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Absence of selected signs of toxicity from TCDD-treated, c-src deficient mice

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Upon entering cells, TCDD is known to initially bind to the cytosolic Ah receptor,[1-4] translocate into the nucleus where it forms a complex with "Arnt" and eventually binds with the dioxin response elements (DRE or XRE) located within the promoter sequence of selected genes and transactivates them. Largely unexplained, however, is the mechanisms by which TCDD causes many of its toxic effects *in vivo,* particularly those causing death.

In 1984 it was initially reported from this laboratory that TCDD causes a spectacular rise in protein kinases in the plasma membrane of the rat hepatocyte[5]. Subsequently, we were able to show that tyrosine kinases are the ones most affected at early stages. Furthermore, we showed that c-Src kinase is the kinase that is particularly activated at a very early stage of TCDD's action and is the one that stays at an elevated state for a very long period.[6] Based on these observations, our group has been investigating this research avenue for the last 15 years.

More recently, we have found in guinea pig adipocytes that c-Src kinase is physically associated with the Ah receptor in cytosol and could be activated as a result of TCDD's action on the Ah receptor. This phenomenon could be demonstrated even in isolated cytosol under a cell free condition.[7] Based on such an observation, a hypothesis[8] has been proposed from our laboratory that a part of TCDD's toxicity could be caused by this concomitant rise in c-Src kinase activity accompanying the reaction of TCDD binding to the Ah receptor.[8] Certainly, additional observations such as the rise in Ras proteins, AP-1 binding activities, down-regulation of the EGF receptor[9], etc. have also helped the formulation of the hypothesis. The key question we raised then was how we can test this hypothesis *in vivo.* After some search, we have decided that the most logical approach would be the use of c-Src knockout mice.

Here we show the results of our preliminary studies on the action of TCDD on c-Src deficient mice, demonstrating that this genetic alteration drastically alters the pattern of toxic actions of TCDD.

MATERIALS AND METHODS

TCDD was obtained originally from the Dow Chemical Co. (Midland, MI) as described previously[9]. All mouse strains were obtained from Jackson Laboratory (Bar Harbor, ME). They were B6, 129-Src^{tm1sor} (stock no. J2381) mice, consisting of homozygous c-src -/-, heterozygous -/+, and wild-type littermate controls, c-src +/+ mice. A preliminary study established that c-src -/- and -/+ mice express approximately 0 and 14.5% of c-Src kinase activity associated with the Ah receptor in hepatic cytosol as compared with that (=100%) of the c-src +/+ wild strain. Only young males were studied. Five mice were used for each batch of controls and treatments. Mice were intraperitoneally injected (i.p.) with 115 or 345 μg/kg of TCDD dissolved in a mixture of corn oil and acetone (9:1). Food and water were given *ad libitum,* and the body weight and the amount of food consumed were measured daily. At the end of 10-day observation period the 115 μg/kg TCDD-treated and the control mice were killed, and various organs were isolated and weighed. The high dose (345 μg/kg) treatment groups were kept for observation. The body weight and the mortality were recorded. The surviving individuals are still kept. c-Src kinase activities were assessed by immunoisolating and ^{32}p-phosphorylating RR-SRC as before[7,9].

RESULTS AND DISCUSSION

The effects of TCDD administration *in vivo* (115 μg/kg, single i.p. injection) on the weights of selected organs and the body were assessed at day 10 of post-treatment in homozygous c-src -/-, heterozygous c-src -/+ mice and their wild-type (c-src +/+) littermates. In the case of c-src +/+ mice, TCDD caused a decrease in body weight, an increase in the weight of liver, and significant reductions in the weight of adipose tissue and thymus (Fig. 1) and modest decreases in the weight of spleen, pancreas, and heart (data not shown). In contrast, the effects of the same TCDD treatment on changes in the weights of the body, adipose tissue and thymus were not significant in c-src -/+ mice. Because of the scarcity of the homozygous c-src deficient mice (c-src -/-) only one animal each, one for control and one for TCDD treatment at 115 μg/kg i.p. were tested. The result (Fig. 1) showed that the trend was similar in c-src -/- mice. On the other hand, the effect of TCDD to cause hepatomegaly (increased liver weight) was observed in all three strains. When the data were expressed in terms of the percentage of liver weight to body weight, this phenomenon of hepatomegaly became much more noticeable. To ascertain the responsiveness of the wild-type, c-src +/+ mice to TCDD, hepatic microsomal preparations from control and TCDD-treated (115 μg/kg after 10 days) mice were tested for EROD (7-ethoxyresorufin-O-desethylase) activities[10]. As expected, EROD activity was found to be induced in TCDD treated wild-type mice, actual values being 500 ± 51 (5 animals) for control and 1,300 ± 115 pmole/ng proteins (5 animals) for TCDD-treated. When the same test was conducted on the c-src -/+ mice, almost the same degree of induction was observed, the actual values being 510 ± 40 (5) and 1500 ± 120 (5) pmole/ng protein for control and TCDD treated -/+ mice, respectively.

To investigate whether the above phenomenon of reduced TCDD toxicity in c-src deficient mice is due to reduced c-Src kinase activities, the c-src +/+ mice were treated with geldanamycin, a tyrosine kinase inhibitor known to specifically prevent activation of c-Src or its very closely related kinases and the same TCDD toxicity tests were repeated. The results clearly showed that this chemical blocking method on c-Src kinase also produced the same trend with respect to the reduction of the body weight and adipose tissue loss as the genetic manipulation to suppress c-src expression. On the other hand, geldanamycin

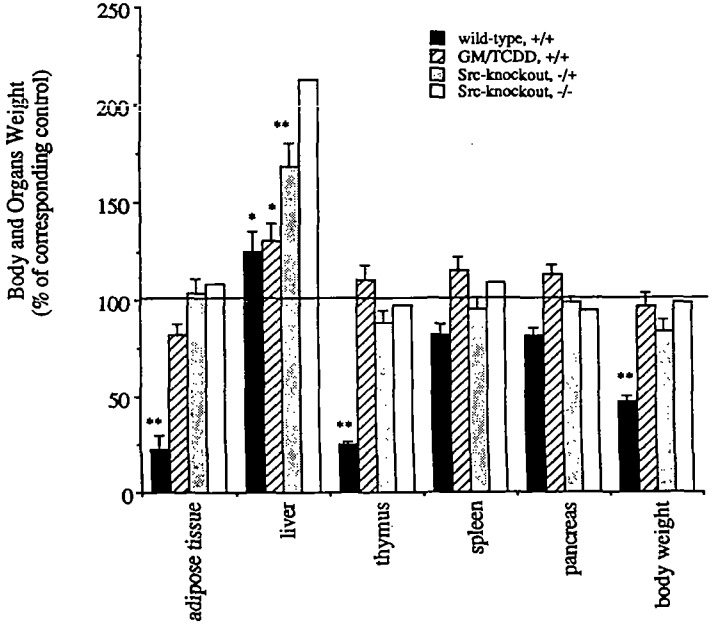

Fig. 1. Differential toxic effects *in vivo* of 115 µg/kg single i.p. treatment of TCDD on body and organ weights expressed after 10 days among wild type (+/+), c-src knockout (-/+ and -/-) and geldanamycin co-treated (200 µg/kg injection at day -1, 3 and 7), +/+ mice. The data are expressed as % of matched control mice (treated with vehicle only) at day 10 for each strain/treatment group which is set as 100. Statistically significant differences from control are shown as *, ** (Cochran t-test p≤0.05 and 0.01, respectively). Each group consists of 10 individuals, except -/- (only 1 animal due to the scarcity of its supply).

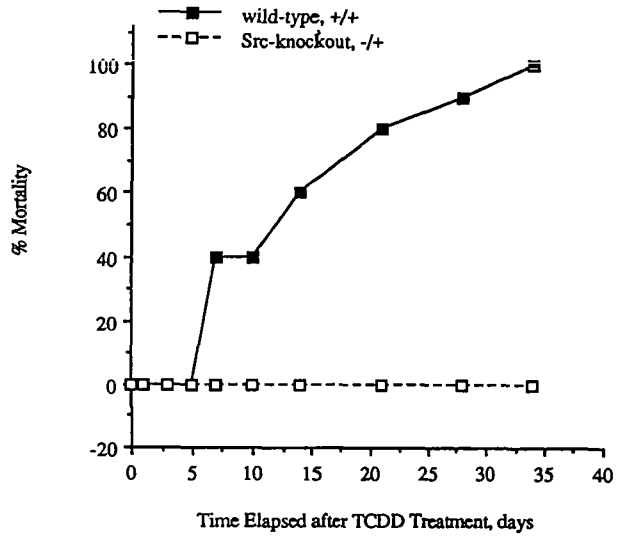

Fig. 2. Time course of lethal effect of a high dose TCDD (345 µg/kg single i.p.) treatment of wild (+/+) mice as compared to the lack of lethal response of c-src knockout (-/+) mice to the same TCDD treatment. The latter showed no overt sign of toxic effects and all are gaining weight (as of 9/5/96, 70 days after TCDD injection).

itself shows the effect of inducing thymic atrophy. Therefore, no conclusion could be made on the effect of TCDD on this organ.

To confirm that the above reduction in the effect of TCDD on organ weights of c-src deficient mice is related to reduction in the *in vivo* toxicity of TCDD, a higher dose of TCDD (345 μg/kg, which is known to cause 100% lethality in C57/Black mice) was tested on c-src -/+ and +/+ mice. As a result of this treatment, all of the c-src +/+ mice died (the last one died on day 34) as expected (Fig 2.), but none of the c-src -/+ mice died during the test period. This experiment is still in progress, and as of the time of the submission of this manuscript (i.e. 280 days post treatment) all c-src -/+ mice are still alive, gaining weight and showing no visible or overt signs of TCDD toxicity.

One of the most noticeable differences between c-src deficient and wild-type mice with respect to their response to TCDD is the selected absence of certain signs of toxicity in the former strains , while other effects of this toxicant are fully expressed. For instance, wasting syndrome appears to be totally lacking in TCDD-treated c-src deficient mice and yet induction of cytochrome P450 and hepatomegaly are fully expressed in the same mice. Such selective alteration of toxicity points to the specific role of c-src in mediating one of the major toxic signal transduction pathways of TCDD.

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REFERENCES

1. Tucker RE, Young AL and Grapy AP (Eds.), *Human and Environmental Risks of Chlorinated Dioxins and Related Compounds,* Plenum Press, New York, 1982.
2. Poland A and Knutson JC, *Annu Rev Pharmacol Toxicol* **22:** 517-554, 1982
3. Poland A and Kimbrough RD (Eds) in Biological Mechanism of Dioxin Action, Banbury Report, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984.
4. Bock KW, *Rev Physiol Biochem Pharmacol* **125**: 1-42, 1994.
5. Madhukar BV, Brewster DW and Matsumura F, *Proc Natl Acad Sci USA* **81**: 7407-7411,1984.
6. Bombick DW and Matsumura F, *J. Biochem Toxicol* **2**: 141-145, 1987.
7. Enan E and Matsumura F, *Biochem Pharmacol* **52**: 1599-1612, 1996.
8. Matsumura, F, *Biochem Pharmacol* **48**: 215-224, 1994.
9. Enan E and Matsumura F, *Biochem Pharmacol* **49**, 249-261, 1995.
10. Kennedy SW, Jones SP, and Bastien LJ, J Analyt Biochem 266: 362-370, 1995.