Inflammation, Vol. 36, No. 6, December 2013 (© 2013)
DOI: 10.1007/s10753-013-9685-0

Comparison of α_2-Macroglobulin Synthesis by Juvenile vs. Mature Rats after Identical Inflammatory Stimulation

Takashi Kuribayashi,[1] Tetsuro Seita,[1] Katsuhito Kawato,[2] Shunsuke Yamazaki,[3] and Shizuo Yamamoto[1,4]

Abstract—Synthesis of α_2-macoglobulin (α2M) by 3-week-old juvenile rats was compared to that of mature 7- and 11-week-old rats. Serum concentrations of α2M, interleukin (IL)-6- and cytokine-induced neutrophil chemoattractant (CINC)-1 were measured by enzyme-linked immunosorbent assay. The area under the concentration vs. time curve (AUC) for α2M was significantly different among the three groups. The synthesis of α2M increased in an age-dependent manner. No significant difference was observed for the AUC of IL-6, but that of CINC-1 in 3-week-old rats was significantly lower than that in 7- or 11-week-old rats. These results suggest that synthesis of α2M was increased in mature compared to juvenile rats, possibly due to differences in liver function. The maximum concentration of CINC-1 in 3-week-old rats was observed 6 h after turpentine oil injection. The serum concentrations of IL-6 and CINC-1 increased more quickly in juvenile rats than in mature rats after inflammatory stimulation.

KEY WORDS: juvenile rats; mature rats; α2M; IL-6; CINC-1.

INTRODUCTION

Acute-phase proteins are useful as inflammatory markers [1–5]. α_2-Macroglobulin (α2M) is a typical acute-phase protein in rats [6–8]. α2M reacted more sensitively than did α_1-acid glycoprotein (AAG) after inflammatory stimulation in rats [9, 10]. The authors previously reported α2M kinetics in rats. On the other hand, C-reactive protein (CRP) is a typical acute-phase protein in dogs [1, 4, 10, 11]. The serum peak concentration of CRP in 1-month-old dogs after injection of turpentine oil was lower than that in 3- or 18-month-old dogs [12]. However, AAG did have a significantly different level among the three groups of dogs [12]. Therefore, characteristic acute-phase protein levels change with age. To our knowledge, differences in synthesis of α2M by juvenile vs. mature rats have not been investigated previously. The aim of this study was to clarify whether such differences exist. Furthermore, levels of interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-1, considered to contribute to the synthesis of α2M [10, 13, 14], were assessed.

MATERIALS AND METHODS

Animals

A total of 15 Sprague–Dawley rats (3, 7, and 11 weeks of age) were purchased from Charles River Laboratories Japan (Yokohama, Kanagawa, Japan). The body weights of adult male rats ranged from 200 g for 7-week-old rats to 400 g at 11 weeks of age [15]. Rats aged 7 and 11 weeks were considered "mature" rats in this

[1] Graduate School of Environmental Health, Azabu University, 1-17-71 Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan
[2] Laboratory of Immunology, Azabu University, 1-17-71 Chuo-ku, Fuchinobe, Sagamihara, Kanagawa 252-5201, Japan
[3] Laboratory of Physiology, Department of Food and Nutrition, Kamakura Women's University, 6-1-3 Ofuna, Kamakura, Kanagawa 247-8512, Japan
[4] To whom correspondence should be addressed at Graduate School of Environmental Health, Azabu University, 1-17-71 Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan. E-mail: kuribaya@azabu-u.ac.jp

study, and rats weaned 3 weeks after delivery [16] were considered "juvenile" rats. Rats were kept in isolation at a temperature of 23±2°C and relative humidity of 55±10 % on a 12/12 dark (20:00–8:00)/light (8:00–20:00) cycle, and the air was exchanged 12 or more times per hour. Rats were fed MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed free access to water. All experiments conformed to Japanese regulations concerning animal care and use, as described in the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, JALAS, 1987). The present animal experiment was approved by the Institutional Animal Care and Use Committee of Azabu University.

Animal Experimental Design

Turpentine oil (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) was intramuscularly injected at 0.2 ml/kg body weight. Turpentine oil has been used to induce inflammation in many previous studies, reliably and with little individual variation [26–28] and, therefore, was chosen to induce inflammation in this study. Blood was collected by ventricular puncture before turpentine oil injection and 6, 12, 24, 48, 72, and 96 h after injection under anesthesia with pentobarbital (Kyoritsu Seiyaku Corporation, Tokyo, Japan).

Measurements of α2M, IL-6, and CINC-1

The serum concentrations of α2M were measured by enzyme-linked immunosorbent assay (ELISA) of samples collected before treatment and 24, 48, 72, and 96 h after turpentine oil injection by the method described by Honjo *et a*l. [7]. The commercial ELISA kits for measurement of serum concentrations of IL-6 and CINC-1 in preinjection and 6, 12, and 24 h postinjection samples were purchased from Invitrogen Corporation (CA, USA) and Panapharm Laboratories Co., Ltd. (Kumamoto, Japan), respectively.

Statistical Analysis

The peak serum concentrations (C_{max}) of α2M, IL-6, and CINC-1 of individual rats were used for analysis. The area under the concentration vs. time curve (AUC) was calculated by the traperizol method. The serum concentrations of α2M, IL-6, and CINC-1 were analyzed using the Student's *t* test. Differences in *p* values <0.05 were considered significant.

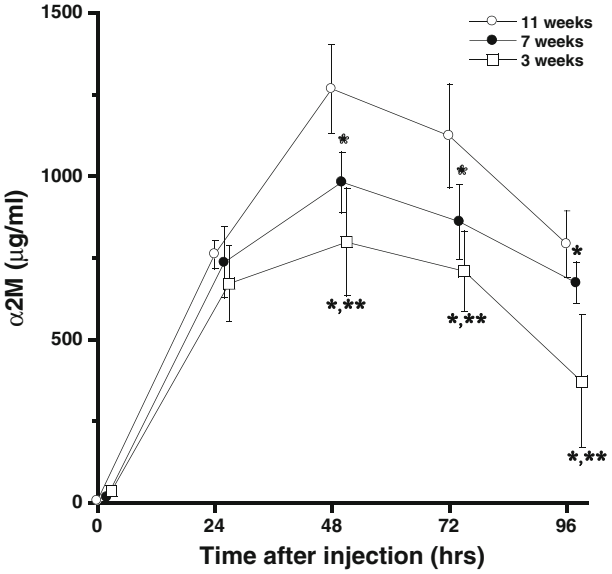

Fig. 1. Changes in serum concentrations of α_2-macroglobulin (α_2M) in 3-, 7-, and 11-week-old rats after injection of turpentine oil. Mean± standard deviation (n=5). *Value differs significantly from that of 11-week-old rats (p<0.05). **Value differs significantly from that of 7-week-old rats (p<0.05).

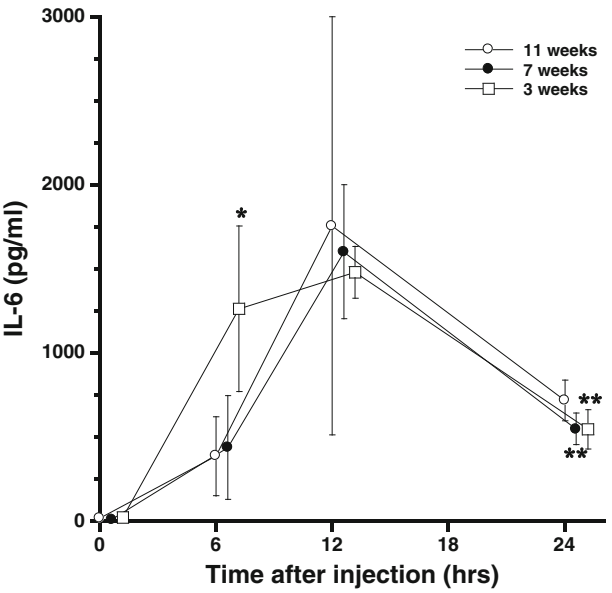

Fig. 2. Changes in serum concentrations of interleukin (IL)-6 in 3-, 7-, and 11-week-old rats after injection of turpentine oil. Mean±standard deviation (n=5). *Value differs significantly from that of 3-week-old rats (p<0.05). **Value differs significantly from that of 11-week-old rats (p<0.05).

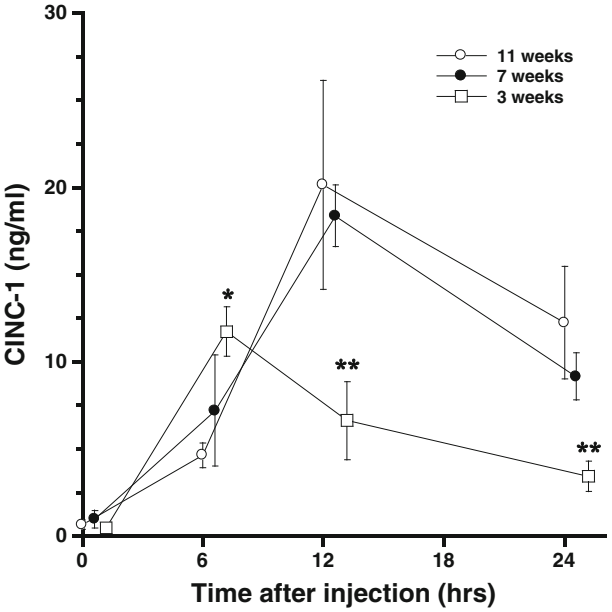

Fig. 3. Changes in serum concentrations of cytokine-induced neutrophil chemoattractant-1 (CINC-1) in 3-, 7-, and 11-week-old rats after injection of turpentine oil. Mean±standard deviation (n=5). *Value differs significantly from that of 3-week-old rats (p<0.05). **Value differs significantly from that of 7- and 11-week-old rats (p<0.05).

RESULTS

Changes of serum concentrations of α2M, IL-6, and CINC-1 are shown in Figs. 1, 2, and 3, respectively. The C_{max} of α2M was observed 48 h after turpentine oil injection. The C_{max} of IL-6 was observed 12 h after turpentine oil injection. The serum concentrations of α2M in 3- and 7-week-old rats at 48, 72, and 96 h after turpentine oil injection were significantly lower than that in 11-week-old rats. The serum concentrations of IL-6 and CINC-1 in 3-week-old rats 6 h after turpentine oil injection were significantly higher than those in 7- and 11-week-old rats.

The C_{max} and AUC of α2M, IL-6, and CINC-1 are shown in Table 1. The AUC of α2M significantly increased in an age-associated manner but that of IL-6 did not differ significantly among the three age groups. The AUC of CINC-1 in 3-week-old rats was significantly lower than that in 7- and 11-week-old rats. The C_{max} was calculated from individual rat data. The C_{max} of α2M and IL-6 increased in an age-associated manner. Significant differences in C_{max} of α2M and IL-6 were observed among 3-, 7-, and 11-week-old rats. The C_{max} of CINC-1 in 3-week-old rats was significantly lower than that in 7- and 11-week-old rats; however, no significant difference was observed between 7- and 11-week-old rats.

DISCUSSION

The synthesis of α2M by juvenile and mature rats subjected to the same inflammatory stimulation was compared. The C_{max} values for α2M in the three rat groups were observed 48 h after turpentine oil injection, and serum concentrations of α2M decreased. The kinetics of α2M were not different among the three groups. The AUC is an essential parameter for comparing the amount of absorption of candidate drug substances from the small intestine [17, 18]. Also, comparisons of AUC are considered to be more appropriate than comparison of serum concentrations at each time point for ascertaining differences in synthesis of α2M. The AUC of α2M increased with age and significant differences were observed among the three groups of rats. The synthesis of α2M in juvenile

Table 1. Kinetic Parameters of α_2-Macroglobulin, Interlekin-6, and Cytokine-Induced Neutrophil Chemoattractant-1 in 3-, 7-, and 11-Week-Old Rats

Parameters		Age (weeks)		
		3	7	11
C_{max}	α2M (μg/ml)	817.8±133.0	993.6*±79.1	1,308.8*,**±107.1
	IL-6 (pg/ml)	413.1±249.5	547.7*±95.3	716.5*,**±119.8
	CINC-1 (ng/ml)	11.7±1.4	20.1*±1.8	21.2*±6.0
AUC	α2M (μg h/ml)	57.0±10.7	70.1†±24.6	85.2†,††±2.8
	IL-6 (ng h/ml)	24.2±3.1	20.4±8.4	22.5±12.9
	CINC-1 (μg h/ml)	151.6±15.8	265.8†±105.7	284.2±71.6

* Value differs statistically from 3-week-old rat value (p<0.05)
** Value differs statistically from 7-week-old rat value (p<0.05)
† Value differs statistically from 3-week-old rat value (p<0.05)
†† Value differs statistically from 7-week-old rat value (p<0.05)

rats was significantly lower than that of mature rats in spite of the identical inflammatory stimulation.

Jinbo *et al*. investigated the serum concentrations of IL-1b, IL-2, IL-4, IL-6, CINC-1, IL-10, and IFN-γ in rats stimulated by injection of turpentine oil [14]. Only the serum concentrations of IL-6 and CINC-1 increased prior to α2M. Other cytokines did not change. Furthermore, Honjo *et al*. administered IL-6 or CINC-1 separated by gel chromatography from rat serum rich in α2M to rats [13]. The serum levels of α2M increased after injection of a solution including both IL-6 and CINC-1 [13]. IL-6 and CINC-1 were presumed to have contributed to the synthesis of α2M in the livers of these rats [19–24]. Only the concentration of CINC-1 in 3-week-old rats was significantly lower than in 7- and 11-week-old rats. Furthermore, in the current study, the AUC of IL-6 was not significantly different among the three groups of rats. We noted that the synthesis of IL-6 and CINC-1 did not differ among the three age groups. That the synthesis of albumin increased in an age-dependent manner has been reported [25]. Rat liver microsomal protein increased and the age-associated hepatocyte binuclear index increased [26–28]. Furthermore, liver protein synthesis in adult rats increased compared to that in young rats [29], and so the capacity of rat liver for synthesis of protein is thought to increase with age. The AUC of α2M in 11-week-old rats was significantly higher than in 7-week-old rats, even though profiles for IL-6 and CINC-1 were similar. These results suggest that the synthesis of α2M is increased with age due to differences in hepatic function at various ages.

A significant difference in the concentration of α 2M was not seen among the three groups at only 6 h after turpentine oil injection. The reason for this phenomenon may be that the concentrations of both IL-6 and CINC-1 in 3-week-old rats were significantly higher than in 7- and 11-week-old rats. However, Serushago *et al*. investigated IFN-γ levels in the media of mononuclear cell cultures from newborn and adult rats [30]. The levels of IFN-γ in cultures of mononuclear cells from newborns reached plateau levels on the third day of culture, whereas levels of IFN-γ from adult cell cultures had still increased after 5 days of culture [30]. On the other hand, levels of proinflammatory cytokines, including IL-6, in the extremely premature infant were higher than that of adult patients with *Bacillus cereus* sepsis [31]. High levels of proinflammatory cytokines means there was a significant response of macrophages/monocytes against *B*. *cereus* [31]. We presumed that the functions of phagocytes were more active in juvenile rats than in mature rats. However, the levels of IL-6 and CINC-1 increased earlier in 3- than in 7- and 11-week-old rats. The reason for this phenomenon has not been clarified by the present study. Further studies are needed to understand why IL-6 and CINC-1 in 3-week-old rats increased more quickly than in 7- and 11-week-old rats.

In conclusion, a greater amount of α2M is synthesized by mature than by juvenile rats, in spite of similar inflammatory stimulation. However, the synthesis of IL-6 and CINC-1 did not differ in an age-dependent manner. We think that this result could be attributable to differences in the synthesis of α2M in the liver. On the other hand, IL-6 and CINC-1 in 3-week-old rats increased more quickly than in 7- and 11-week-old rats. Further investigations will be needed to determine why these cytokines increase in juvenile rats.

ACKNOWLEDGMENTS

This research was partially supported by a research project grant awarded by Azabu University.

REFERENCES

1. Capsi, D., M.L. Baltz, F. Snel, E. Gruys, D. Niv, R.M. Batt, *et al*. 1984. Isolation and characterization of C-reactive protein from the dog. *Immunology* 53: 307–313.
2. Ceron, J.J., P.D. Eckersall, and S. Martinez-Subiela. 2005. Acute phase in dogs and cats: current knowledge and future perspectives. *Veterinaria Clinicology Pathology* 34: 85–99.
3. T.W., Du-Clos. 2000. Function of C-reactive protein. *Animal Medicine* 32: 274–278.
4. Eckersall, P.D., S. Duthie, M. Toussaint, E. Gruys, P. Heegaars, M.A.C. Lipperheide, *et al*. 1994. Standardization of diagnostic assays for animal acute phase proteins. *Advances in Veterinary Medicine* 41: 643–655.
5. Eckersall, P.D., M.J.A. Harvey, J.M. Ferguson, J.P. Renton, D.A. Nickson, and J.S. Boyd. 1993. Acute phase proteins in canine pregnancy (*Canis familiaris*). *Journl of Reproductive Fertility* 47: 159–164.
6. Inoue, S., T. Jinbo, M. Shino, K. Iguchi, M. Nomura, K. Kawato, *et al*. 2001. Determination of α_2-macroglobulin concentrations in healthy rats of various ages and rats inoculated with turpentine oil by enzyme-linked immunosorbent assay. *Journal of Experimental Animal Science* 42: 44–49.
7. Honjo, T., T. Kuribayashi, M. Matsumoto, S. Yamazaki, and S. Yamamoto. 2010. Kinetics of α_2-macrogobulin and α_1-acid glycoprotein in rats subjected to repeated acute inflammatory stimulation. *Laboratory Animals* 44: 150–154.
8. Fisher, N.A., H.E. Poulsen, B.A. Hansen, N. Tygstrup, and S. Keiding. 1989. Age dependence of rat liver function measurements. *Journal of Hepatology* 9: 190–197.
9. Jinbo, T., M. Motoki, and S. Yamamoto. 2001. Variation of serum α_2-macroglobulin concentration in healthy rats and rats inoculated *Staphylococcus aureus* or subjected to surgery. *Comparative Medicine* 51: 332–335.
10. Kuribayashi, T., M. Tomizawa, T. Seita, K. Tagata, and S. Yamamoto. 2011. Relationship between production of acute-phase

proteins and strength of inflammatory stimulation in rats. *Laboratory Animals* 45: 215–218.
11. Kuribayashi, T., T. Shimada, M. Matsumoto, K. Kawato, T. Honjo, M. Fukuyama, *et al*. 2003. Determination of serum C-reactive protein (CRP) in healthy beagle dogs of various ages and pregnant beagle dogs. *Experimental Animals* 52: 387–390.
12. Karadag, F., S. Kirdar, A.B. Karul, and E. Ceylan. 2008. The value of C-reactive protein as a marker of systemic inflammation in stable chronic obstructive pulmonary disease. *European Journal of Internal Medicine* 19: 104–108.
13. Hayashi, S., T. Jinbo, K. Iguchi, M. Shimizu, T. Shimada, M. Nomura, *et al*. 2001. A comparison of the concentrations of C-reactive protein and alpha1-acid glycoprotein in the serum of young and adult dogs with acute inflammation. *Veterinary Research Communications* 25: 117–126.
14. Honjo, T., T. Kuribayashi, Y. Mokonuma, A. Yamaga, S. Yamazaki, and S. Yamamoto. 2010. The effects of interleukin-6 and cytokine-induced neutrophil chemoattractant-1 on α_2-macroglobulin production in rat. *Experimental Animals* 59: 589–594.
15. Jinbo, T., T. Sakamoto, and S. Yamamoto. 2002. Serum α_2-macroglobulin and cytokine measurements in an cute inflammation model in rats. *Laboratory Animals* 36: 153–157.
16. Charles River Laboratories Japan Inc., Research and development data, body weight curve, CD(SD), Available at: http://www.crj.co.jp, Accessed May 23, 2013.
17. Okaichi, Y., and H. Okaichi. 1995. Development of spatial task performance in the radial arm maze in weaning rats. *Japan Journal Animal Pyschology* 45: 1–12. Japanese with English abstract.
18. Gao, P., M.E. Guyton, T. Huang, J.M. Bauer, K.J. Stefanski, and Q. Lu. 2004. Enhanced oral bioavailability of a poorly water soluble drug PNU-91325 by supersaturatable formulations. *Drug Development and Industrial Pharmacy* 30: 221–229.
19. Atef, E., and A.A. Belmonte. 2008. Formulation and *in vitro* and *in vivo* characterization of phenytoin self-emulsifying drug delivery system (SEDDS). *European Journal of Pharmaceutical Sciences* 35: 257–263.
20. Horn, F., U.M. Wegenka, C. Lutticken, J. Yuan, E. Roeb, W. Boers, *et al*. 1982. Regulation of α_2-macroglobulin gene expression by interleukin-6. *Animals N Y Academic Science* 737: 308–322.
21. Ling, P.P., R.J. Smith, S. Kie, P. Boyce, and B.R. Bistrian. 2004. Effects of protein malnutrition on IL-6-mediated signaling in the liver and the systemic acute-phase response in rats. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 287: 801–808.
22. Nijsten, M.W.N., E.R. DeGroot, H.J. TenDuis, J.H. Klaren, C.E. Hack, and L.A. Arden. 1987. Serum levels of interleukin-6 and acute phase response. *Lancet* 330: 921.
23. Sheikh, N., K. Tron, J. Dudas, and G. Ramadori. 2006. Cytokine induced neutrophil chmoattractant-1 is released by the noninjured liver in a rat acute phase model. *Laboratory Investigation* 86: 800–814.
24. Kuribayashi, T.,Seita, T., Momotani, E., Honjo, T., Yamazaki, S., Yamamoto, S. Correlation between synthesis of α_2-macroglobulin in hepatocytes and changes in serum cytokine levels in rats after inflammatory stimulation. Scand. J. Lab. Anim. Sci.(in press).
25. Van Bezooijen, C.F.A., C.F. Grell, and D.L. Knook. 1976. Albumin by liver parenchymal cells from young, adult and old rats. *Biochemical and Biophysical Research Communications* 71: 513–519.
26. Schmucker, D.L. 1998. Aging and the liver: an update. *Journal of Gerontology* 53A: B315–B320.
27. Pieri, C., I.Z.S. Nagy, G. Mazzufferi, and C. Giuli. 1975. The aging of rat liver as revealed by electron microscopic morphometry—I. Basic parameters. *Experimental Gerontology* 10: 291–304.
28. Pieri, C., I. Zs-Nagy, and G. Mazzufferi. 1975. The aging of rat liver as revealed by electron microscopic morphometry—II. Parameters of regenerated old liver. *Experimental Gerontology* 10: 341–349.
29. Monsoni, L., P.P. Mirand, M.L. Houlier, and M. Arnal. 1993. Age-related changes in protein synthesis measured in vivo in rat liver and gastrocnemius muscle. *Mechanics Aging Development* 68: 209–220.
30. Serushago, B., C. Macdonald, S.H.S. Lee, A. Stadnyk, and R. Bortolussi. 1995. Interferon-gamma detection in cultures of newborn cells exposed to *Listera* monocytogenes. *Journal of Interferon and Cytokine Research* 15: 633–635.
31. Saito, M., N. Takahashi, S. Ueda, Y. Kuwabara, M. Komiyama, Y. Koike, *et al*. 2010. Cytokine profile in a premature infant with systemic *Bacillus cereus* infection. *Paediatrics International* 52: e34–e36.