大韓獸醫學會誌(2005) 第45卷 第4號
Korean J Vet Res(2005) 45(4) : 517~525

Lack of connexin 32 does not enhance the benzene-induced hematotoxicity and hemopoietic tumor incidence in mice

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(Accepted: November 28, 2005)

Abstract : This study was performed to evaluate using wild-type (WT) and C×32 knockout (KO) mice if lack of cell to cell communication by connexin 32 gap junction enhances the benzene-induced hematotoxicity and hemopoietic tumor development. The WT and C×32 KO mice were exposed to 300 ppm of benzene for 6 hours/day, 5 days/week for a total of 26 weeks by inhalation, and then sacrificed to evaluate the toxicities of hemopoietic organs or allowed to live out their life span to evaluate the hemopoietic tumor incidence. The significant increase and decrease of organ weight were respectively noted in spleen and thymus of both WT and C×32 KO mice without significant difference between the genotypes. Histopathologically, benzene exposure for 26 weeks induced the morphological changes in hemopoietic organs, characterized by fat cell accumulation in the bone marrow and extramedullary hemopoiesis in the spleen. The fat cell accumulation was, compared with that of WT mice, considerably exacerbated in the C×32 KO mice. However, no significant difference was observed in the changes of hematological values and bone marrow cellularity as well as in the onset and incidence of hemopoietic tumors between WT and C×32 KO mice. In conclusion, this study indicated little significant role of the cellular communication by C×32 gap junction in the action mechanism of benzene hematotoxicity and leukemogenicity.

Key words : benzene, connexin 32, hematotoxicity, hemopoietic tumor

Introduction

Benzene, which has widely been used as an industrial solvent and is a component of gasoline, is an environmental contaminant that can induce hematotoxicity and hemopoietic neoplasia in humans and mice [1, 2, 4-6, 8]. Its hematotoxicity includes depression of bone marrow cells including hemopoietic progenitor (CFU-GM, E) and stem cells (CFU-S) [5, 6, 26], resulting in aplastic anemia [5]. The mechanism underlying benzene-induced hematotoxicity and leukemia is, however, still not fully understood, although DNA damage by benzene metabolites and free radicals produced and aberrant cell cycle regulation of hemopoietic progenitor cells have been proposed to be associated with the mechanism [16, 25, 26].

Hemopoiesis and its regulation are achieved in the microenvironment where bone marrow-stromal cells are closely contacting with hematopoietic stem and progenitor cells and they are dynamically interacting each other for blood cell proliferation and differentiation [28]. The mechanism by which bone marrow stromal cells interact with hematopoietic cells is not fully understood, but production of growth factors has been believed as one mechanism and direct cell to cell communication was proposed as another possible one [13, 19-22].

The cell-cell communication through gap junctions may also play an important role in hemopoiesis at normal and regenerating status [13, 19-22]. The presence of connexin 43 (C×43) gap junction protein and its active participation in hemopoiesis at the normal and regenerating states had been reported in the bone marrow [3, 13, 21, 22]. C×37 and C×26 proteins

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were also detected in the arteriolar endothelium, even their contributions in association with hemopoiesis have not yet been clarified [13, 22]. On the other hand, other connexins including C×32 have been undetectable in bone marrow at protein level [13] and thus their presence and function are still controversial.

It has been postulated in various organs that disorder of GJICs including C×32 is closely associated with tumorigenesis [7, 9, 10, 12, 29-30], but it has not yet been studied if C×32 plays any role in the homeostasis of hemopoiesis in bone marrow and its disorder contributes to development of hemopoietic neoplasm.

Based on the background, in the present study, a possible role of C×32 in the benzene-induced hematotoxic and leukemogenic mechanism was investigated. For the purpose of study, we evaluated bone marrow toxicity and hemopoietic tumor incidence using C×32 KO mice after exposing to 300 ppm benzene for 26 weeks by inhalation and then compared the results with those from the WT mice under same experimental protocol.

Materials and Methods

Animals

C×32 KO mice, from the Institute für Genetik, Universität, Germany [18], were maintained as heterozygous KO mice at the animal facility of National Institute of Health Sciences (NIHS), Japan. C×32 WT and KO mice were obtained by breeding the heterozygous mice at each experiment. The resulting neonates were identified by PCR for their homozygosity. The WT and C×32 KO mice aged 8th to 9th weeks were used for the study. During the study, the mice were housed within stainless-steel wire-cages in the inhalation chambers and kept on a 12-hr light-dark cycle. The basal pellet diet (Funabashi Farm, Japan) was provided *ad libitum*, except during a 6-hour daily inhalation of benzene food was withdrawn. Water was supplied automatically throughout the study.

Benzene exposure

The mice were exposed 1.3 m^3 inhalation chambers. Benzene was purchased from Wako (Japan). The benzene atmospheres were generated by heating liquid benzene to 16°C to form a vapor (Sibata Scientific, Japan). The benzene-laden air then was directed into the inhalation chambers. The benzene concentration in the chambers was measured at half-hourly intervals during daily exposures using a gas chromatograph (Shimadzu, Japan). The temperature and humidity in the chambers were automatically controlled to 24 ± 1°C and 55±10%, respectively.

The WT and C×32 KO mice were respectively divided into sham-control and benzene-exposed groups; each group was composed of 10 to 12 mice. The experimental groups were exposed to 300 ppm of benzene for 6 hours/day, 5 days/week for a total of 26 weeks; the sham-control mice were maintained under the same conditions without benzene inhalation. Five to six mice from each group were first sacrificed after the 26 week exposure to evaluate the toxic effects of benzene on blood and bone marrow and the remaining five to seven mice from each group were allowed to live out their lives to further evaluate the incidence of the hemopoietic neoplasia.

Clinical signs

The clinical sign was examined twice a day, every morning and evening, before and after daily inhalation during the 26-week exposure.

Hematology and bone marrow cellularity

After 26-week benzene inhalation, peripheral blood was collected from orbital plexus, and then blood count and its parameters were assessed using a Sysmax M-2000 blood cell counter (Sysmex, Japan). For differential count, Giemsa stain was carried out on the smeared blood samples.

To evaluate bone marrow cellularity, bone marrow cells were harvested from one femur of five mice per group according to the protocol described previously [26]. Briefly, bone marrow cells were flushed out of the bone shafts with 2 ml Dulbeco Minimum Essential Medium (DMEM) without phenol, using a 27-gauge hypodermic needle syringe, and then counted using Sysmax M-2000 blood cell counter.

Necropsy and histopathology

After 26-week benzene exposure, five to six mice from each group were sacrificed for autopsy. The mice were grossly examined, and hemopoietic tissues including bone marrow, thymus and spleen and other solid organs were fixed in 10% neutral buffered formaldehyde for 48 hours. After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and examined under the light microscope.

Mortality and Tumor incidence

Mortality of animal was evaluated throughout the study. The died animals were immediately autopsied and, after recording the date and gross examination, all the organs including bone marrow were formalin fixed for histopathological examination.

Statistical analysis

Student t-test was used to evaluate the significant differences of food consumption and body weight between the non-exposed and benzene-exposed group of wild-type and C×32 KO mice as well as between wild-type and C×32 KO mice of each group.

Results

Clinical signs and survival ratio for life time observation

No animal died during the 26-week benzene exposure, but a systemic cyanosis and the increase of activity was found in both of benzene-exposed WT and C×32 KO mice. After the exposure, most of the benzene-exposed WT and C×32 died of various cancers including hemopoietic malignancies (Fig. 1). The benzene exposure to the mice for 26 weeks considerably shortened their life span of both WT and C×32 KO mice. Interestingly, in the benzene-exposed groups, the life span of the C×32 KO mice was longer than that of the WT mice (Fig. 1).

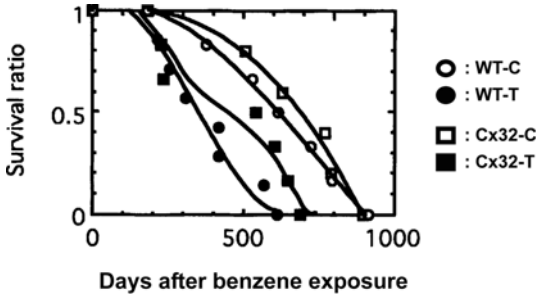

Fig. 1. Survival ratio of WT and C×32 KO mice for whole life span. WT-C, sham-control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32-C, sham-control group of C×32 KO mice; C×32-T, benzene-exposed group of C×32 KO mice.

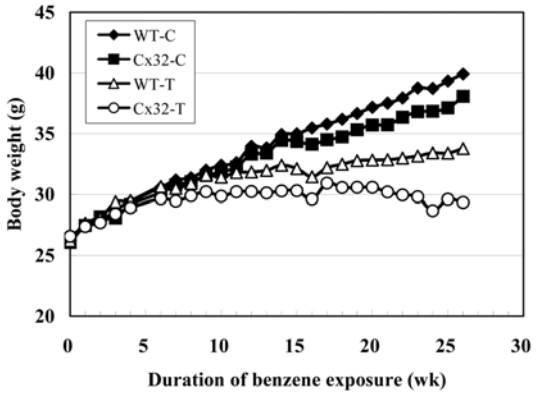

Fig. 2. Change of body weight in the wild-type (WT) and C×32 KO mice exposed to 300 ppm of benzene for 26 weeks. Note the marked decreases of body weights in WT and C×32 KO mice during the benzene exposure. WT-C, sham-control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32-C, sham-control group of C×32 KO mice; C×32-T, benzene-exposed group of C×32 KO mice.

Food consumption and body weight

During the benzene inhalation for 26 weeks, there was no difference in food consumption between non-exposed group and benzene-exposed group of both WT and C×32 KO mice and between WT and C×32 KO mice of both groups. In body weight, significant difference was not observed between WT and C×32 KO mice of non-exposed group throughout the study, even if the mean values of C×32 KO mice was a little less than those of wild-type mice at the late stage of this study (Fig. 2).

Benzene exposure induced a significant decrease in the body weight during benzene exposure in the benzene-exposed groups of both WT and C×32 KO mice. However, the decrease was much more marked in C×32 KO mice. In C×32 KO mice, significant difference appeared from after seven weeks of exposure ($p<0.05$). On the other hand, in WT mice, the significant difference was observed from after fourteen weeks of exposure. Furthermore, from after the twelfth week of exposure, the mean value of body weight showed a significant difference between benzene-exposed wild-type and C×32 KO mice ($p<0.05$) and the surprising decrease of body weight was noted in benzene-exposed C×32 KO mice from after 24-week exposure.

Absolute organ weights

Significant increases were noted in the organ weight

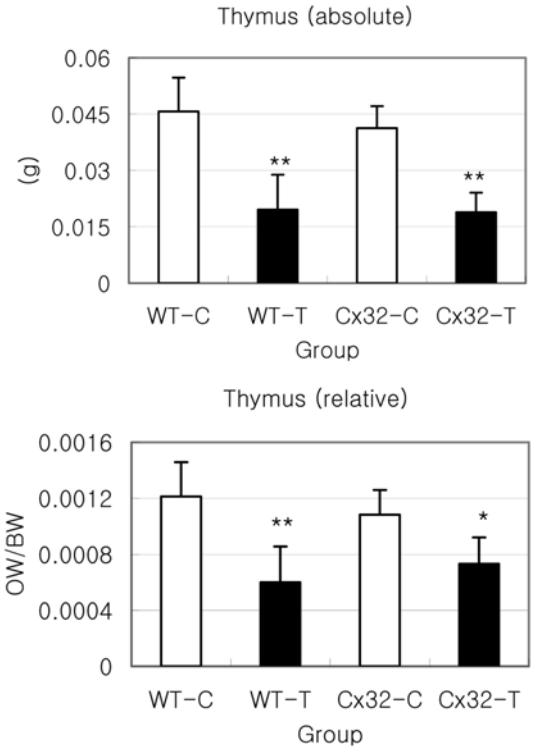

Fig. 3. Changes of absolute and relative organ weight of thymus in the wild-type (WT) and C×32 KO mice exposed to 300 ppm of benzene for 26 weeks. *, significantly different from each control value at $p<0.05$; **, significantly different from each control value at $p<0.01$. WT-C, sham-control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32-C, sham-control group of C×32 KO mice; C×32-T, benzene-exposed group of C×32 KO mice.

values of lung in both benzene-exposed wild-type ($p < 0.05$) and C×32 KO mice ($p < 0.05$). On the other hand, the organ weights of testes of the benzene-exposed C×32 KO mice ($p < 0.05$) and of thymus of both benzene-exposed wild-type ($p < 0.01$) and C×32 KO mice ($p < 0.01$) were significantly decreased by 26-week exposure of 300 ppm of benzene (Fig. 3).

Relative organ weights

The organ weights of lung and spleen were significantly increased in both benzene-exposed WT ($p < 0.05$) and C×32 KO mice ($p < 0.05$) after benzene exposure for 26 weeks. Significant increases in the organ weights of brain and kidney of C×32 KO mice ($p < 0.05$) and the organ weight of heart of WT mice ($p < 0.05$) were also noted after the 26 week-exposure of benzene. On the other hand, the organ weight of thymus was significantly decreased in the WT mice exposed to benzene for 26 weeks ($p < 0.01$) and markedly decreased in the benzene-exposed C×32 KO mice (Fig. 3).

Blood values, differential count, and bone marrow cellularity

Significant decreases in the number of peripheral leukocytes (WBC) ($p<0.05$), red blood cells (RBC) ($p < 0.01$ and $p < 0.05$), and platelet ($p < 0.05$) were noted in both wild-type and C×32 KO mice after the exposure of benzene for 26 weeks (Fig. 4). The values of hemoglobin (HGB) in both benzene-exposed WT ($p < 0.01$) and C×32 KO mice ($p < 0.05$), hematocrit (HCT) in benzene-exposed wild-type mice ($p < 0.01$) were significantly decreased (Fig. 4). Mean corpuscular volume (MCV) was significantly increased in both WT ($p < 0.05$) and C×32 KO mice ($p < 0.05$) after 26-week benzene exposure (Fig. 4). In differential count, a significant decrease in the portion of lymphocytes ($p < 0.01$, $p < 0.01$) and a significant counteracting increase ($p < 0.01$, $p < 0.01$) in the portion of neutrophils were noted in both WT and C×32 KO mice exposed to benzene for 26 weeks. On the other hand, in the bone-marrow cellularity, no changes were observed in the benzene-exposed group of both WT and C×32 KO mice (Fig. 4). Consequently, there was no notable difference in the toxic effects of benzene on hematological parameters and bone marrow cellularity between WT and C×32 KO mice when they were exposed to 300 ppm benzene for 26 weeks (Fig. 4).

Histopathology

After a 26-week exposure of 300 ppm of benzene, notable histopathological lesions in association with benzene exposure were observed in the bone marrow, spleen, and lung of the benzene-exposed WT and C×32 KO mice (Table 2). However, those findings were in general severe in C×32 KO mice compared with those noted in WT mice, as shown in Table 2. In bone marrow, hypoplastic change characterized by moderate to severe accumulation of fat cells was found in the medullary areas close to epiphyses with a diffuse and prominent increase of brown-pigmented macrophages (Fig. 5). In spleen, extramedullary hemopoiesis was very active in red pulps with depletion of lymphoid cells in the white pulps, resulting in atrophy of the

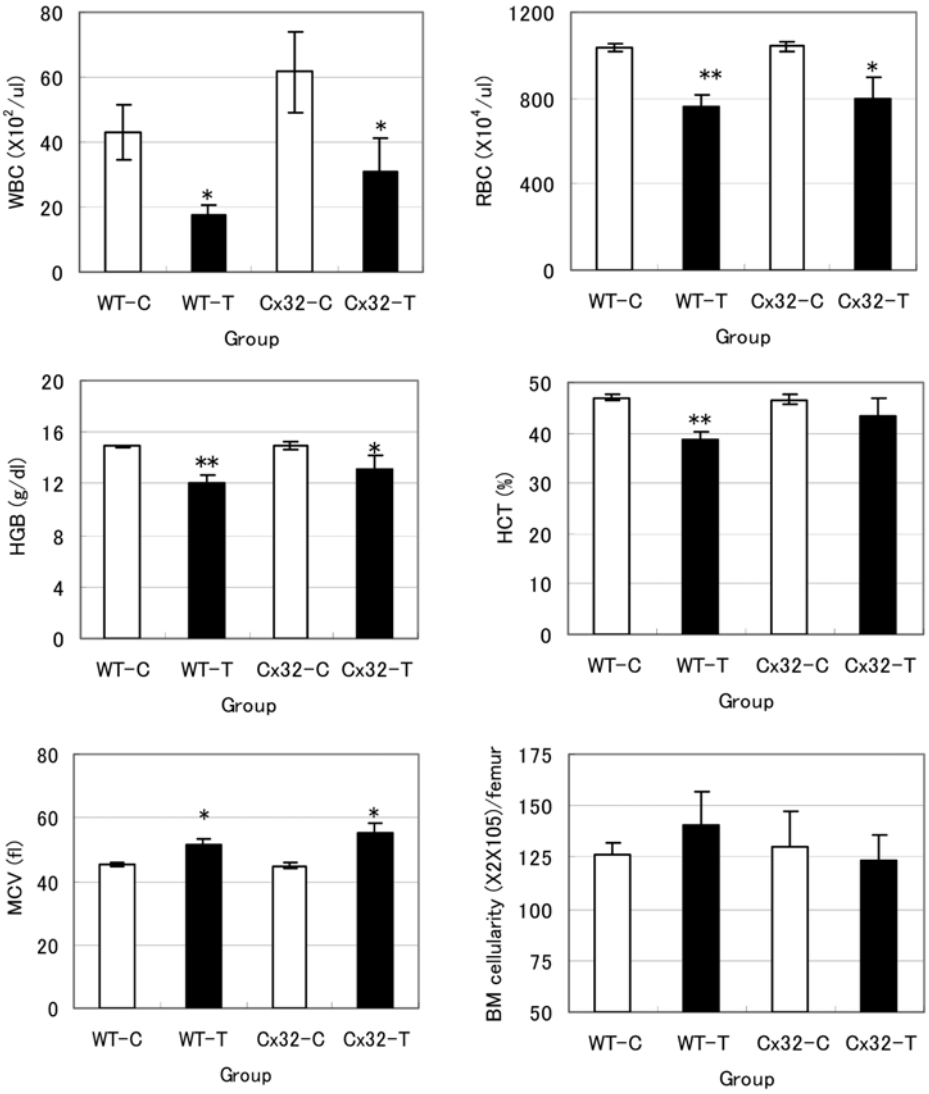

Fig. 4. Changes in hematological values and bone marrow cellularity in the wild-type and C×32KO mice exposed 300 ppm of benzene for 26 weeks. *, significantly different from each control value at $p<0.05$ **, significantly different from each control value at $p<0.01$ WT-C, sham-control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32-C, sham-control group of C×32 KO mice; C×32-T, benzene-exposed group of C×32 KO mice.

white pulps (Table 1). Diffuse interstitial pneumonia was noted in both benzene-exposed WT and C×32 KO mice, but much more prominent in the lungs of C×32 KO mice. A notable increase in the number of mucus-secreting cells was observed in the bronchi and bronchioles of benzene-exposed WT and C×32 KO mice and, sometimes, in the proliferating alveolar epithelial cells of C×32 KO mice. The proliferation of basophilic alveolar epithelial cells in terminal bronchioles and alveolar ducts was frequently noted in the lungs of benzene-exposed C×32 KO mice (Table 2).

Tumor incidence

No tumor was observed in both WT and C×32 KO mice exposed to 300 ppm benzene for 26 weeks. After cessation of benzene exposure, most of the mice in both sham-control and benzene-exposed group died of tumors, but long-term benzene exposure was characterized

by earlier onset of hemopoietic tumors in both benzene-exposed groups of WT and C×32 KO mice independent of the presence of C×32 (Fig. 6).

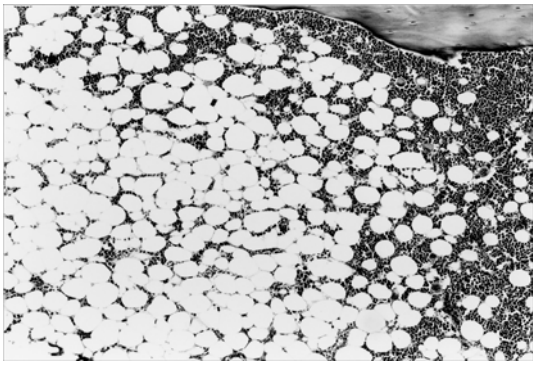

Fig. 5. Accumulation of fat cells in the bone marrow of C×32 KO mice exposed to 300 ppm of benzene for 26 weeks. This histological change was, compared with that of WT mice, exacerbated by lacking of C×32. H&E. ×100.

Discussion

Although the underlying mechanism by which benzene induces leukemia is still not fully understood, both of genetic and epigenetic effects by reactive benzene metabolites and reactive oxygen species produced have been implicated [11, 16, 23, 16]. Disruption of gap junction intercellular communication coupled with such kinds of proliferating stimuli has been considered as a tumorigenic mechanism of various epigenetic carcinogens [12, 17, 29, 30]. In case of benzene, repeated on-and-off exposure pattern as well as exposure dosage has been shown to be an important encouraging factor to induce hemopoietic neoplasm [5], by providing the disturbing microenvironment for stem cell proliferation and differentiation resulting in unstability of tumor suppressor genes such as p53 [26]. However, it has not yet been studied how important the intercellular communication by connexins is in the process of hemopoietic carcinogenesis of benzene, even

Table 1. Histopathological findings in the wild-type and C×32 knockout mice exposed to 300 ppm of benzene for 26 weeks

Histopathology / Group	WT-C	WT-T	C×32KO-C	C×32KO-T
No. of mice examined	6	5	5	5
Bone marrow				
Accumulation of fat cells	0 (0.00)*	3 (60.0)	0 (0.00)	4 (80.0)
Moderate		2 (40.0)		
Severe		1 (20.0)		4 (80.0)
Brown pigmented macrophages	0 (0.00)	3 (60.0)	0 (0.00)	3 (60.0)
Mild				1 (20.0)
Moderate		3 (60.0)		1 (20.0)
Severe				1 (20.0)
Spleen				
Hemopoiesis	0 (0.00)	5 (100.0)	0 (0.00)	5 (100.0)
Moderate		2 (40.0)		1 (20.0)
Severe		3 (60.0)		4 (80.0)
Atrophy of white pulp	0 (0.00)	3 (60.0)	0 (0.00)	4 (80.0)
Severe		3 (60.0)		4 (80.0)
Lung				
Interstitial pneumonia	6 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)
Mild	6 (100.0)	1 (20.0)	5 (100.0)	1 (20.0)
Moderate		4 (80.0)		
Severe				4 (80.0)
Hyperplasitic foci of basophilic alveolar epithelial cells	0 (0.00)	1 (20.0)	0 (0.00)	3 (60.0)

WT-C, control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32KO-C, control group of C×32KO mice; C×32KO-T, benzene-exposed group of C×32KO mice.
*, No. of mice with pathological lesions and its percentage in parentheses.

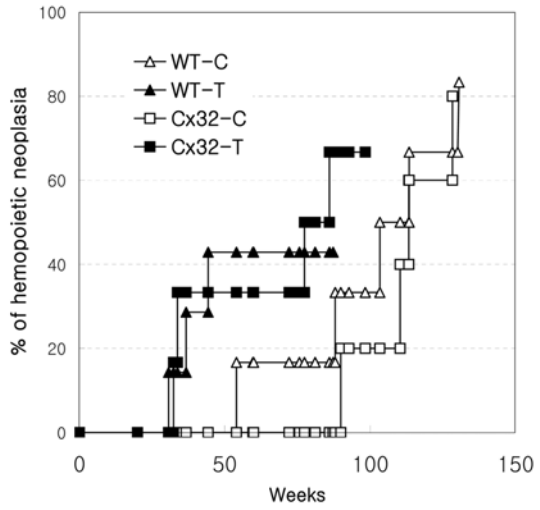

Fig. 6. Accumulated incidence of the hemopoietic neoplasia developed for whole life span in the wild-type and C×32 KO mice after the exposure of 300 ppm of benzene for 26 weeks. WT-C, sham-control group of wild-type mice; WT-T, benzene-exposed group of wild-type mice; C×32-C, sham-control group of C×32 KO mice; C×32-T, benzene-exposed group of C×32 KO mice.

if there have been studies evidencing a functional role of direct cell-cell communication in regulating hemopoiesis at normal and pathological statuses in human and mouse bone marrow [13, 20, 22].

Hemopoietic stem cells lodge and grow in a delicate space within bone marrow stroma, so called 'niche', where dynamic cell:cell communication is present between the stem cells and stromal cells [22]. So far, C×43 and C×37 were shown to be detectable in bone marrow [13]. In particular, C×43 is believed to be a major gap junction protein regulating cell to cell communication in human and mouse bone marrow [3, 13, 21, 22]. On the other hand, the expression of C×32 in bone marrow and its functional role for blood formation has been controversial. We hypothesized that only small number of primitive hemopoietic stem cells might be present in bone marrow with C×32 gap junction, resulting in those controversial data. On the point of view, benzene leukemogenicity study using the mouse lacking C×32 might give an answer to our questions concerning a functional role of the gap junction protein in hemopoiesis. Furthermore, if C×32 plays a role in hemopoietic stem cell physiology, it would be quite of interest to see if lack of C×32-mediated cell communication affects the benzene-induced hematotoxicity and leukemogenicity.

Long-term benzene exposure for 26 weeks in the present study gave mild to significant effects on the hemopoietic organs such as the bone marrow, spleen, thymus and lung with or without histopathological changes both in the WT and C×32 KO mice. Among the organs histopathologically examined, in the bone marrow and lung tissues, lack of C×32 gap junction protein considerably exacerbated the toxic effects of benzene. We discussed in the previous paper about the exacerbated peumotoxicity and its plausible mechanism, which was associated with proliferation of the alveolar epithelia expressing CYP2E1, a major enzyme for benzene metabolism, in the lung of the benzene-exposed C×32 KO mice [27]. The marked decreases of body weights noted in the WT and C×32 KO mice during the benzene exposure were in a good correlation with the pulmonary lesions [27].

The exacerbating effect of benzene on the bone marrow lacking of C×32 was characterized by accumulated fat cells, possibly associated with the decrease of bone marrow cellularity comparable with its increase in the WT mice. It is, however, interesting here that hematological analyses including serum chemistry did not indicate any notable difference in their parameters between WT and C×32 KO mice, suggesting that, without cell to cell communication by C×32, hemopoietic stem cells in bone marrow were capable of compensating the continuous blood cell loss by benzene exposure throughout 26 weeks. Furthermore, in the onset and incidence of hemopoietic tumors, no difference was noted between WT and C×32 KO mice as well. Therefore, our results may indicate little significant role of the cellular communication by C×32 gap junction in the action mechanism of benzene with regard to its hematotoxicity and leukemogenicity. Nevertheless, the marrow atrophic change exacerbated in C×32 KO mice might suggest a possible role of C×32 in maintaining the physiological environment of bone marrow, although we could not determine the exact role of C×32.

In conclusion, in this study, lack of C×32 in the bone marrow did not enhance the hematotoxicity and hemopoietic tumor incidence induced by long-term benzene exposure. However, it still remains to elucidate a potential physiological role of C×32 in the microenvironment of bone marrow.

Acknowledgements

The author thanks Drs. Tohru Inoue, Yoko Hirabayashi, Yukio Kodama, Yasushi Kawasaki and Jun Kanno at NIHS of Japan for their collaboration in this study.

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