Influence of Age on Susceptibility to *Pseudomonas aeruginosa* Exotoxin A-induced Hepatotoxicity in Long-Evans Rats

Hsiao-Li CHUANG1)*, Yen-Te HUANG2)*, Chien-Chao CHIU1), Hans Hsien-Chuan CHEN3), Ying-Yueh CHU4) and Ter-Hsin CHEN5)**

1)Department of Veterinary Medicine, National Chung Hsing University, Taichung 402, 2)National Laboratory Animal Center, National Applied Research Laboratories, Taipei 115, 3)Yushu BiotechPath Consulting, Ltd., Taipei 115, 4)Department of Food, Health and Nutrition Science, Chinese Culture University, Taipei 119 and 5)Institute of Veterinary Public Health, National Chung Hsing University, Taichung 402, Taiwan

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ABSTRACT. Epidemiological investigations suggest that increased age is associated with susceptibility to infection. *Pseudomonas aeruginosa* (*P. aeruginosa*) infection and associated exotoxin A (PEA) toxicity have been reported in hospitalized elderly patients and young children with cystic fibrosis. The present study investigated age-related differences in PEA-induced hepatotoxicity in post weaning (PW, 3 weeks), young adult (YA, 12 weeks), and mature adult (MA, 60–64 weeks) rats. PEA (20 µg/kg) was injected intravenously and mortality, clinical chemistry, hepatic histopathology, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) and PCNA (Proliferating cellular nuclear antigen) staining, and serum cytokine levels were assessed at specific time points, up to 72 hr post-exposure (HPE). Mortality in MA rats was 100% at less than 48 HPE. Serum ALT levels in MA rats were approximately 5-fold greater than levels in PW and YA rats at 36 HPE. MA rat liver histological sections showed diffuse hepatocellular necrosis. In contrast, hepatocellular apoptosis, demonstrable by the TUNEL method, was noted simply in the perportal and midzonal regions from 36 to 48 HPE. Increased morphological mitoses and PCNA-positive hepatocytes were seen in PW and YA rats at 72 HPE. These parameters were correlated with age-dependent significant increases in TNF-α, IL-2, IL-6, and IL-18 levels. These data suggest that inflammatory cytokines play an important role in age-related differences in PEA-induced hepatotoxicity. Moreover, these cytokines might correlate with different patterns histopathologic features at various ages.

KEY WORDS: age, cytokines, PCNA, *Pseudomonas aeruginosa* exotoxin A, TUNEL.


Age-related changes in immune and physiological functions in humans and laboratory rodents have been extensively investigated [13, 20]. Many studies have noted that subject age is a critical factor in many experimental models of liver disease, such as acetaminophen, carbon tetrachloride, lipopolysaccharide (LPS), α-galactosylceramide, and cadmium-induced liver injury [5, 9, 15, 22, 23, 26]. In these model systems, mature adults exhibit greater susceptibility than young adults to injury by xenotoxic substances that induce various cytokines released from Kupffer cells (KCs) and T-cells. Several studies have shown that tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and interleukin-6 (IL-6) are the critical inflammatory mediators in the development of acute liver damage [15, 17]. The impact of age on cytokine production has been examined in 2 animal models that provided possible mechanisms of age-associated changes involving immune system activation. One model is the LPS-induced hepatotoxicity seen in senescent rats that exhibit higher levels of TNF-α, IL-1β, and IL-6 compared with young rats [23]. The other model is the cadmium-induced liver injury where an effect of increased age on inflammatory cytokines expression was noted [25, 26].

These studies suggest that increased age is strongly correlated with the degree of liver disease severity. Compromised innate immune responses to infection may contribute to increased *Pseudomonas aeruginosa* (*P. aeruginosa*) infections in the elderly animals [10]. In contrast, the primary cause of morbidity and mortality in children with cystic fibrosis is mainly by *P. aeruginosa* [6]. *P. aeruginosa* exotoxin A (PEA) is regarded as the major and most lethal virulence factor produced by the majority of *P. aeruginosa* clinical isolates [14]. *In vitro* studies have shown that PEA induces peritoneal macrophages to synthesize interleukin-1alpha (IL-1α) and KCs to secrete TNF-α [4, 8]. Reports from Schumann et al. [18, 19] and our own research [3] have shown that in mice and rats, PEA activates T-cells, natural killer cells, and KCs resulting in changes in the production of a variety of cytokines, including not only TNF-α, but also IL-2 and IL-6. These cytokines are regarded as important mediators of PEA-induced hepatotoxicity.

In the present study, we investigated the effects of age and the role of cytokine induction on animal survival, clinical chemistry, histopathology, immunostaining, and serum cytokines concentrations in a rat model of PEA-mediated liver injury.

MATERIALS AND METHODS

Reagent: PEA was obtained from Calbiochem Chemical...
Company (La Jolla, CA). PEA was dissolved in Dulbecco’s phosphate buffered saline (DPBS) (Gibco, Grand Island, NY) to obtain a final injection concentration of 10 µg/ml.

**Animals:** A total of 109 specific pathogen-free male Long-Evans (Narl: LE) rats, that included post weaning (PW, 3-wk old); young adult (YA, 12-wk old); and mature adult group (MA, 60–64-wk old), were obtained from the National Laboratory Animal Center (NLAC, Taipei, Taiwan). Animals were maintained at 22 ± 1°C, with 55–65% relative humidity and a 12 hr light-dark cycle, and were given a standard laboratory rat diet and water *ad libitum*. All experiments were performed by approval of the institutional animal care and use committee.

**Experimental designs:** The first experiment evaluated survival rates of PEA-treated PW, YA, and MA rats (n=8 per group). Rats were intravenously injected with PEA (20 µg/kg) and observed up to 72 hr post-exposure (HPE). The second experiment evaluated 20 µg/kg PEA-induced hepatotoxicity in PW (n=30), YA (n=30), and MA (n=25) rats at various time-points based on correlation of clinical chemistry, histopathology, and cytokine levels. Five rats each were sacrificed in the PW and YA groups at 0, 12, 24, 36, and 48 HPE, and in the MA group at 0, 12, 24, 36, and 48 HPE. Animals were euthanatized with CO2 asphyxiation followed by exsanguination and blood was withdrawn by cardiac puncture. In order to minimize animal use, 5 rats each in the 3 age group were injected with DPBS only. Age-specific liver samples were used as controls for TUNEL and PCNA immunostaining.

**Clinical chemistry:** Blood sample of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and icterus index levels were assayed using an automatic analyzer (HITACHI 717, Hitachi, Tokyo, Japan).

**Histopathological examination:** Livers removed from sacrificed rats at appropriate time points outlined above were fixed in 10% buffered neutral formalin solution, embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E) for histopathological examination.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) stain:** A portion of liver was fixed with Tissue Embedding Medium and rapidly frozen in a hexane environment with dry ice plus acetone prior to storage at –70°C. Tissues were sectioned at 4 µm for TUNEL staining. TUNEL assay was performed according to the manufacturer’s protocol using an *in situ* Cell Death/AP Detection kit (Roche Diagnostics, Mannheim, Germany). TUNEL-positive hepatocytes at periportal and pericentral areas were quantitated by counting the number of TUNEL-positive hepatocytes in 5 similar sized fields at higher power (×200).

**Proliferating cell nuclear antigen staining (PCNA stain):** Four µm liver sections were deparaffinized and blocked endogenous peroxidase by 3% hydrogen peroxide, incubated at 37°C for 30 min with a mouse monoclonal anti-PCNA antibody (Zymed, South San Francisco, CA), and then incubated with Picture™ HRP Polymer conjugate (Zymed, South San Francisco, CA) at room temperature for 20 min. HRP localization was visualized using a Single Solution AEC kit (Zymed, South San Francisco, CA). The PCNA-positive hepatocytes at periportal and pericentral areas were quantitated by counting the number of PCNA-positive hepatocytes in 5 similar sized fields at higher power (×200).

**Quantitative determination of serum cytokines:** Serum TNF-α, IL-2, IL-6, and IL-18 cytokines levels were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (Biosource, Camarillo, California).

**Statistical analysis:** Data obtained were expressed as mean ± standard deviation. The results were analyzed using one-way ANOVA followed by the Scheffé test for multiple groups. Results were considered significant at p<0.05. The log-rank test was applied for comparison of survival times between the different age groups.

**RESULTS**

**Animal survival:** Survival curves for PW, YA, and MA rats at various time-points following intravenous injection of 20 µg/kg PEA are presented in Fig. 1. Survival times were significantly greater in the PW and YA groups compared with the MA group. The mortality in MA rats was 100% at less than 48 HPE, whereas in the PW and YA rats, no death was noted until 72 HPE.

**Clinical chemistry:** No significant elevations in serum levels of ALT, AST, and icterus indices were noted in the control rats of any age group. In PEA-treated MA rats, ALT and AST levels increased significantly, peaking at 36 HPE. In the PW and YA groups, ALT and AST increased only moderately, peaking at 48 HPE and then declining thereafter (Fig. 2A, B). The serum icterus index as well as ALT also increased in an age-dependent manner at 36 HPE (Fig. 2C), with the MA group showing a significant elevation. No difference in these measures was seen between the PW and YA groups.
groups at any time point.

**Histopathology:** At 24 HPE, pronounced periportal hepatocytes necrosis was observed in the MA rats (Fig. 3A). The lesion extended to midzonal and even to the entire lobules at 36 HPE (Fig. 3D). A few apoptotic hepatocytes and single cell necrosis were also noted. In these rats, the hepatic cords appeared to be dissociated and the bile ducts were hyperplastic. In addition, some shrunken, necrotic hepatocytes with pyknotic nuclei were also noted. In the livers of YA rats, only mild apoptotic hepatocytes were observed in the periportal regions at 24 HPE (Fig. 3B). Then, numerous apoptotic bodies, swollen hepatocytes, and single cell necrosis were observed from 36 (Fig. 3E) to 48 HPE. These lesions started in the periportal areas and extended to the midzonal region were upon time dependant. These affected hepatic parenchymal cells exhibited predominantly apoptotic morphology, although some parenchymal cells had oncocytic characteristics. In the PW rats, liver histopathologic changes were similar to those observed in the YA group (Fig. 3C, F). However, only small numbers of degenerated hepatocytes but numerous mitotic figures, were observed in PW and YA groups at 72 HPE. No histopathological alterations were observed in PW, YA, or MA rats treated with DPBS.

**TUNEL and PCNA staining:** In agreement with the observations noted for H&E-stained liver tissue sections, the MA rat livers rarely showed TUNEL-positive hepatocytes in the periportal or pericentral areas at 24 and 36 HPE. In contrast, in the PW and YA groups, numerous TUNEL-positive hepatocytes and apoptotic bodies were present. In the periportal areas, TUNEL-positive hepatocytes in the PW and YA groups were significantly greater than MA group at 24 and 36 HPE (Fig. 4). No TUNEL-positive hepatocyte was present in the PW and YA control groups, and a few were present in the MA control group. The control rats at 0 HPE were used as co-controls for age-matched PEA-treated rats at 36 HPE.

Significantly greater numbers of PCNA-positive hepatocyte nuclei were noted in PW rats compared with YA rats in the periportal areas but not in pericentral areas (Fig. 5) at 72 HPE.

**Serum cytokines levels:** TNF-α increased following PEA treatment and peaked in the MA group at 36 HPE. In the YA group, increased TNF-α was noted at 24 HPE and reached a peak at 48 HPE, followed by a progressive decline until 72 HPE. In the PW group, TNF-α was increased at 36 HPE and reached a peak at 48 HPE (Fig. 6A). A significantly greater increase in serum IL-6 was observed in the MA group in comparison with the PW and YA groups at 36 HPE (Fig. 6B). Serum IL-2 levels in the MA group were increased at 24 HPE and continued to rise at 36 HPE. Trace amounts of IL-2 were observed in the YA and PW groups, which reached a peak at 36 HPE and 48 HPE, respectively (Fig. 6C). IL-18 was detected in the MA group at 24 and 36 HPE at levels that were significantly (p<0.05) higher than those of the PW or YA groups (Fig. 6D). Serum TNF-α, IL-2, IL-6, and IL-18 were not detected in the control rats from any age group.
DISCUSSION

PEA is one of the most important virulence factors produced by *P. aeruginosa* and is known to cause severe liver damage in experimental animals [3, 18, 19]. However, the age-related alterations in the PEA-induced hepatotoxicity...
are not well understood. Based on our previously reported findings, a non-lethal dose of PEA at 20 $\mu$g/kg was utilized in the present study to determine the influence of age on the susceptibility to the PEA-induced hepatotoxicity in rats.

The present results revealed several significant age-related differences in response to PEA injection. For mortality, up to 100% mortality was observed in MA rats at less than 48 HPE. In contrast, all PW and YA rats survived up to 72 HPE. These data suggest that the MA group was more sensitive to PEA treatment than the PW and YA groups.

Periportal hepatocytes necrosis developed earlier in the MA rats starting at 24 HPE. Thereafter, the MA rat livers presented with diffuse necrosis concomitant with only a few apoptotic hepatocytes. Paucity morphological apoptosis was confirmed by TUNEL staining. Moreover, significantly increased serum ALT and AST also confirmed the severe hepatic necrosis. In the YA and PW groups, there were numerous apoptotic bodies, swollen hepatocytes, single cell necrosis, and TUNEL-positive hepatocytes. The MA group presented a pattern of diffuse necrosis that was distinctly different from the apoptosis observed in the YA and PW groups. Hepatocyte cell death typically follows one of two patterns: necrosis or apoptosis [12]. In signaling pathway of TNF-\(\alpha\)-induced hepatocyte cell death, caspase contributes to apoptosis, but reactive oxygen species contribute to necrosis [22, 24]. Hence, it is possible that different pattern of cell death in MA rats (diffuse necrosis) compared with YA and PW rats (apoptosis and single cell necrosis) might be correlated with levels of inflammatory cytokines and oxidative stress in PEA-induced hepatotoxicity. However, the underlying mechanism for these differences needs to be clarified through further study. In the PW and YA groups, no significant differences were observed in the abundance of TUNEL-positive hepatocytes in the periportal or pericentral areas. However, we observed more mitotic hepatocytes and PCNA-positive hepatic nuclei (an index of mitosis) in PW rats than in YA rats at 72 HPE. The association of hepatocyte mitosis with age has been noted previously [1, 16].

Age-related effects on inflammatory cytokine production induced by PEA have not been previously reported. We investigated the possible relationship between hepatic inju-
rious indications (Fig. 2A-C) and cytokines levels (Fig. 6A-D). PEA treatment significantly increased serum levels of the cytokines TNF-α, IL-2, IL-6, and IL-18 in MA rats. Cytokine responses in rats are known to vary with age [2, 21] and functional differences have been shown to be responsible for increases in the severity of hepatotoxicity observed in aging animals [2, 25]. In agreement with these reports, we found that age-related differences were associated with KCs-related cytokines levels in PEA-induced liver injury. Serum TNF-α and IL-6 increased to a greater degree in MA rats than in PW or YA rats. The role of TNF-α in PEA-induced hepatotoxicity has been demonstrated using an anti-TNF-α antibody and TNF-α receptor 1 and/or 2 knockout mice [18, 19]. Moreover, pretreatment of rats with either GdCl3 or an anti-TNF-α antibody eliminated the hepatotoxicity induced by 40 µg/kg PEA (unpublished data). Laithwaite et al. also reported that LPS can inhibit PEA-induced macrophage cytotoxicity in vitro [11]. It has been suggested that PEA toxicity may be related to expression of low-density lipoprotein receptor-related protein on KCs. The IL-6 is involved in hepatic inflammatory responses [17, 18, 23]. Our previous study also showed that IL-6 levels were PEA dose dependent and were correlated with liver injury [3]. The involvement of levels of TNF-α and IL-6 in age-related differences in hepatotoxicity has been found in this study.

T-cell-related cytokines, including IL-2 and IL-18, were significantly increased in the MA group compared to the PW and YA groups. Both Fagioni et al. [7] and our research group have reported that levels of IL-2 and IL-18 were correlated in PEA-treated mice and rats. Significant increases in IL-2 and IL-18 has also been reported in conjunction with severe liver injury [7, 17, 18]. Taken together, increased levels of KC- and T-cell-related cytokines suggests that MA rats may be more sensitive than YA and PW rats to PEA-induced liver injury.

In summary, the present study demonstrated that age is an important factor determining the susceptibility of rats to hepatotoxicity. Moreover, we found differences in different patterns of PEA-induced histopathologic features, with predominant liver necrosis in older rats and apoptosis in younger rats. Significantly increased serum levels of TNF-α, IFN-γ, IL-2, and IL-6 were also age-related. Our results support the concept that there are specific differences between pediatric and adult populations in the response to liver toxicants such as PEA.

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