6価クロムによるマウスの生体内脂質過酸化促進と毒性との関係

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Induction of Lipid Peroxidation in Mice by Hexavalent Chromium and its Relation to the Toxicity

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ABSTRACT. Comparative effects of hexavalent ($K_2Cr_2O_7$: Cr(VI)) and trivalent chromium ($Cr(NO_3)_3$: Cr(III)) on the development of lipid peroxidation, and the relationship between the lipid peroxidation and damage to tissues were studied using male ddY strain mice. The animals were administered with either of two chemicals at a dose of 20 mg Cr/kg by a single intraperitoneal injection. The results obtained were as follows: (1) Lipid peroxidation in the liver, as measured by the synthesis of thiobarbituric acid reactive substances (TBARS), showed a significant increase at 24 and 48 hr after Cr(VI) injection, while in the kidney it was observed only at 48 hr. In the mice administered with Cr(III), TBARS formation in the liver went down below the control levels, while no change was observed in the kidney. (2) Chromium contents in the liver and kidney showed a maximum level at 6 hr after injection of Cr(VI) and then those declined to the half of the maximum level at 48hr, respectively. Chromium contents in the liver and kidney of the mice injected with Cr(III) were lower than those injected with Cr(VI) during the experimental period. (3) Increases of TBARS formation in the liver, chromium content in the liver and kidney, and ornithine carbamyl transferase (OCT) activity indicative of the liver cell damage, and urea nitrogen content in the serum, indicative of the kidney damage, observed at 24 hr after injection of Cr(VI) were inhibited by simultaneous injection of 100 mg/kg of L-ascorbic acid, as antichrome agent, respectively. These observations might suggest a possible causative role of lipid peroxidation in Cr(VI) toxicity. (4) Incremental formation of TBARS in the liver after Cr(VI) injection was inhibited by simultaneous injection of N,N-diphenyl-p-phenylenediamine (DPPD), an antioxidant, but DPPD did not suppress the increase of chromium content of the organs examined (the liver and kidney), serum OCT activity and urea nitrogen content observed after Cr(VI) injection. This finding contradicted with the conclusion described above. The results of this study confirmed Cr(VI) induces lipid peroxidatioin in the liver and kidney of the mice, while lipid peroxidation is not responsible for the tissue damage induced by Cr(VI)—KEY WORDS: chromium, lipid peroxidation, mouse, ornithine carbamyl transferase, urea nitrogen.

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Chromium, in traces, is known as an essential factor for the maintenance of life as well as the growth of animals [16]. However, its excessive intake is known as being toxic, mainly to the liver and kidney [12]. It is generally known that the acute toxicity of hexavalent chromium (Cr(VI)) to animals is higher than that of trivalent one (Cr(III)) [15]. In our previous experiment, it was also found that Cr(VI) compounds had more stronger inhibitory effect to the growth of cultured HeLa cells than Cr(III) compounds [26].

Lipid peroxidation *in vivo* is thought to cause membrane damage and to play an important role in the inductioin of tissue injuries by some chemicals. Its involvement in lung damage caused by air pollutants and herbicides, as well as in acute liver injury caused by carbon tetrachloride and ethanol has been reported [18]. Induction of lipid peroxidation in various tissues by heavy

metals such as cadmium, mercury, copper, nickel, cobalt, thallium, has also been reported [4, 8, 9, 19, 33]. On the other hand, it has also been proposed that the increase of lipid peroxidation in isolated hepatocytes, induced by heavy metals such as cadmium, mercury or vanadium, is not necessarily responsible for the cell injury induced by these metals [24]. Yonaha *et al.* [32] reported that Cr(VI) above 1 mM facilitated lipid peroxidation in rat liver microsomes, while Cr(III) showed an inhibitory effect. Ueno *et al.* [28] reported that the Cr(VI) facilitated lipid peroxidation in isolated rat hepatocytes, and that antioxidants such as N,N'-diphenyl-*p*-phenylenediamine(DPPD) or α-tocopherol inhibited the chromium-induced lipid peroxidation, while these antioxidants failed to reduce the decrease of cell viability induced by Cr(VI). However very little is known about how the faciliation of lipid peroxidation induced by heavy metals is associated with the manifestation of damaged tissues or cells.

The present study aimed to compare the effects of Cr(VI) and Cr(III) exposure on *in vivo* lipid peroxidation in mice. Furthermore, the relationship between lipid peroxidation and chromium-induced tissue injury was examined using L-ascorbic acid, an antichrome agent [11, 17, 20, 21] and DPPD, an potent antioxidant [30].

MATERIALS AND METHODS

Animals: Male ddY mice weighing 25 to 30 g were used for the study. The animals were allowed free access to commercial chow (mouse chow MF: Oriental Yeast Co., Ltd.) and water. Ambient temperature was maintained between 21 and 23°C, and the mice were exposured to a 12-hr light and 12-hr dark cycle during the study period.

Chemicals: The chemicals used were; potassium dichromate ($K_2Cr_2O_7$) as Cr(VI), chromium nitrate ($Cr(NO_3)_3$) as Cr(III) (Kanto Chemical Co., Inc.), L-ascorbic acid as an antichrome agent (Kanto Chemical Co., Inc.) and DPPD as an antioxidant (Tokyo Chemical Ind., Co., Ltd.). All chemicals and reagents employed were of commercial reagent-grade quality.

Injection: Potassium dichromate and chromium nitrate were dissolved in saline, and injected intraperitoneally into each group of five mice at a dose of 20 mg Cr/kg, respectively. In the other experiments, mice were injected intraperitoneally with 100 mg/kg of L-ascorbic acid in saline or 600 mg/kg of DPPD in corn oil immediately after the injection of Cr(VI).

Determination of lipid peroxidation: At the time required after injected of chromium, the mice were killed by decapitation, and the liver and kidney were quickly excised and washed in ice-cold 1.15% KCl. A 10% (W/V) homogenate of each tissue was prepared in ice-cold 1.15% KCl. These homogenates were used for the determination of lipid peroxidation, according to the thiobarbituric acid (TBA) method by Uchiyama and Mihara [27]. As an indicator of lipid peroxidation, amounts of TBA-reactive substances (TBARS) were expressed in terms of nmol malondialdehyde (MDA)/g wet wt. tissue.

Determination of chromium: Chromium was estimated in the tissue homogenates following digestion with HNO_3 using atomic absorption spectrophotometer (Shimazu Model AA-630-01).

Biochemical assay: Blood samples were collected at the decapitation in centrifuging tubes, and then centrifuged. The serum obtained were used for the biochemical assay which will be described below. As indicators of the liver and kidney damage, ornithine carbamyl transferase (OCT) activity [5] and urea nitrogen content [25] in the serum were measured using a test-kit from Wako Pure Chemical Ind.

In this paper, the data were expressed as

the mean±standard error of the results from the five mice. Comparisons between the mean values of the control and the treated mice were made by Student's *t*-test with $p<0.05$ as a level of significance.

RESULTS

TBARS formation in the liver and kidney of the mice injected with 20 mg/kg of Cr(VI) or Cr(III) is shown in Fig. 1. TBARS in the liver showed a marked increase after the injection of Cr(VI), showing a level about three times higher than the control level at 24 hr after the injection. Even at 48 hr after injection, higher levels above the control level were observed. Significant increase of TBARS in the kidney was also observed at 48 hr after injection of Cr(VI), however the degree of increase was lower than that in the liver. On the other hand, significant decrease of TBARS in the liver was observed at 6, 12, and 48 hr after injection of Cr(III). No significant change of TBARS in the kidney of the mice injected with Cr(III) was observed during the experimental period.

Chromium contents in the liver and kidney of mice injected with 20 mg/kg of Cr(VI) or Cr(III) are shown in Fig. 2. Chromium contents in the liver reached a maximum level (80 μg Cr/g wet wt. tissue) at 6 hr after injection of Cr(VI), and then decreased to the half of the maximum level at 48 hr. Chromium contents in the kidney was similar to those observed in the liver. In the mice injected with Cr(III), chromium contents in the liver and kidney were lower than those observed in the mice injected with Cr(VI) throughout the experimental period.

The relationship between lipid peroxidation and tissue injury induced by an injection of Cr(VI) was examined with L-ascorbic acid and DPPD. Figs 3–6 show TBARS, chromium content in the liver and kidney, serum OCT activity indicative of the liver cell damage, and serum urea nitrogen content indicative of the kidney damage, at 24 hr after injection of Cr(VI) (20 mg/kg) with or without L-ascorbic acid (100 mg/kg) or DPPD (200 mg/kg). Both L-ascorbic acid and DPPD showed a marked inhibition of the increase of TBARS levels in the liver

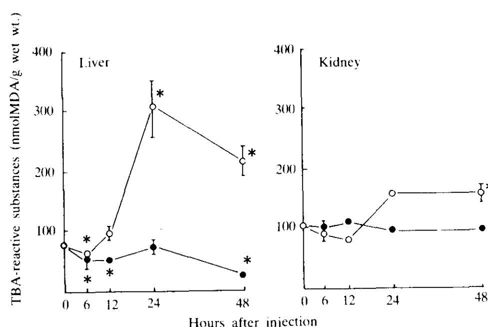

Fig. 1. Effects of hexavalent ($K_2Cr_2O_7$) and trivalent chromium ($Cr(NO_3)_3$) on lipid peroxidation in the liver and kidney of the mice. $K_2Cr_2O_7$ and $Cr(NO_3)_3$ were injected intraperitoneally into the mice at a single dose of 20 mg Cr/kg. As an indicator of lipid peroxidation, amounts of TBARS were expressed in terms of nmol malondialdehyde(MDA)/g wet wt. tissue. Each point represents the mean ± standard error for five mice. –○–, $K_2Cr_2O_7$; –●–, $Cr(NO_3)_3$.
*Denotes a significant change ($p<0.05$) from corresponding control ("0" time) value.

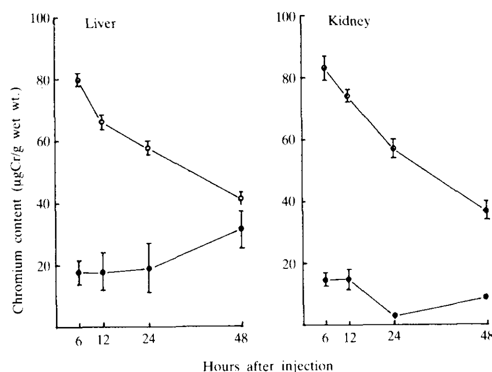

Fig. 2. Chromium contents in the liver and kidney of the mice injected with hexavalent and trivalent chromium. See Fig. 1 for experimental conditions. –○–, $K_2Cr_2O_7$; –●–, $Cr(NO_3)_3$.

that could be induced by Cr(VI), but of TBARS formation in the kidney was not inhibited by L-ascorbic acid (Fig. 3). Although L-ascorbic acid diminished the chromium contents in the liver and kidney of the mice that had been injected with Cr(VI), DPPD was found to have no effect on the chromium content either in the liver or kidney (Fig. 4). L-ascorbic acid suppressed the increase of serum OCT activity in the mice injected with Cr(VI), while DPPD enhanced that increase (Fig. 5). Similarly, L-ascorbic acid suppressed the increase of serum urea nitrogen content induced with Cr(VI), while DPPD did not affect it (Fig. 6).

DISCUSSION

In the present study, the injection of Cr(VI) to the mice caused significant increases in lipid peroxidation estimated by of TBARS synthesis in the liver and kidney. The injection of Cr(III) to the mice caused a significant decrease in lipid peroxidation in the liver, whereas no significant differences

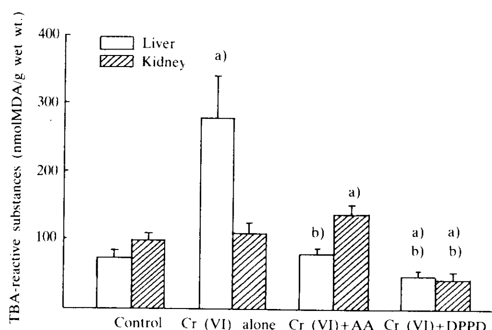

Fig. 3. Effects of L-ascorbic acid (AA) and N, N'-diphenyl-*p*-phenylenediamine (DPPD) on increases of TBARS formation in the liver and kidney of the mice induced by injection of hexavalent chromium. $K_2Cr_2O_7$ (20 mg Cr/kg) was injected intraperitoneally into the mice with or without AA (100 mg/kg) or DPPD (600 mg/kg).Control groups of animals were injected only saline instead of the chemicals tested. Each data were obtained 24 hr after injection.
a) Denotes a significant change ($p<0.05$) from corresponding control mice.
b) Denotes a significant change ($p<0.05$) from corresponding mice injected chromium alone.

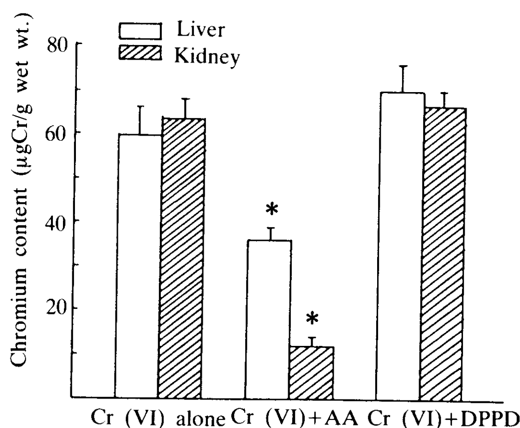

Fig. 4. Effect of L-ascorbic acid (AA) and N, N'-diphenyl-*p*-phenylenediamine (DPPD) on increase of chromium contents in the liver and kidney of the mice induced by injection of hexavalent chromium. See Fig. 3 for experimental conditions.
*Denotes a significant change ($p<0.05$) from corresponding mice injected chromium alone.

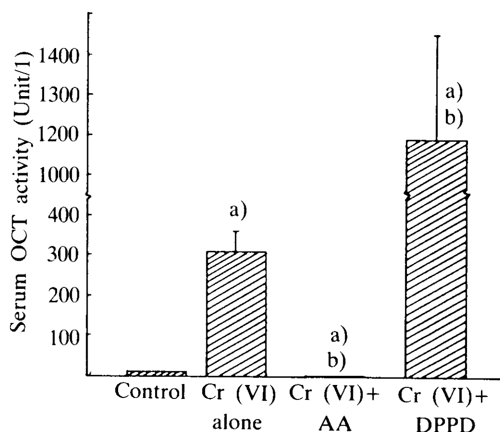

Fig. 5. Effects of L-ascorbic acid (AA) and N, N'-diphenyl-*p*-phenylenediamine (DPPD) on increase in serum ornithine carbamyl transferase (OCT) activity, as an indicator of liver cell damage, of the mice induced by injection of hexavalent chromium. See Fig. 3 for experimental conditions.
a) Denotes a significant change ($p<0.05$) from corresponding control mice.
b) Denotes a significant change ($p<0.05$) from corresponding mice injected chromium alone.

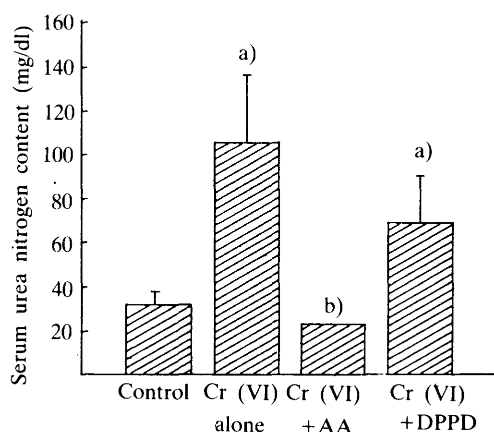

Fig. 6. Effects of L-ascorbic acid (AA) and N, N'-diphenyl-*p*-phenylenediamine (DPPD) on increase in serum urea nitrogen content, as indicator of kidney damage, of the mice induced by injection of hexavalent chromium. See Fig. 3 for experimental conditions.
a) Denotes a significant change ($p<0.05$) from corresponding control mice.
b) Denotes a significant change ($p<0.05$) from corresponding mice injected chromium alone.

were observed in the kidney. Chromium contents in the liver and kidney of the mice injected with Cr(VI) were higher than those injected with Cr(III).

Toxicity difference between Cr(VI) and Cr(III) has recently been discussed in several reviews [2, 10, 13, 17]. Generally, Cr(VI) compounds seem to be more toxic than Cr(III) compounds, and lower toxicity of Cr(III) might be related with relative impermeability of biological membranes to Cr(III) compounds. It has been reported that lipid peroxidation was induced by Cr(VI) in the rat liver microsomes [32] and isolated rat hepatocytes [28] *in vitro*, and in the rat liver *in vivo* [18]. However, it is not known whether Cr(VI)-induced lipid peroxidation is associated with the cellular or tissue injury induced by Cr(VI). In this paper, Cr(VI) caused an increase of lipid peroxidation in the liver and kidney, while Cr(III) caused decreases of lipid peroxidation below the control level in the liver, and did not affect on that in the kidney. These results suggest the association between the lipid peroxidation and toxicity of Cr(VI).

Ascorbic acid, reducing agent, was employed as an antichrome agent with effects of the prevention of chrome ulcers and treatment of systemic chromium poisoning [11, 17, 20, 21]. It is also known that ascorbic acid forms more stable Cr(III) complexes by the reductive chelate formation with Cr(VI) [21]. In this paper, L-ascorbic acid was able to inhibit not only the increase of the formation of TBARS in the liver but also that of chromium content in the liver and serum OCT activity which could be caused by Cr(VI) injection. From these results, it is assumed that inhibitory effect of L-ascorbic acid on Cr(VI)-induced lipid peroxidation and liver injury occurs as a result of the decrease in chromium transfer into the liver in the reducing process. L-ascorbic acid also suppressed increases in chromium content of the kidney and serum urea nitrogen content by Cr(VI) injection, however it facilitated TBARS formation in the kidney. These results contradict with the above results of experiments for the liver. It has been reported that the peroxidation in the microsomes induced by $HgCl_2$ or $FeSO_4$ is increased by ascorbic acid [28]. Ascorbic acid catalyzed peroxidation in the presence of metal ions is thought to involve the monodehydroascorbate radical [7]. Other evidence that ascorbic acid acts as synergism on antioxidant function of vitamin E [14]. Considering the above points, it is assumed that the increase of TBARS formation in the kidney of the mice injected with Cr(VI) and L-ascorbic acid simultaneously was induced by the effect of ascorbic acid in the presence of chromium or decrease in vitamin E in the kidney. Further study will be required to explain the mechanism of the increase of TBARS formation observed in the kidney of the mice injected with Cr(VI) and L-ascorbic acid.

Stacey and Kappus [23] reported that DPPD as antioxidant was able to block the Hg^{2+}-induced elevation in lipid peroxidation completely, but did not cause any reduction of the Hg^{2+}-induced loss of cellular lactate dehydrogenase in the isolated rat hepatocytes. They concluded from these results that the lipid peroxidation associated with the loss of viability of isolated rat hepatocytes is not directly responsible for this cell injury. Stacey *et al.* [22] also reported similar results for the cadmium. These results do not support the hypothesis that lipid peroxidation is responsible for the cellular injury observed with these chemicals. On the contrary, it has been reported that DPPD reduces the toxic effects of methylmercury or mercuric chloride *in vivo* [30, 31]. In this paper, a more critical evaluation of the matter by an injection of an antioxidant, DPPD administered simultaneously with Cr(VI) to the mice has shown a contrary result to the conclusion described above. That is, DPPD was able to suppress the increase of TBARS formation that could be induced by Cr(VI), however caused no reduction in the increase of serum OCT activity, serum urea nitrogen content, or tissue chromium content induced by injection of Cr(VI). These results indicate that the Cr(VI)-induced tissue injury is not associated with lipid peroxidation.

It has been known that lipid peroxidation occur as a result of the decrease in intracellular glutathione (GSH) [1, 3, 6, 23]. Ueno *et al.* [28] reported that the contents of intracellular GSH in isolated rat hepatocytes were diminished by Cr(VI) treatment, and lipid peroxidation in these cells induced by Cr(VI) were inhibited by addition of GSH. Thus, GSH may influence the metabolism and toxicity of chromium. Consequently, it would be necessary to make a detailed investigatioin on the effects of chromium in other systems related to lipid peroxidation such as GSH.

Our conclusion is that Cr(VI)-induced tissue injury is not involved in the concurrently observed lipid peroxidation.

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要　　約

6価クロムによるマウスの生体内脂質過酸化促進と毒性との関係：諏佐信行・上野俊治・古川義宣・道場尚輝・箕浦清二郎（北里大学獣医畜産学部獣医公衆衛生学教室）――6価（$K_2Cr_2O_7$）および3価クロム（$Cr(NO_3)_3$）を腹腔内投与（20mgCr/kg）したマウスにおける，組織内の脂質過酸化（チオバルビツール酸反応物質）と組織障害との関連性について検討し，以下の成績を得た.（1）6価クロムは，肝臓内脂質過酸化を著明に促進し，腎臓内のこれを僅かに促進したが，3価クロムは，これらを促進し得なかった.（2）6価クロム投与マウスの肝および腎臓内クロム含量は，3価クロム投与マウスのそれらより高かった.（3）還元剤であるL-アスコルビン酸は，6価クロムによって誘起された組織内脂質過酸化の促進，組織内クロム蓄積，肝臓（血清オルニチン・カルバミル・トランスフェラーゼ活性の増加）および腎臓障害（血清尿素窒素量の増加）をそれぞれ抑制した.（4）抗酸化剤であるN, N'-ジフェニル-p-フェニレンジアミンは，6価クロムによる組織内の脂質過酸化促進を抑制したが，クロムの蓄積および組織障害を抑制し得なかった. 以上の成績から，6価クロムは組織内の脂質過酸化を促進することが明らかとなったが，認められた脂質過酸化の促進は，6価クロムの毒性発現に直接関与しないことが示唆された.