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PSK, A BIOLOGICAL RESPONSE MODIFIER, INDUCES A SYSTEMIC DEVELOPMENTAL DELAY IN X-RAY IRRADIATED MOUSE EMBRYOS: COINCIDENCE WITH ITS ANTI-TERATOGENIC EFFECT

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Some biological response modifiers (BRMs) have been shown to suppress teratogenesis. We previously showed that PSK, a BRM and a protein-bound polysaccharide extracted from *Basidiomycetes*, suppressed X-irradiation-induced ocular anomalies in mouse embryos. In those studies, we noticed that the irradiated and PSK-treated embryos tended to be smaller in body size than the controls that were irradiated but not treated with PSK, in spite of the reduced teratogenesis. In this study, therefore, we analyzed in detail systemic development from embryonic day (E)7.75 to E9.0 of embryos that were irradiated and treated with PSK at E7.5, following the same experimental protocol used in the previous study. PSK treatment not only enhanced the X-irradiation-induced developmental delay, but also delayed development, albeit less extensively, as compared with controls. These developmentally delayed embryos generally showed proportional and symmetric size reduction. Possible mechanisms of the PSK-induced delay in systemic development and a relation with its anti-teratogenic effect are discussed.

Key words: PSK, irradiation, growth retardation, anti-teratogenic effect, mouse embryo

INTRODUCTION

Teratogen-induced defects can be suppressed by several chemicals and this effect is categorized as the "interference effect" in the teratological literature (1), as reviewed in our previous report (2). These previous studies suggested that these anti-teratogenic agents did not work by direct interaction with teratogenic chemicals, but reduced teratogenesis via modifications to the biological response, such as activation of drug metabolism and/or excretion, induction of metalothionein and activation of the immune system (1, 3). However, the detailed mechanism of each effect at the cytological or molecular level still remains unclear.

PSK, a protein-bound polysaccharide extracted from a fungus, *Basidiomycetes*, is a biological response modifier (BRM) which is used in cancer therapy (4, 5). We previously showed that PSK suppressed abnormal ocular development induced by X-irradiation at embryonic day (E) 7.5, when administered immediately after irradiation, not only externally but also histologically at 72 hours (hr) after the treatment (E10.5)(6). We further showed that PSK regulated p53 level and modified cell proliferation and apoptosis within 12 hr after irradiation, and suggested that PSK may modify endogenous restoration mechanisms for DNA damages by X-ray irradiation in early organogenetic period and contribute to decrease the incidence of malformation (2).

In that series of experiments (2, 6), we also noticed that PSK-treated embryos tended to show a smaller body size than the control embryos either during fetal period or at term. Smaller body size than the standard during the prenatal period is called intrauterine growth retardation and has been generally considered as a defective condition that may relate to postnatal diseases as their predisposition (7). The causes are diverse from genetic to environmental including maternal malnutrition, diabetes, infections, smoking and so on (7, 8) as well as their complex interaction (9). However, it remains difficult to

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distinguish the normal but slow growth of the fetus from growth restriction, and symmetric intrauterine growth retardation with normal interval rate of growth may simply represent a constitutionally small yet otherwise normal fetus (7, 8). In our previous studies (2, 6), tendency of decreased body size coincided with the reduced teratogenicity, a favorable condition, further suggested that smaller body size should not be simply assumed to be abnormal.

In this study, therefore, we aimed to confirm and analyze possible mechanism of the PSK-induced developmental delay for a better understanding of smaller body size during prenatal development. Using the same protocol as the previous study (2), we examined in detail the external features of embryos after irradiation and PSK administration, and defined the developmental stages of the treated embryos. We confirmed the systemic developmental delay in the treated embryos and observed that the body size reduction was generally proportional and symmetric, and showed no specific disproportional phenotypes.

MATERIALS AND METHODS

Animals

Jcl: ICR mice (CLEA Japan, Tokyo, Japan) from 8 to 13 weeks old were used in this study. They were maintained at the Institute of Experimental Animals of Shimane Medical University and handled in accordance with the institutional animal care guidelines. An estrous female was placed overnight in the same cage with a potent male, and when a vaginal plug was found in the morning after mating, the noon of the plug day was designated as E0.5.

Experimental treatments

X-ray irradiation and PSK administration were carried out at E7.5. Pregnant mice were settled in a plastic box and whole body irradiation at 2 Gy was carried out with an irradiation generator (ML-15 MDX, Mitsubishi, Tokyo, Japan). Within ten minutes of irradiation, PSK (Krestin®, Kureha, Tokyo, Japan) dissolved in physiological saline (50 mg/ml) was injected intraperitoneally. The dose of PSK was 200 mg/kg body weight, as determined on the basis of our previous data (2, 6). Physiological saline was similarly injected into the control mice. As shown in Table 1, embryos were divided into four groups according to treatments and sacrificed at four time points. Embryos of non-irradiated and PSK-treated (P group), irradiated and saline-treated (X group), irradiated and PSK-treated (X+P group), non-irradiated and saline-treated control (C group) groups were sacrificed at 6 hr (E7.75), 12 hr (E8.0), 24 hr (E8.5), and 36 hr (E9.0) after the treatments (Table 1). Two to four embryos were randomly selected from each litter for the present morphological analyses (Table 1), while the other embryos were prepared for RNA and other analyses (data not shown).

Table 1. Numbers of embryos examined

Treatment group	Number of embryos (dams)			
	E7.75	E8.0	E8.5	E9.0
X+P	15(5)	12(5)	10(4)	12(3)
X	20(5)	13(5)	12(3)	8(4)
P	14(5)	11(5)	10(3)	10(4)
C	15(5)	15(4)	10(4)	11(4)

X: irradiation, P: PSK, C: control

External observation and staging of embryos

Embryos were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) or Bouin's solution (formalin 25 ml, saturated picric acid aqueous solution 75 ml, and acetic acid 5 ml). External morphology of the embryo was inspected under a dissecting microscope. We determined the developmental stage of each embryo by calculating the number of the somites and comparing the external features with those of the standard defined by Kaufman (10). We sometimes observed embryos with phenotypes of intermediate stages such as those with phenotypes of both E7.5 and E8.0, for example. These embryos were designated as E7.5-8.0 in the result (Table 2).

Histological observation of embryos

To confirm the developmental delay at the histological level, embryos fixed as above were dehydrated in a graded series of ethanol, embedded in stylene- or epoxy-resin, and cross-sections of 500 nm

Table 2. Result of external observation of embryo from E7.75 to E9.0

Date of observation (E)	Treatment group	Numbers of embryos: Determined developmental stage (E) 7.5	7.5-8.0	8.0	8.0-8.5	8.5	8.5-9.0	9.0
7.75	X+P	15						
	X	11	9					
	P	8	6					
	C	7	8					
8.0	X+P		11	1				
	X		11	2				
	P	2	9					
	C		12	3				
8.5	X+P		8	2				
	X			9	3			
	P			4	6			
	C				10			
9.0	X+P		1	7	4			
	X				5	3		
	P				2	2	2	4
	C						2	9

P: PSK, X: irradiation, C: control *p<0.05 **p<0.01

thickness were made at the forebrain region. Sections were stained with toluidine blue and observed under a light microscope.

Statistical analysis

The Mann-Whitney's U-test was used in statistical analyses, and a level of $p<0.05$ was regarded as significant.

RESULTS

We examined in detail the external features of embryos after the irradiation and/or PSK treatment and determined the developmental stage of each embryo as described in the method (Table 2). In Table 2, 'the date of observation' indicates the days of gestation when the embryos were sacrificed and fixed for observation. However, the observed and determined developmental stage of these embryos, judging from the standard (10), was frequently different from the date of observation in the treated groups.

While all embryos of the C group which were sacrificed at E8.5 showed developmental stage of E8.0-8.5, the developmental stage for the embryos of the X group observed at E8.5 was mainly at E8.0 and significantly younger than the C group ($p<0.05$ for X vs. C, Table 2). Embryos of the X group observed at E9.0 showed a significantly younger developmental stage of E8.0-8.5 or E8.5 than embryos of the C group observed at E9.0, whose developmental stage was E8.5-9.0 or E9.0 ($p<0.01$ for X vs. C, Table 2). Therefore, irradiation caused a developmental delay in embryos as compared with the control (X vs. C) as previously reported (11).

As suggested in our previous studies (2, 6), PSK treatment further delayed the development of the irradiated embryos (Table 2). At E7.75, E8.5, and E9.0, embryos of the X+P group showed significantly younger developmental stages than embryos of the X group ($p<0.05$ for X+P vs. X at E7.75, E8.5, and E9.0, Table 2). Therefore, the X+P group exhibited the latest developmental stage among the treated groups as compared with the C group except at E8.0. Figure 1 gives an example that an embryo of the X+P group sacrificed at E9.0 showed developmental stage of E8.0, while an embryo of the C group was defined as developmental stage of E9.0 (Fig. 1A, B). Delay in the development was also confirmed histologically at the forebrain region (Fig. 1C). The neural tube was still wide open and the neuroepithelial layer was thinner in the delayed embryos, whereas the embryos that developed similarly to the standard (10) showed the closed forebrain with the thicker, thus more proliferated, neuroepithelium (Fig. 1C).

Interestingly, PSK treated group (P group) also induced the developmental delay as compared with the C group, albeit less extensively than in the X or

X+P group, at E8.0, E8.5 and E9.0 (P vs. C, $p<0.05$). We observed some developmental delay in the C group at each time point from the standard, probably due to individual variations and/or strain-based variations, i.e. ICR in the present study vs. (C57BL x CBA) F1 in the standard (10).

In this study, the observed embryos including those exhibited a systemic developmental delay generally showed proportional and symmetrical body parts.

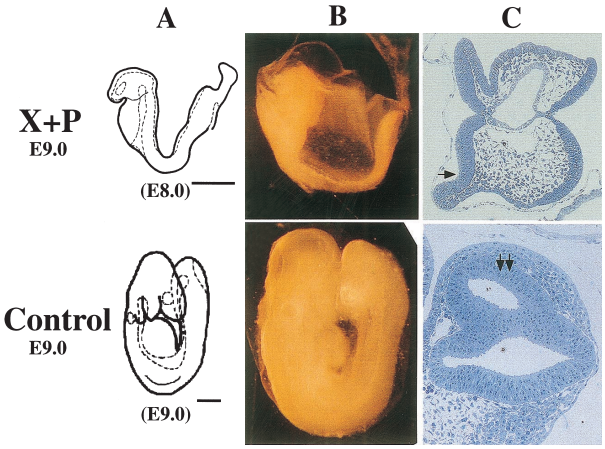

Fig. 1. Schematic representation (A), photos (B), and cross sections at the forebrain region (C) of an E9.0 embryo of the X+P group (upper panels) and an E9.0 control embryo (lower panels). Stages in parentheses indicate the determined developmental stages. The neural tube remains open and the neuroepithelium is thinner (arrow) in the X+P embryo, whereas the control embryo has an closed neural tube/brain vesicle and the thicker neuroepithelium (double arrow). Bar: 500 μm.

DISCUSSION

In this study, we determined the developmental stage based on the standard (10) of embryos after X-irradiation and/or PSK-treatment (Table 2), because we observed in our previous studies (2, 6) that these embryos frequently showed a smaller body size than controls. X-irradiation caused a significant developmental delay in embryos (X group). PSK treatment not only further enhanced the X-irradiation-induced developmental delay (X+P group), but also caused a significant, albeit less, delay in the non-irradiated embryos (P group).

X-ray, a purely physical teratogen, causes apoptosis in embryonic tissues after 6 to 12 hr of irradiation (12). One can assume that the massive apoptosis induced by X-irradiation would cause a systemic developmental delay. Then, what about the PSK effect to enhance the developmental delay? In our previous study (2), PSK, when administered immediately after irradiation at E7.5, suppressed mitosis and increased apoptosis at 12 hr after irradiation, as compared with embryos that were not treated with PSK. Therefore, decreased cell number by cell-cycle arrest and apoptosis at 12 hr after irradiation and PSK-treatment (E8.0) might cause systemic development at later time points (E8.5 and E9.0). However, at E7.75, since apoptosis was less in the X+P group than in the X group whereas mitosis occurred similarly in both groups (2), simple balance between mitosis and apoptosis can not explain the PSK effect on the developmental delay at E7.75 observed in the present study.

We also previously observed that p53 level was reduced by X-irradiation and modified by PSK treatment. While p53 was shown to be expressed at a high level in embryos (13), the p53 level was increased at 6 hr after X-irradiation, reduced at 12 hr, and then returned to the original level at 24 hr. Interestingly, PSK treatment delayed this X-irradiation induced change in the p53 level by approximately 12 hr (2). The high level of p53 has been suggested to play a role in the development, especially in chondrocyte proliferation, from a knockout mouse study (14). Aside from the gross changes associated with null status such as exencephaly, the knockout 17-day-old fetal mice showed a reduced body size, alterations in bone length and width, and delayed chondrogenesis (14). Although the function of p53 in the earlier developmental stages remains unknown, its high expression level suggests an important role in the development via its cell cycle regulation. Therefore, in the present study using the experimental protocol identical to the previous study (2), the PSK-induced modification of the change in p53 level by X-irradiation during early organogenetic period might have caused the further delay in the systemic development.

In the present study, many PSK-treated embryos exhibited a systemic developmental delay. However, they were generally proportional and symmetrical in the body parts. Therefore, the developmental delay in these embryos may represent a constitutional size

reduction and may not necessarily be a defective sign. The coincidence of the developmental delay with the reduced teratogenesis in the PSK-treated embryos is intriguing but the relation and detailed mechanisms remain speculative. Defects caused by teratogens result in malformation when the repair or recovery mechanisms can not work not only effectively but also not in time, since the prenatal development is a fairy strictly scheduled chronological process (15). PSK itself showed no significant developmental toxicity in mice or rats at the dose up to 1000 mg/kg, five times higher than that used in the present study (16). If the PSK-induced systemic and proportional developmental delay could spare time for the embryo to recover the damage in time, it might help to reduce the incidence of malformations.

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