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Potentiation of iron accumulation in cardiac myocytes during the treatment of iron overload in gerbils with the hydroxypyridinone iron chelator CP94

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Gerbils administered iron dextran are the only animal species which have been shown to develop hemochromatosis of the liver and heart in the same manner as transfusion dependent homozygous thalassemics. The iron chelating hydroxypyridinone, CP94, has been administered prophylactically to iron overloaded gerbils in a dosing regime which favors the formation of bidentate chelated iron, to examine the possibility of additional toxicity being caused to the liver and heart by the bidentate chelated iron complex. Hepatic iron accumulation was inhibited by CP94 administration for up to 6 weeks, but not after 20 weeks. Iron accumulation in the heart was increased significantly after 6 and 20 weeks of chelator treatment. Pathological changes in both organs were markedly more severe after 20 weeks in chelator treated animals. There was a higher incidence of cardiofibrosis and more extensive liver fibrosis in iron overloaded, chelator treated animals after 20 weeks.

Keywords: cardiac myocytes, CP94, gerbil, iron

Introduction

Great interest has focused in recent years on the development of orally active iron chelators for the treatment of transfusion related hemochromatosis in cases of β-thalassemia. The ease of oral administration and lower cost of the hydroxypyridin-4-ones has led to the development and testing of new derivatives which might be used to supplement or replace desferroxamine (DFO), the currently used therapeutic. DFO, given as a subcutaneous infusion, chelates iron as a hexadentate complex with very high affinity, whereas the orally active hydroxypyridin-4-ones are bidentate chelators which may have very different properties of cell and tissue distribution which have not been investigated fully (Porter *et al.* 1989).

We have recently described a new animal model of human hemochromatosis which can produce both hepatic fibrosis (Carthew *et al.* 1991, 1992) and also cardiofibrosis (Carthew *et al.* 1993) in response to the parenteral administration of iron dextran to gerbils. The hepatic fibrosis and cardiofibrosis were dose dependent and occured at similar levels of tissue iron as those causing hemochromatosis of the liver (Bassett *et al.* 1986) and heart (Parkes *et al.* 1992) in man. As the liver and heart are the two of the principal target organs for the toxicity of iron in either human genetic hemochromatosis (Bothwell *et al.* 1989) or secondary transfusion related hemochromatosis due to β-thalassemia (Kan 1983) we have attempted to use this model to modify the onset of the pathology of iron overload in the gerbil with the orally active iron chelator 1,2-diethyl-3-hydroxypyridin-4-one (CP94) (Porter *et al.* 1990) and to examine for any toxicity due to the iron chelated complex formed during treatment.

In cases of transfusion dependent thalassemia, blood is given at regular intervals. Reticuloendothelial cells have been shown to release iron from catabolized red cells at a relatively constant rate and that with saturation of transferrin, a significant proportion of this iron is transferred from the spleen to the liver in small molecular weight complexes or in ferritin (Siegenberg *et al.* 1990). The presence of non-protein bound iron in particular has been consistently demonstrated in thalassemia (Graham *et al.* 1979) and is thought to be important in terms of organ toxicity due to free radical generation (Burkitt & Mason 1991). The usual method of testing the ability of iron chelators to reduce body iron stores has been to administer

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iron dextran as a single or small number of intraperitoneal injections followed by a period to allow the iron to be stabilized as ferritin and hemosiderin in tissues, before attempting to remove it with chelator (Porter *et al.* 1990). We have chosen to give iron dextran repeatedly during the attempt at chelation therapy as this was felt to be more applicable to the human therapeutic situation where serum ferritin levels are highly elevated (Pootrakul *et al.* 1988) as are levels of non-protein bound iron measured in the serum or plasma of patients with hemochromatosis (Grootveld *et al.* 1989).

By administering iron at regular intervals at a dose in excess of the amount which could be theoretically bound as the hexadentate chelate by CP94, we have favored the possibility of the bidentate iron chelate being formed. This allows us to examine the possible additional toxicity of the bidentate chelate of CP94 and iron in gerbils, which could increase damage to the liver and heart.

Materials and methods

Chelator treatment of iron overloaded gerbils

Two groups of four and single groups of 10 and 12 female Mongolian gerbils, 6–8 weeks of age, were given weekly injections of 200 mg kg^{-1} of iron dextran (Sigma, Poole, UK) in a volume of 4 ml kg^{-1}, subcutaneously in the back of the neck. One of the groups of four animals and the group of 12 were also given CP94 2 days after each iron injection, for 5 days a week at a dose of 125 mg kg^{-1} (free base equivalent) in saline, by gavage. This dose of CP94 chelator was the bidentate molar equivalent required to bind the iron administered. Two groups of four animals per group were dosed with control dextran and chelator by gavage as chelator toxicity controls.

Autopsy and examination of tissues

Six weeks after the commencement of treatment the groups of four animals given iron dextran and iron dextran plus chelator treatment were sacrificed in a rising concentration of CO_2 and cardiac bled for serum preparation. A full autopsy was performed and tissue was taken from liver and heart for freeze drying, prior to the determination of non-heme iron levels. Representative slices of liver tissue (all lobes) and heart tissue as well as spleen, kidney, pancreas, intestine, stomach and lungs (inflated) were immersion fixed in 10% neutral buffered formalin for histopathological examination. Paraffin wax sections (5 μm) were prepared of tissues and stained with hematoxylin & eosin, by the Perls' reaction of iron and van Geison stain for collagen (Bancroft & Stevens, 1977).

Iron determinations

Frozen tissue from the liver and heart was freeze dried and powdered prior to determination of non-heme iron content, as described previously (Carthew *et al.* 1993). All tissue iron values are means ± SEMs.

Statistics

Statistical evaluation of the results was performed using one way analysis of variance, with the Dunnett test for significance at the 5% level. Linear regression analysis was performed on the hepatic and cardiac non-heme iron levels for chelator and non-chelator treated animals at 20 weeks to determine any treatment related effects. This was carried out after fitting the data to a second-degree polynomial equation, which showed no evidence of non-linearity.

Results

The short-term effect of administering the oral chelator CP94 daily (5 days a week) on the accumulation of iron given once a week was to reduce significantly (at the 5% level) the iron levels in the liver after 6 weeks of treatment (see Table 1). The amount of non-heme iron present in the liver was only 50% (approximately) of that of the controls given only iron dextran, while the cardiac iron levels were surprisingly increased to statistical significance at the 5% level. The histological examination of liver tissues taken at this time point showed that there was less stainable hemosiderin iron in hepatocytes in the chelator treated group compared with the non-chelator iron dextran treated group. There was no histochemically demonstrable iron in myocytes in either group after 6 weeks of treatment.

By 19 weeks of treatment with iron dextran and chelator two animals in the chelator group had died and the rest of the animals were losing weight, or appeared clinically unwell (hunched, with reduced physical activity). At this point it was decided to terminate the study and the remaining animals were sacrificed at 20 weeks for comparative evaluation.

At autopsy it was evident that the chelator treated gerbils had a high proportion of livers which appeared micronodular. This was confirmed by subsequent histopathological examination (see Table 1). It was also apparent that the non-heme iron levels in the livers of chelator treated gerbils had increased to similar levels in the iron dextran treated animals and there was no longer a statistically significant difference in liver iron levels between the two groups (although the chelator group was lower). Detailed histopathological scoring of the liver pathology in the two groups showed that the severity of the hepatic fibrosis in the iron dextran treated group corresponded mainly to that of a bridging fibrosis (see Table 1), whereas the majority of liver pathology of the chelator group was considerably more severe with the development of micronodular livers (see Table 1) due to a progression of the hepatic fibrosis.

The cardiac pathology also differed between the chelator and non-chelator treated groups. Cardiac non-heme iron levels were significantly different in the two groups (at the 5% level), with the chelator treated group having higher iron levels than the group treated only with iron dextran. Histological examination for iron also showed

Table 1. Summary of the pathological effects in the liver and heart after CP94 chelator treatment in Mongolian gerbils with iron overload for 6 or 20 weeks

Treatment	Treatment period (weeks)	No. of animals examined	Hepatic iron levels (nmol mg $^{-1}$)	Hepatic fibrosis[a] (no. animals affected out of no. examined)	Cardiac iron levels (nmol mg^{-1})	Iron detected histologically in myocytes	Cardiac fibrosis (no. animals affected out of no. examined)
CP94 (Con)	6	4	13 ± 4	0/4	5.9 ± 0.6	0/4	0/4
CP94 + Fe/Dex	6	4	162 ± 12[b]	0/4	31.8 ± 2.9[b]	0/4	0/4
Fe/Dex	6	4	312 ± 19	0/4	24.8 ± 0.6	0/4	0/4
CP94 (Con)	20	5	12 ± 1	0/5	7.3 ± 1.4	0/5	0/5
CP94 + Fe/Dex	20	12	514 ± 53	+4 (8/12) +3 (2/12) +2 (2/12)	56.7 ± 4.9[b]	8/12	9/12
Fe/Dex	20	10	574 ± 38	+4 (2/10) +3 (4/10) +2 (4/10)	43.2 ± 3.3	1/10	0/10

[a]Hepatic fibrosis was graded as +2 focal, +3 bridging fibrosis, +4 micronodular fibrosis.
[b]Significantly different from the corresponding controls (not treated with chelator) at the 5% level.

that the predominant difference between the animals in the two groups was the accumulation of iron as hemosiderin in myocytes in the chelator treated group, which did not occur in the non-chelator group. The appearance of iron in myocytes in the chelator treated groups was parallelled by an increase in the cardiac pathology due to myocyte iron with cardiac fibrosis apparent in most animals, as has been shown to be the sequelae of the accumulation of myocyte iron in the gerbil previously (Carthew *et al.* 1993). The control chelator treated groups did not not show any significant changes of iron levels in the livers and hearts: the levels found were comparable to the normal background levels (data not shown).

To determine whether there was any relationship between the hepatic and cardiac iron levels in the different treatment groups, the comparable cardiac and hepatic iron levels were plotted by linear regression analysis. The correlation for the iron dextran treated animals was $r = 0.25$ while for the iron dextran and chelator treated animals the correlation was $r = 0.84$.

Thus the increase in liver iron levels did not relate to a corresponding increase in cardiac iron levels where there was no chelator treatment, whereas in the chelator treated animals the cardiac iron levels increased with increasing hepatic iron levels.

Discussion

The use of an orally active iron chelator in the prophylactic treatment of transfusion dependent patients with β-thalassemia would undoubtedly improve not only the compliance problems in young patients but also offer the prospect of extending the treatment to countries where the current expense of DFO treatment precludes therapy. Such a therapy continues for life and so there are stringent requirements for the effectiveness of such chelators over a period of years. There is also concern regarding the possible long-term toxicity of any compound used to replace the proven effective current pretreatment, DFO, which has some undesirable, but limited, toxic effects (Olivieri *et al.* 1986).

The normal regulatory toxicological testing which is required of all drugs prior to clinical use could be inadequate in the case of metal chelators, as the toxicity of a metal chelated complex in the iron overloaded state would not necessarily be detected by routine testing in the non-iron overloaded state. The chelator CP94 we have examined in the present study has been used in the Mongolian gerbil, which is the only known animal which responds to prolonged iron overload in the same manner as man, developing both hepatic and cardiac fibrosis. Although we found that CP94 was effective in the short-term treatment of gerbils, over 6 weeks, at removing iron which was being administered during the same period, the amount of iron in the heart was increased over the same period. Other short-term studies using rats and mice have also shown that the hydroxypyridin-4-ones are effective at removing hepatic tissue iron (Gyparaki *et al.* 1987, Kontoghiorges *et al.* 1987, Porter *et al.* 1990, Florence *et al.* 1992). However, rats and mice are not suitable models for prolonged iron overload as on the whole they do not respond with the same pathology as occurs in man, i.e. the development of either hepatic or cardiofibrosis in response to continued long-term iron overload. The only published exception to this is the dietary carbonyl iron fed rat model (Iancu *et al.* 1987, Park *et al.* 1987) which takes up to 9 months to produce any hepatic fibrosis and apparently does not have any associated cardiotoxicity.

By extending the chelator and iron dosing regime over a longer term using the gerbil, and favoring the amount of the bidentate chelated complex of iron being formed, we have shown that not only does there appear to be a problem in the continued reduction of hepatic iron levels, but that the chronic toxicity of chelator treatment in the iron loaded gerbil leads to an increased severity of the liver pathology and an accumulation of iron in the heart, in the myocyte cells in particular. As the myocyte is the target cell for the toxicity in cardiac iron overload (Buja & Roberts 1971), the increased cardiac pathology found in the present study is consistent with previous findings using this model (Carthew *et al.* 1993). Clearly the mechanism by which the accumulation of myocyte iron occurs in the chelator treated gerbil needs to be determined. This could occur due to the increased toxicity of iron chelator complexes in the liver exacerbating hepatocellular necrosis and releasing either free, low molecular weight iron, or the chelated iron, which are subsequently taken up by myocytes. Certainly the increased damage to the liver in chelator treated animals could indicate that the iron chelator complex itself may be toxic when present in high concentrations in the liver. It may also be possible for relatively low molecular weight bidentate complexes of iron and CP94 to persist in the circulation before excretion, increasing the likelihood of their being taken up by myocytes and subsequently degraded intracellularly to form the insoluble iron complex hemosiderin, which was so evident in the myocytes of chelator treated animals. The repeated weekly dosing of iron dextran may lead to higher levels of non-protein bound iron in the plasma than are usually present when long periods for equilibration of iron in tissues are used experimentally. A period of 2 days was allowed before chelator administration for iron equilibration, to minimize any such effect in the present study. The amount of iron administered to the gerbils was also greater than that accumulated by transfusion dependent thalassemia patients, which is of the order of up to 10 mg kg^{-1} per week (Porter *et al.* 1989). This may have increased the levels of non-protein bound iron in the plasma as well as the formation of bidendate iron complex with CP94 which could be taken up by hepatocytes or myocytes increasing the toxicity to both organs.

The best evaluation of the chronic toxicity of future iron chelators would be carried out in a suitable model of iron overload which responds in the same way as man does to chronic iron overload. That is to say a model which develops the hepatic and cardiotoxicity and fibrosis as occurs in the Mongolian gerbil. Our results also demon-

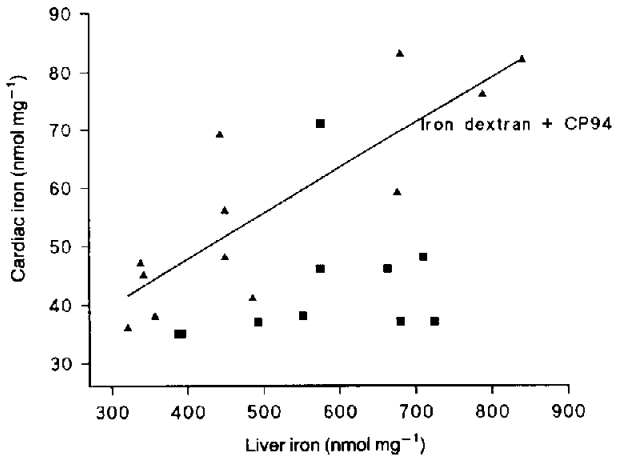

Figure 1. Correlation of hepatic and cardiac iron levels in iron overloaded Mongolian gerbils with and without CP94 chelator treatment: ■, iron dextran, no chelator; ▲, iron dextran, CP94 chelator treated.

strate the importance of carrying out reasonably long-term chronic toxicity testing with iron overloaded animals for a period of several months, rather than just the short-term testing of a few weeks which has not previously detected the kind of adverse response seen in the present study.

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