development of the hematopoietic neoplasms, MDS or AML, has not previously been demonstrated. Activated CD8+ cells and TNF-α have been described in MDS [19], and severe inflammation and clonal expansion of activated T lymphocytes are prominent features of the bone marrow pathology encountered in BID [1].

Several studies have implicated altered regulation of NF-κB and pro-inflammatory cytokines, such as TNF-α, in the development of cancers driven by inflammation [25–28]. However, the role of TNF-α in inflammation and cancer is complex, being pro-tumorigenic or anti-tumorigenic, depending on the cell type, microenvironment, and intracellular signaling triggered in response to the cytokine [26,30–33]. Even though TNF-α is generally understood to be an important mediator in inflammation and cancer, interpretation of the precise role individual TNF-α polymorphisms play in biology and disease is complicated by the enormous linkage disequilibrium associated within the MHC Class III site. For example, the −308A and −238A polymorphisms have been linked in extended haplotypes. However, these two polymorphisms almost never occur in tandem. Further, the −308A haplotype is associated with higher TNF-α production and is implicated in acute solid organ transplant rejection while the −238A haplotype is not. Finally, although −308A has been linked with certain HLA haplotypes, the association of −238A with severity of chronic infection and autoimmune disease has not been linked to specific HLA genes [12,13].

In this study, only −238A (AA/AG) of the four single nucleotide TNF-α polymorphisms analyzed was significantly associated with the development of BID. The number of cases of BID available for analysis in this study was relatively small. However, the relationship between the −238A polymorphism and BID was robust and did not diminish when cumulative exposure, average exposure intensity or confounders were accounted for. Moreover, the association with −238A was specific for persistent BID but was not observed for *de novo* MDS, or BP. Therefore our findings suggest that the −238A polymorphism may be over-represented in a subpopulation of individuals with increased risk of selectively developing persistent bone marrow disease and dysplasia following chronic exposure to high concentrations of benzene.

An explanation for the role of −238A in the pathogenesis of BID may be forthcoming from studies that implicate TNF-α in hematopoietic stem cell regulation. The −238A allele appears to be unique in that it is associated with cell-specific de-repression of TNF-α production [6,12,14]. Membrane bound TNF-α expressed on CD34+ cells also has been implicated in the induction of tolerance in haploidentical bone marrow transplantation [34], suggesting a role for regulation of TNF-α expression in hematopoietic stem cell survival. These findings suggest the possibility that cell-specific alter-

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ations in TNF-α expression linked to this polymorphism may facilitate the escape of damaged hematopoietic progenitor cells from CD8+ T cell targeting and promote clonal selection in the evolution of neoplastic hematopoietic disease. It is also possible that −238A may be linked in an extended haplotype with other genes that play a role in influencing TNF-α expression in hematopoietic progenitor cells.

The severe inflammatory changes and altered immune parameters that characterize BID, together with an association with the TNF-α −238A haplotype in affected individuals, provides independent evidence to support a role for inflammation and altered immune response in the evolution of benzene-induced bone marrow dysplasia. Further studies are needed to elucidate the precise role of −238A or other genes in the pathogenesis of chemical induced bone marrow injury. However, these results suggest that benzene should be added to the list of agents for which chronic inflammation may play an important or predisposing role in the development of human neoplasia.

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