# Rapid Induction of Hepatic Fibrosis in the Gerbil after the Parenteral Administration of Iron-Dextran Complex

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**The parenteral administration of iron-dextran complex to gerbils caused hepatic hemosiderosis and fibrosis after 6 wk. Type I and III collagen synthesis in the liver developed from perisinusoidal stellate cells that are often referred to as myofibroblasts. Immunohistologically these cells were shown to have large intracellular deposits of ferritin. The hepatic fibrosis appeared to be associated with aggregates of these cells rather than the aggregates of Kupffer cells, which also occur in hemosiderosis in the liver. No appreciable necrosis of hepatocytes to trigger the fibrotic response was found, so that the fibrosis appeared to be related to the accumulation of ferritin in the perisinusoidal stellate cells. In contrast, rats and mice did not accumulate ferritin in their perisinusoidal cells or develop hepatic fibrosis in response to parenterally administered iron, although they accumulated similar or greater amounts of total iron in their livers. The rapid induction of hepatic fibrosis in gerbils in response to parenterally administered iron will provide a model to investigate the mechanism of induction of collagen deposition in response to iron overload and a means of quickly evaluating therapeutic treatments for iron overload–induced fibrosis *in vivo* using iron-chelating drugs. (HEPATOLOGY 1991;13:534-539.)**

One of the major problems in the understanding and treatment of both primary and secondary hemochromatosis is the lack of an *in vivo* model producing fibrosis of the liver in response to the accumulation of iron. The parenteral administration of iron to numerous species has been used in attempts to provoke a fibrotic response with limited success (1-4). Recently, the administration of carbonyl iron in the diet of rats has been found to produce hemosiderosis (5) and liver fibrosis resembling hemochromatosis after 8 mo or more (6). The *in vivo* testing and development of iron chelators, which could prove effective in the treatment of both primary and secondary hemochromatosis, have lacked a model that would satisfy the criterion of rapidly producing hepatic (or pancreatic) fibrosis in the absence of the usual massive liver parenchymal necrosis associated with fibrosis caused by structural remodeling after lobular damage. Recently, we have observed numerous cases of hepatic fibrosis with associated iron overload in gerbils, which have occurred spontaneously at 6 or more mo of age. An investigation of this phenomenon (to be reported in detail separately) showed this to be due to gut bacterial endotoxin lipopolysaccharides causing hemorrhages in the liver and hence subsequent focal accumulation of iron in hepatic cells. This in turn led to the persistence of focal and general iron overload in the liver, resembling secondary hemochromatosis, with a fibrosis that developed into a micronodular cirrhosis in advanced cases (7). Because hepatic fibrosis does not develop in mice and rats easily or quickly in response to hepatic iron overload (1), we have used the parenteral administration of iron to gerbils, rats and mice to determine whether we can produce a form of iron overload resembling primary or secondary hemochromatosis and accelerate a fibrotic response in the gerbil, which is so obviously lacking in other rodents.

## MATERIALS AND METHODS

***Animals Dosed with Iron-Dextran Complex.*** MF1 mice (male and female) 6 to 8 wk old were given a subcutaneous injection of iron-dextran complex (Sigma Chemical Co., Fancy Road, Poole, Dorset, UK; 100 mg iron/ml) at a dose of 10 mg iron/10 gm body weight. Porton rats (male and female) 6 wk of age and male and female gerbils (6 wk of age) were also subcutaneously dosed with 10 mg iron/10 gm body weight of the Sigma iron-dextran complex. Duplicate animals were killed at 2, 4, 6, 8 and 12 wk and 6 mo after dosing with the iron dextran, and all major organs were immersion-fixed in 10% neutral buffered formalin for histological examination. Control animals of all three species (in duplicate) were also subcutaneously injected with control dextran (mol wt = 5,000) solution of a concentration of 114 mg/ml (11.4 mg/10 gm body weight). Control animals were killed at 0, 4, 6, 8 and 12 wk after injection and treated the same as iron-dextran–dosed animals. All animals were treated in accordance with the statutory regulations required by the Home Office (United Kingdom) Animal (Scientific Procedures) Act 1986.

***Histological Stains.*** Five micrometer paraffin processed sections were routinely stained with hematoxylin and eosin, van Gieson's stain for collagen, Martius scarlet blue for fibrin and collagen and Perls' Prussian blue reaction for iron.

***Antibody Production.*** Type I, type III and type IV collagens were obtained from Sigma Chemical Company (Poole, Dorset).

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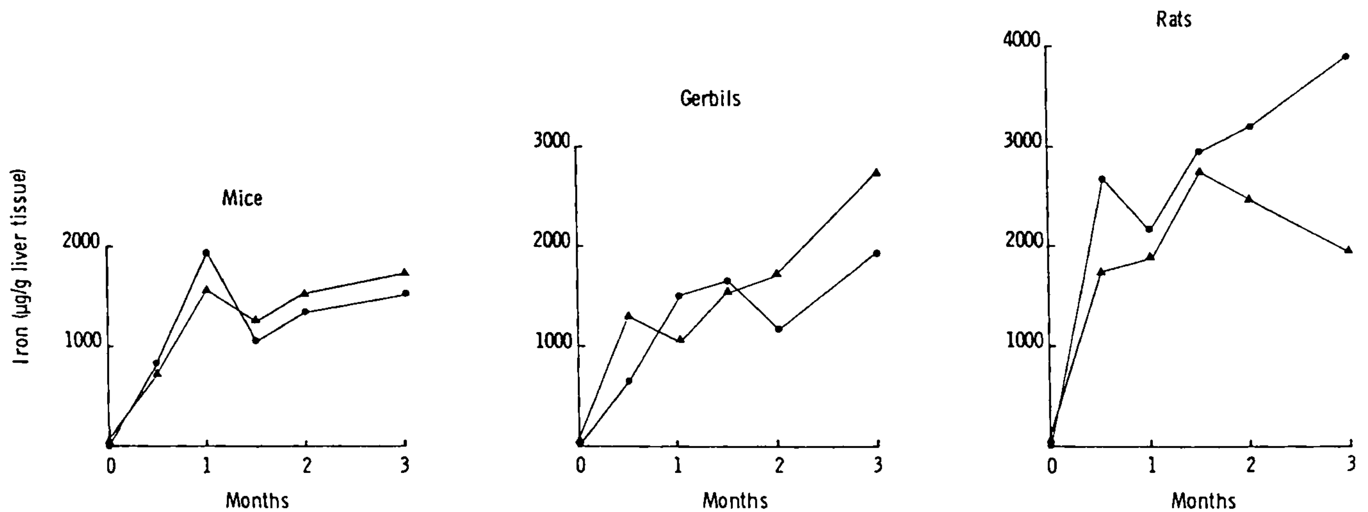


FIG. 1. Iron levels in micrograms per gram in the livers of mice, gerbils and rats at different times after dosing subcutaneously with iron-dextran complex (Sigma, 100 mg iron/ml) 10 mg iron/10 gm body weight. Females (●—●); males (▲—▲).

Three Dunkin-Hartley guinea pigs were injected with each of the collagen types. For the first injection the collagens were suspended in sterile saline and emulsified with Freund's complete adjuvant. Each guinea pig received 1 ml of the collagen emulsion intraperitoneally for the first injection. In the second and subsequent injections the collagen was emulsified with incomplete Freund's adjuvant; 0.5 ml was injected intraperitoneally and 0.5 ml subcutaneously. A test bleed was carried out after the third injection. If antibodies were present, a final booster injection was given. The guinea pigs were killed with $CO_2$ and exsanguinated immediately.

The antibody to rat liver ferritin was raised as described above. The rat liver ferritin was obtained from Sigma Chemical Company (Poole, Dorset, UK).

***Immunohistochemistry.*** The antibodies raised in the guinea pig were incorporated into an indirect peroxidase method. The peroxidase-conjugated rabbit anti-guinea pig antisera was obtained from Dako Ltd. (High Wycombe, Bucks, UK).

The anti-human ferritin antibody was obtained from Dako Ltd. and was incorporated in a standard peroxidase-antiperoxidase method. Both antibodies were unreactive with hemosiderin as has been previously found (8).

***Iron Determinations.*** Nonheme iron concentrations in liver tissues were assayed in homogenates by a modification of the colorimetric method of Torrance and Bothwell (9) using bathophenanthroline disulfonate to complex the iron.

## RESULTS

***Hepatic Iron Content.*** After the subcutaneous administration of iron-dextran complex to gerbils, rats and mice, iron accumulated in the liver at substantial levels in mice, rats and male gerbils by 2 wk and to a lesser extent in female gerbils (Fig. 1). Iron visualized histochemically with the Perls' Prussian blue reaction, which localizes hemosiderin, demonstrated that large amounts were deposited initially in the sinusoidal cells, in particular Kupffer cells, with a later uptake of iron by hepatocytes in substantial amounts. At this stage in hepatic iron overload little difference between the uptake and response of the three rodent species was noted. All three showed the typical iron-laden giant cell formation, which is characteristic of the fusion of iron-containing macrophages in the liver. There was little evidence of iron toxicity especially in hepatocytes. Four weeks after the iron injections, a drop in hepatic iron levels in both sexes of mice was seen; however, the general trend after this time was for rodents of both sexes to accumulate iron in the liver, with the exception of the male rats at 2 and 3 mo and the female gerbils at 2 mo after dosing. By 6 wk of iron overload, focal aggregates of macrophages had formed in the rodent livers. These were more prominent in the gerbil. Among the macrophages, or adjacent to them, stellate cells with oval-shaped nuclei were seen, often present as focal proliferations. The cytoplasma of these cells stained

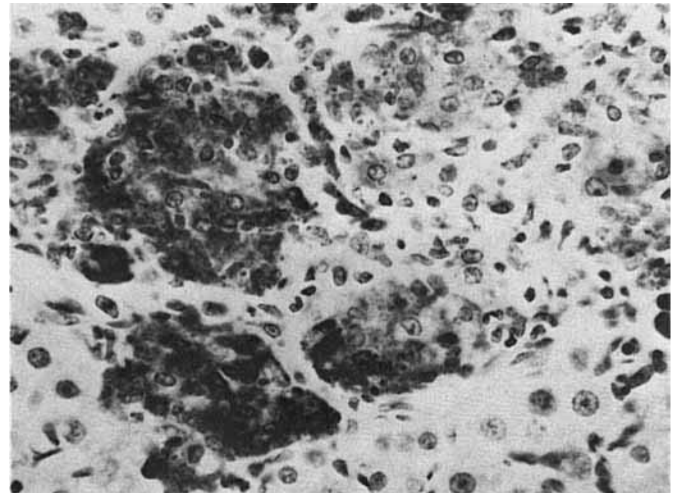

FIG. 2. Iron-stained stellate cells in an inflammatory focus in the liver of a gerbil with hemosiderosis. Note the relative even staining of the cytoplasmic iron ferritin in the stellate cells. (Perls' reaction for iron; original magnification ×330.)

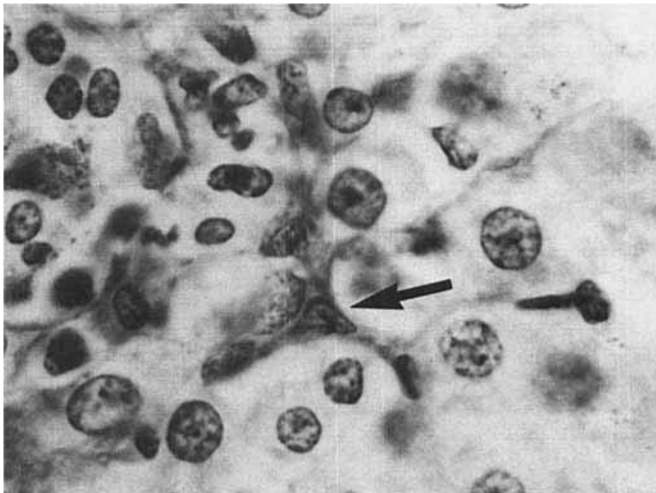

FIG. 3. A stellate cell *(arrow)* at the edge of a focus of inflammation showing collagen synthesis in the cytoplasm. (Martius scarlet blue; original magnification ×660.)

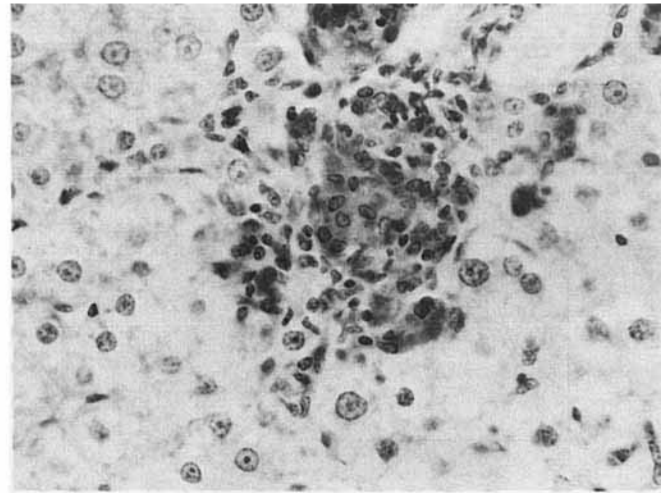

FIG. 5. Type I collagen demonstrated intracytoplasmically in the stellate cells of an inflammatory focus in a gerbil liver 6 wk after iron administration. (Immunoperoxidase stain; original magnification ×330.)

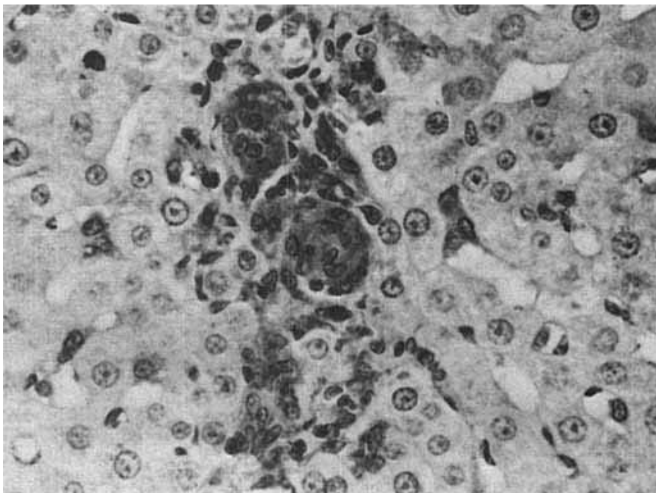

FIG. 4. Cytoplasmic immunostaining of a stellate cell focus in a gerbil's liver for ferritin 4 wk after iron administration. (Immunoperoxidase; original magnification ×330.)

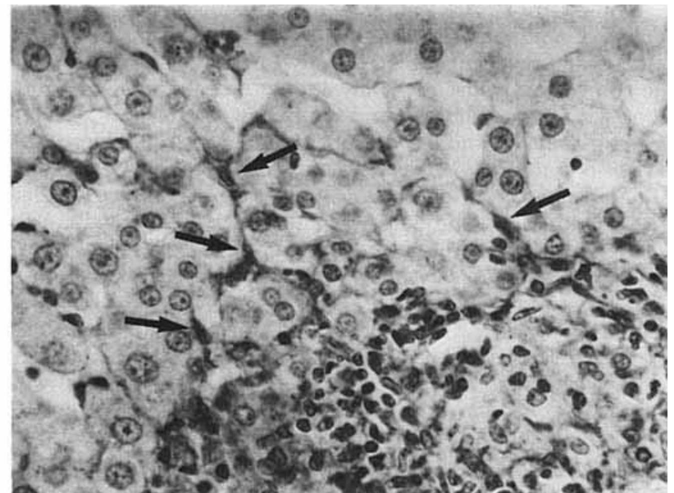

FIG. 6. Type III collagen being synthesized by the perisinusoidal stellate cells *(arrows)* at the edge of a chronic inflammatory focus. (Immunoperoxidase; original magnification ×330.)

**TABLE 1. Species and sex response with time to iron-dextran complex given subcutaneously to mice, rats and gerbils in terms of the development of hepatic fibrosis and micronodular cirrhosis for 3 mo**

| Species | Sex | Dose (mg/gm body wt) | Mean hepatic iron concentration at 3 mo (μ/gm liver tissues) | Fibrosis after dosing | Micronodular cirrhosis |
|---|---|---|---|---|---|
| Mice | M | 1 | 1,749 | None | None |
| | F | 1 | 1,542 | None | None |
| Rats | M | 1 | 1,968 | None | None |
| | F | 1 | 3,916 | None | None |
| Gerbils | M | 0.25 | 366 | Small amounts of fibrosis at 3 mo | None |
| | F | 0.25 | 241 | Small amounts of fibrosis from 3 mo | None |
| Gerbils | M | 1 | 2,732 | From 6 wk | From 3 mo |
| | F | 1 | 1,916 | From 6 wk | From 3 mo |

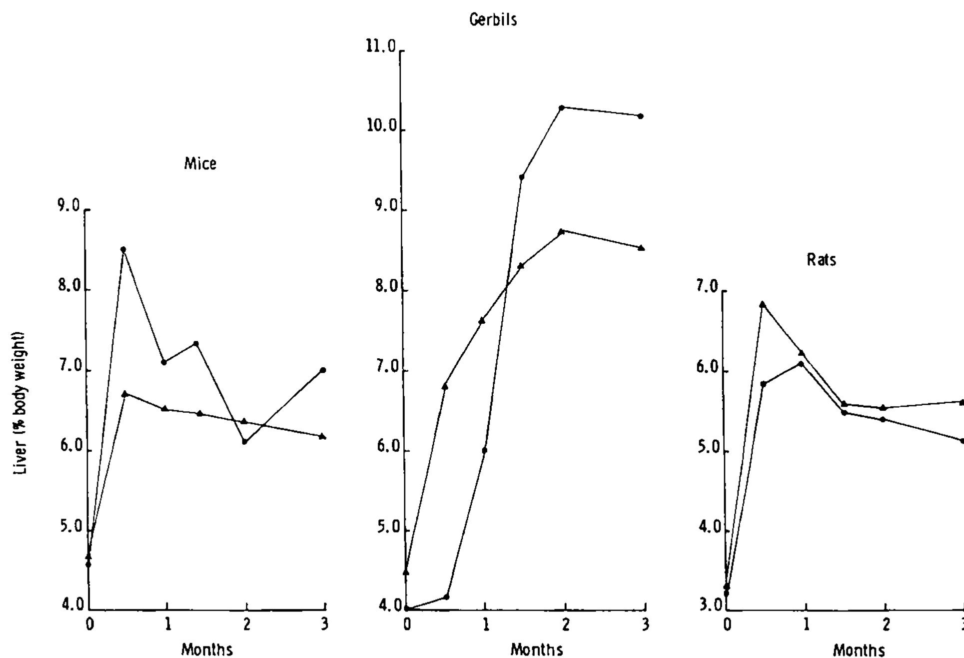


FIG. 7. Increase in liver weight (as percent of body weight) of mice, gerbils and rats given subcutaneous iron-dextran complex (Sigma, 100 mg iron/ml) 10 mg iron/10 gm body weight with time. Females(●—●); males (▲—▲).

homogeneously by the Perls' Prussian blue reaction rather than the granular color associated with hemosiderin-containing cells (Fig. 2). Van Gieson's and Martius scarlet blue stains for connective tissue collagen showed that in gerbils these cells were synthesizing collagen in the areas around the focal aggregates of macrophages and stellate cells (Fig. 3).

***Ferritin Deposition.*** Only the stellate cells were immunostained with antibody to ferritin (Fig. 4). Hepatocytes, endothelial cells and Kupffer cells were not stained appreciably for ferritin but rather for varying amounts of granular hemosiderin with the Prussian blue reaction of the Perls' stain.

***Collagen Deposition.*** The nature of the collagen deposition was investigated with immunostaining for type I, III and IV collagens. Type I collagen was first demonstrated by immunostaining intracytoplasmically in stellate cell aggregates 6 wk after dosing with the iron-dextran complex (Fig. 5). Type III collagen deposition was apparent after 6 wk in the perisinusoidal spaces (Fig. 6) and gradually increased in amounts along with type I collagen over the subsequent period as the fibrotic response increased, leading to fibrosis bridging the portal triads by 8 wk after dosing. An increase in detectable type IV collagen staining was only apparent in the neovascularized areas of the fibrotic response in gerbil livers. None of the livers of rats, mice or control gerbils (dosed with dextran only) had any evidence of stellate cell aggregates with ferritin or hemosiderin deposits present or any evidence of collagen synthesis at any time after the administration of iron or control dextran. The relative weights of the gerbil livers were substantially increased (Fig. 7) from approximately 4%

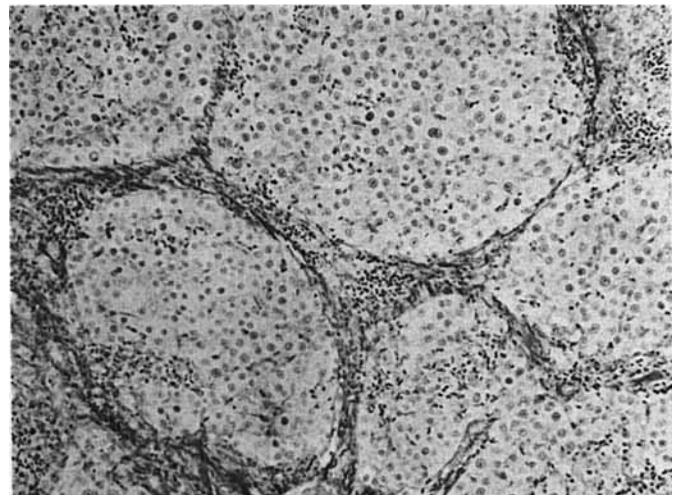

FIG. 8. Micronodular cirrhosis in the gerbil liver 3 mo after iron administration. (Reticulin silver stain; original magnification ×80.)

to 10% of body weight by 12 wk after iron administration. At this time the livers were macroscopically micronodular in appearance with some diffuse inflammatory cell infiltrates (Fig. 8), whereas the livers of rats and mice were enlarged but not fibrotic or micronodular in appearance (Table 1).

## DISCUSSION

The pattern of iron accumulation in gerbils, initially in Kupffer cells, then in perisinusoidal cells and to a lesser (but appreciable) extent in hepatocytes is similar to

that seen in secondary hemochromatosis rather than primary (genetic) hemochromatosis. The major difference in terms of hepatic fibrosis between the gerbil and rats or mice is the uptake of iron into the stellate perisinusoidal cells in the liver and its appearance in the form of ferritin intracellularly. The involvement of perisinusoidal stellate cells in the fibrotic response of the liver to injury is well established both *in vivo* (10, 11) and *in vitro* (12-14). Hepatic fibrosis did not occur in the rat and mouse to any extent, although both these species are known to have stellate or Ito cells perisinusoidally in the liver.

One of the key assertions in the interpretation of hepatic fibrosis caused by iron overload in humans is that a critical amount of iron must be present in the liver before the fibrotic response is triggered (4, 8). This is based on the observation that humans with primary hemochromatosis have relatively higher levels of hepatic iron than rodents (15), which seem to have a limit of approximately 4,000 μg/gm.wt (wet weight) for rats after a single parenteral administration of iron (Fig. 1). However, it is clear that the hepatic iron levels needed to produce fibrosis in the gerbil are only comparable to those achieved in the mouse and less than those in the rat, where there is no fibrosis even 6 mo or 1 yr after the parenteral administration of iron (Personal observation, 1990).

The mechanism by which fibrosis subsequently ensues is most probably a free radical one based on numerous reports of free radical involvement in the lipid peroxidation that could precede the chronic inflammatory fibrosis (16, 17). It has already been demonstrated that ferritin is more effective in generating free radicals than the relatively safer storage form of iron, hemosiderin (18). This would explain why the gerbil is more responsive than rats and mice to hepatic fibrosis even at relatively low levels of iron deposition in the liver. The accumulation of ferritin in stellate cells of the liver is thus more causally related to subsequent fibrosis than a large relative amount of total iron in the liver. The accumulation of ferritin and its relative hepatic disposition has received little attention in investigations of the factors associated with any form of hemochromatosis in humans (19). Some investigations have examined the relative ferritin content of cells in the intestine and linked a lack of ferritin content in duodenal absorptive epithelial cells to cases of idiopathic hemochromatosis (20). Other investigations of cases of primary hemochromatosis have concentrated on the regulation of transferrin receptors in the liver (21-23) as a possible mechanistic explanation for the particular accumulation of iron in the form of hemosiderin in hepatocytes. Comparatively, the response of the gerbil to hepatic iron overload is analogous to that of human secondary hemochromatosis of the type commonly seen in β-thalassemia, where the necessity for repeated blood transfusions commonly leads to excessive hepatic iron overload and fibrosis, although death is usually due to cardiac failure caused by iron overload.

In terms of the treatment of secondary hemochroma tosis, the major difficulty is the chelation and excretion of sufficient iron to reduce the total body burden to an amount that does not trigger fibrosis. The problem here seems to be that only unbound iron can be chelated by drugs such as deferoxamine, and very little free iron is present at any time. Some investigators believe that the toxic form of iron is a complex of iron bound to serum proteins, but this has not been isolated and characterized (24-26).

The gerbil's rapid hepatic fibrosing response to parenteral iron could provide an animal model to examine more precisely the relative amounts of iron necessary to provoke fibrosis and offers the possibility of examining the effectiveness of clinical intervention therapies with iron-chelating drugs, which may be useful in controlling iron overload caused by repeated blood transfusions in β-thalassemia.

It will also allow a detailed examination of the changes in perisinusoidal cells when isolated *in vitro* in response to a range of possibly collagen-inducing forms of iron, so that the cellular mechanism triggering the fibrotic response can be examined. It has already been shown that fat storage (Ito or stellate) cells, which were isolated from livers and kept in culture for a period of days, undergo a form of limited differentiation to fibroblast-like cells (13) referred to as myofibroblasts, and it is commonly thought that these cells are responsible for the collagen synthesis associated with chronic hepatic fibrosis (27-29). Our results certainly implicate this type of cell in the mechanism of hepatic fibrosis in the gerbil in response to iron overload, and it will be interesting to see whether this can also be examined *in vitro*. Finally, it will also be important to see whether this form of ferritin-stimulated fibrosis (perisinusoidal cell) rather than fibrosis caused by hepatocyte stimulation of collagen synthesis occurs in the analogous human cases of secondary hemochromatosis associated with β-thalassemia.

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