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Susceptibility of cultured rat hepatocytes to oxidative stress by peroxides and iron. The extracellular matrix affects the toxicity of *tert*-butyl hydroperoxide

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Abstract

The aim of this study was to set up an in vitro model for studying the importance of an altered extra-cellular matrix composition and its importance for the resistance to oxidative stress, in hepatocytes from normal and iron loaded rats.

Primary cultures of hepatocytes from iron loaded and normal rats were plated on a laminin rich extracellular matrix or on collagen type I, and incubated with *tert*-butyl hydroperoxide (TBH). Malon dialdehyde (MDA) and the activities of lactate dehydrogenase (LDH) in cell culture medium were analyzed. The protein synthesis, the concentrations of glutathione and the expression of manganese-superoxide dismutase and ferritin genes were measured.

All hepatocytes contained lower concentrations of glutathione when plated on collagen than on EHS. Ferritin H and Mn-SOD gene expression showed no difference. The rate of lipid peroxidation in iron loaded hepatocytes exposed to TBH was higher on collagen than in those plated on EHS (0.95 ± 0.28 μM MDA vs. 1.62 ± 0.22 μM MDA, $p < 0.05$). Iron loaded cells were in general more susceptible to TBH than were normal hepatocytes (MDA, LDH, protein synthesis and glutathione content). Lipid peroxidation could be prevented by adding desferrioxamine.

In conclusion, we show that the combination of iron overload and collagen matrix in rat hepatocytes leads to an increased susceptibility to oxidative stress. These findings may be of interest for the further studies on effects of iron overload and the altered matrix composition in liver fibrosis.

Keywords: Extracellular matrix; Iron; Rat hepatocytes; Oxidative stress; Glutathione; Collagen

1. Introduction

Iron overload in humans caused by a defect in iron absorption (genetic hemochromatosis) [1] leads to liver damage and cirrhosis in some of the patients – mainly males [2–4]. Hepatic stellate cells are responsible for the deposition of

Abbreviations: EDTA, ethylenediaminetetraacetic acid, EHS, Engelbreth–Holm–Swarm, GSH, glutathione, HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid], HPLC, high performance liquid chromatography, LDH, lactate dehydrogenase, MDA, malon dialdehyde, MEM, minimal essential medium, Mn-SOD, Manganese-superoxide dismutase, PBS, phosphate buffered saline, TBH, *tert*-butyl hydroperoxide.

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collagen type I, and the degradation of the laminin rich basement membrane, both in fibrosis and cirrhosis [5]. The composition of the extracellular matrix is of importance for maintaining cellular phenotype and cell functions. The cellular protection against free radicals depends on the availability of many scavenging systems, including reduced glutathione (GSH) and superoxide dismutase (SOD [6, 7]).

Models for iron overload have clarified the importance of free radicals in the process of hepatocyte death [8–10]. Chronic feeding with either carbonyl-iron or TMH-ferrocene to rats [11] has been used to create an animal model for experimental hemochromatosis. Chelatable iron can participate in the formation of free radicals by way out Fenton and Haber–Weiss reactions. To avoid harmful effects of iron, the iron homeostasis is carefully monitored. Ferritin is the major intracellular iron binding complex and consists of two subunits, the heavy (H) and the light (L) chain.

The damage caused by the increased pool of chelatable iron in situations with iron overload seems to be potentiated if other liver toxic agents, like alcohol or infection with hepatitis C virus, are present. However, it is not known in what way the altered extracellular matrix in the cirrhotic liver affects the cellular resistance to oxidative stress.

In the present study, an in vitro model, comparing primary cultures of hepatocytes from iron loaded and normal rats, plated on a laminin rich gel or on collagen type I, has been used to shed light on this problem. We have studied the effect of an agent causing oxidative stress, *tert*-butyl hydroperoxide (TBH), as well as the role of chelatable iron, in this system. In addition, some of the cellular scavenging systems under the different culture conditions were assayed.

2. Materials and methods

2.1. Cell isolation

Male Sprague–Dawley rats (B&K Universal AB, Sollentuna, Sweden) were fed a normal rat diet (Altromin International, Lage, Germany) supplemented with 2.5% (w/w) carbonyl iron (Sigma, St. Louis, MO) for 8 weeks following weaning. Control animals that were given the same diet without extra iron were termed normal animals. The rats were kept under 12 h light–dark cycles.

Hepatocytes were isolated using an in situ collagenase perfusion technique. The liver was perfused for 15 min with a balanced salt solution (S-MEM, Eagle (GIBCO Laboratories, England) with 0.01% (w/v) EGTA, 1 mM HEPES 100 U/ml penicillin and 100 μg/ml streptomycin, equilibrated with 96% oxygen) followed after 15 min by a collagenase containing buffer (Dulbecco's MEM/Nutmix F 12 (GIBCO), with 0.025% (w/v) collagenase type A from Clostridium histiolyticum (Boehringer Mannheim, Mannheim, Germany), with HEPES, antibiotics and equilibrated as above).

The liver was excised and the resulting cell suspension incubated for 10 min in a warm buffer containing DNAse (0.013% (w/v) DNAse type I from bovine pancreas, grade I (Boehringer, Mannheim), in Dulbecco's MEM/Nutmix F 12 (GIBCO), with HEPES, antibiotics and equilibrated as above) in order to prevent aggregation of cells.

The cell suspension was filtered and centrifuged at $50 \times g$ (IEC Centra-8R, International Equipment Company, USA). The hepatocyte rich pellet was resuspended in S-MEM and washed three times at $50 \times g$ at RT. The cells were then resuspended in medium 199 (GIBCO) to a concentration of 10×10^6 cells/ml and further purified by a Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

Viable hepatocytes found in the pellet were washed and the final pellet was resuspended in medium 199 containing 1 μM hydrocortisone (Sigma), 4 U/ml insulin (Sigma bovine pancreas) and 5% (v/v) foetal calf serum (GIBCO).

Cell viability was checked by exclusion of Trypan blue, and always exceeded 95%. Cell purity was 99%. 0.75×10^6 cells were seeded in 35 mm ∅ plastic culture dishes (Costar, USA) coated with collagen (collagen type I, Sigma, 0.01% collagen solution in 0.1 M acetic acid) or extracellular matrix derived from Engelbreth–Holm–Swarm (EHS) tumors in mice. In some

experiments, larger culture dishes were used, but cells were seeded at the same density as above.

Cells were incubated for 4 h at 37°C in a humid 96% oxygen atmosphere to allow attachment and spreading. The medium was subsequently changed, and the foetal calf serum was replaced by 0.1% bovine serum albumin (Sigma). All incubations were made 16 h after the cells were plated.

2.2. Incubations

Hepatocytes were incubated with the standard incubation medium, with the addition of 0–0.5 mM *tert*-butyl hydroperoxide (TBH, Sigma) or TBH and 1 mM desferrioxamine (Ciba-Geigy AG, Basel, Switzerland). Incubation times were 2.5 and 5 h, unless otherwise stated.

2.3. Analyses

Cell protein concentrations were measured using Bio-Rad® (Bio-Rad Laboratories, Hercules, CA), while iron concentrations were

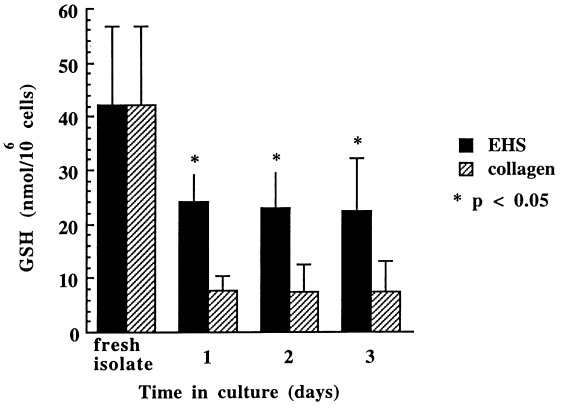

Fig. 1. Changes in glutathione concentrations between freshly isolated normal hepatocytes and cells in culture. Hepatocytes were isolated, and glutathione measured in cell homogenates from the fresh isolation (nmol GSH/10^6 cells). Cells were plated on collagen or EHS, and glutathione concentrations in cell homogenates were measured during the ensuring 1–3 days in culture. Results are mean of 3–5 independent experiments with 2 cell culture plates for each measuring occasion and type of matrix.

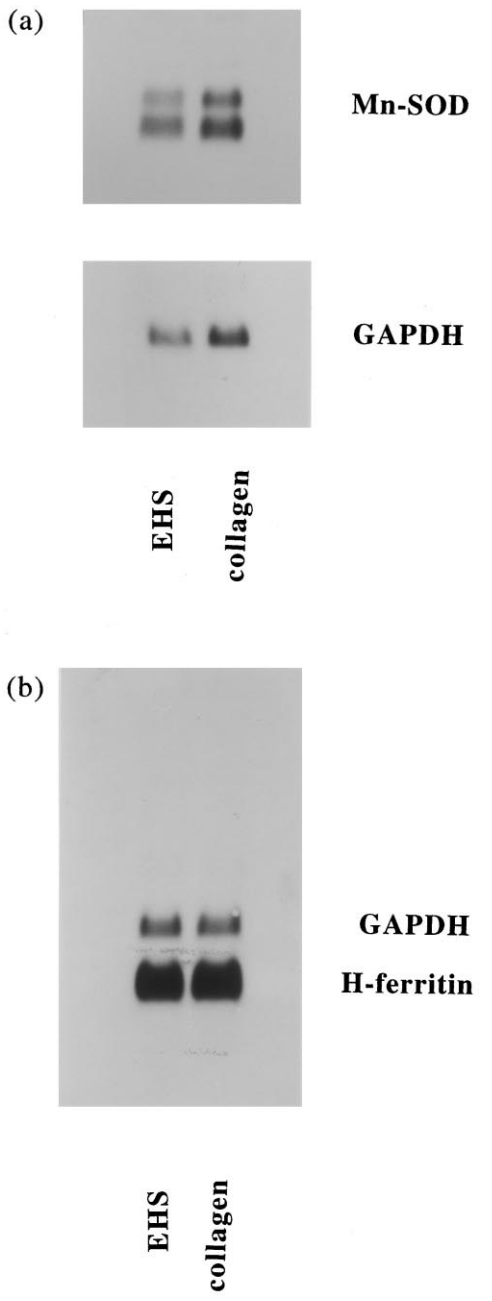

Fig. 2. Northern blot analysis of manganese-superoxide dismutase (Mn-SOD, A) and heavy chain (H) ferritin (B) mRNA levels in normal hepatocytes plated on collagen or EHS. RNA was prepared as indicated in Section 2, and equal amounts of total RNA was fractionated on an 1.2% agarose/2.2 M formaldehyde gel and transferred to a nylon filter. The filter was hybridized with probes for rat ferritin H chain, Mn-SOD and GAPDH.

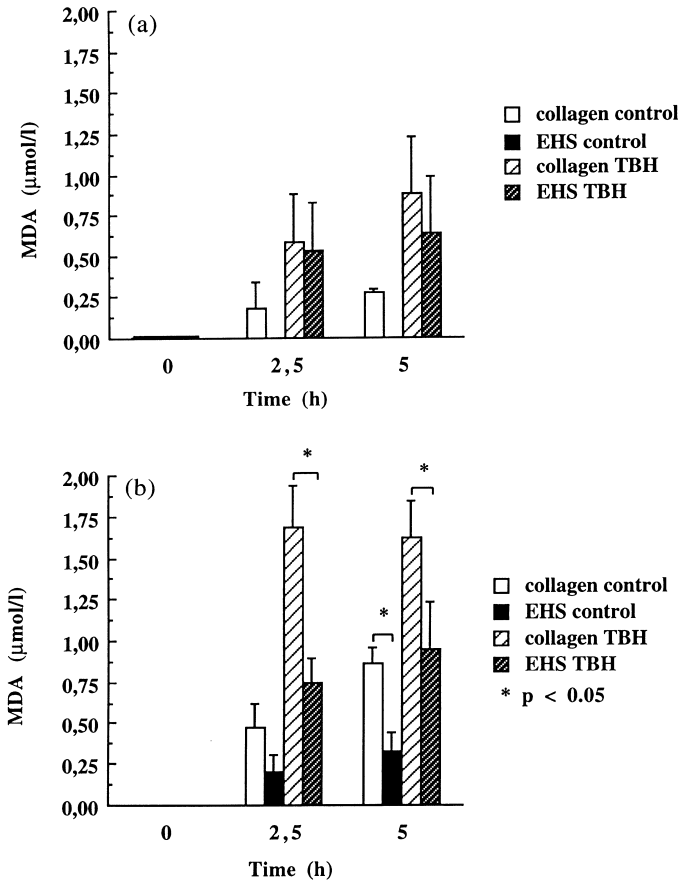

Fig. 3. The rate of lipid peroxidation measured as malon dialdehyde (MDA) concentrations (μM) in cell culture medium from hepatocytes in culture. Hepatocytes from normal (A) or iron loaded (B) rats were isolated and plated on collagen or EHS. Cells were then incubated with or without the addition of 0.5 mM *tert*-butyl hydroperoxide (TBH) for 0–5 h. Samples from cell culture medium were collected, and the concentrations of MDA were measured as described in Section 2. The data represent mean ± SD of three separate experiments with two cell culture dishes at each point.

assayed using a Varian atomic absorption spectrophotometer [12].

Cellular GSH was measured using the GSH-400 assay kit (Bioxytech® S.A. Bonneuil sur Marne, France). Cell damage was determined by measuring lactate dehydrogenase (LDH) activities in the culture medium, with the Sigma LDH Optimized kit. LDH activities were expressed as percent of total LDH activity in medium from cells lysed by Triton X-100 (Sigma). LDH activities were measured immediately after incubations.

For Northern blots, RNA was prepared by phenol/guanidium thiocyanate extraction (AGS GmbH, Heidelberg, Germany). Rat ferritin heavy chain (H) and manganese superoxide dismutase (Mn-SOD) cDNA fragments were cloned into the pBS ± phagemid vector (Stratagene, USA). Anti-sense probes were generated with digoxigenin-UTP by in vitro transcription with SP 6 or T 7 RNA polymerase using the DIG-RNA labelling kit (Boehringer, Mannheim). Equal amounts of total RNA were fractionated on 1.2% agarose/2.2 M formaldehyde gels and transferred to nylon filters. Filters were hybridized with probes in Church buffer (0.5 M sodium phosphate, pH 7.2, 1 mM Na_2EDTA, 7% (w/v) SDS). To determine the

size of the mRNA bands that were obtained, the 0.24–9.5 kb RNA Ladder (Life Technologies, USA) was used. GAPDH was used as control blot.

Free malon dialdehyde (MDA) concentrations were measured to estimate the level of lipid peroxidation in cell culture medium according to a method described earlier [13]. Briefly, medium from cell culture dishes was ultrafiltrated, snap frozen in liquid nitrogen and stored at −70°C pending analysis. A free MDA standard was prepared to corresponding MDA concentrations of 0.1–4.0 μM, using 1,1,3,3-tetraethoxypropane (Sigma). The concentrations of MDA in samples were then calculated from the standard curve obtained in this interval. Acetonitrile/0.03 M Tris–HCl (pH 7.4), 4/1 was used as the HPLC eluant. The HPLC system consisted of a Waters 600 delivery system (Waters Chromatography Division, Millipore Corporation, Milford, MA) with a flow rate of 2.4 ml/min. A Waters 486 UV detector was used at 267 nm. The column consisted of a Waters carbohydrate analyzer column (125 Å, 10 μm), and the data were recorded in a Waters Millenium computerized chromatography system.

Protein synthesizing capacity was used as a measure of cell function and viability, and was estimated by [^{35}S] incorporation into proteins. Briefly, cells were incubated with different concentrations of TBH (0–0.5 mM) for 2.5 h in the standard incubation medium. Cells were then washed twice, and the incubation with TBH was continued for 2.5 h in a methionine free MEM medium (Nordcell, Stockholm, Sweden) supplemented with 25 μCi/ml [^{35}S] methionine (Amersham, UK, #SJ 1015, 3000 Ci/mmol). Dishes were washed and cells harvested. Cells were lysed, and following vortexing and centrifugation, cell debris was pelleted and protein concentration of the supernatant was determined. Total protein synthesis was measured by the amount of methionine incorporated into trichloroacetic acid-precitable material normalized with the total protein content.

2.4. Statistical analysis

Data is presented as means and standard deviations. Statistical significance between groups was calculated by the Student's *t* test. For the analyses of GSH, MDA and LDH, each time indication or concentration point represents data from three to five animals, with two cell culture dishes from each animal. Northern blots and protein synthesis capacity experiments were repeated 3 times, and were all representative. The results from one experiment are presented.

3. Results

3.1. Measurements of GSH

Hepatocytes were plated on collagen type I or EHS matrix. The cell density was carefully measured, since earlier investigations demonstrated that cell density affects the GSH concentrations in cells [14]. Cells in culture showed a decrease in GSH concentrations compared with freshly isolated cells. The GSH concentration for normal hepatocytes plated on EHS was reduced to 60% after 24 h, whereas the concentration was reduced to 20% for cells plated on collagen after the same time in culture (Fig. 1). The reductions of GSH concentrations were more pronounced for iron loaded cells, with a reduction to 32% after 24 h in culture on EHS and to 23% when cells were plated on collagen (not shown in Fig. 1). Northern blot analysis of Mn-SOD and H ferritin mRNA levels showed that there were no differences between the levels of Mn-SOD and H ferritin mRNAs in isolated normal hepatocytes plated on collagen or EHS (Fig. 2A and B).

3.2. Effects of oxidative stress on hepatocytes

The differences in MDA concentrations in normal hepatocytes plated on collagen or EHS and exposed to 0.5 mM TBH for 0–5 h (Fig. 3A), were not significant, although lower figures were recorded in cells on EHS. There was a significantly higher MDA concentration in iron loaded hepatocytes (Fig. 3B) exposed to

TBH in cell culture medium from cells plated on collagen than in medium from cells plated on EHS.

MDA concentrations in cell culture medium were higher in iron loaded hepatocytes (Fig. 4A), when iron loaded and normal hepatocytes were plated on collagen and exposed to 0–0.5 mM TBH for 5 h. Methionine incorporation into cells was lower in iron loaded cells (Fig. 4B) as was the amount of GSH (Fig. 4C). LDH activities in cell culture medium were higher in iron loaded cells (Fig. 4D) than in controls.

Normal and iron loaded hepatocytes plated on EHS or collagen were – to further investigate the role of chelatable iron – exposed to 0.5 mM TBH with or without the addition of 1 mM desferrioxamine (Fig. 5). Addition of desferrioxamine significantly reduced the concentrations of MDA in cell culture medium when cells were pla-

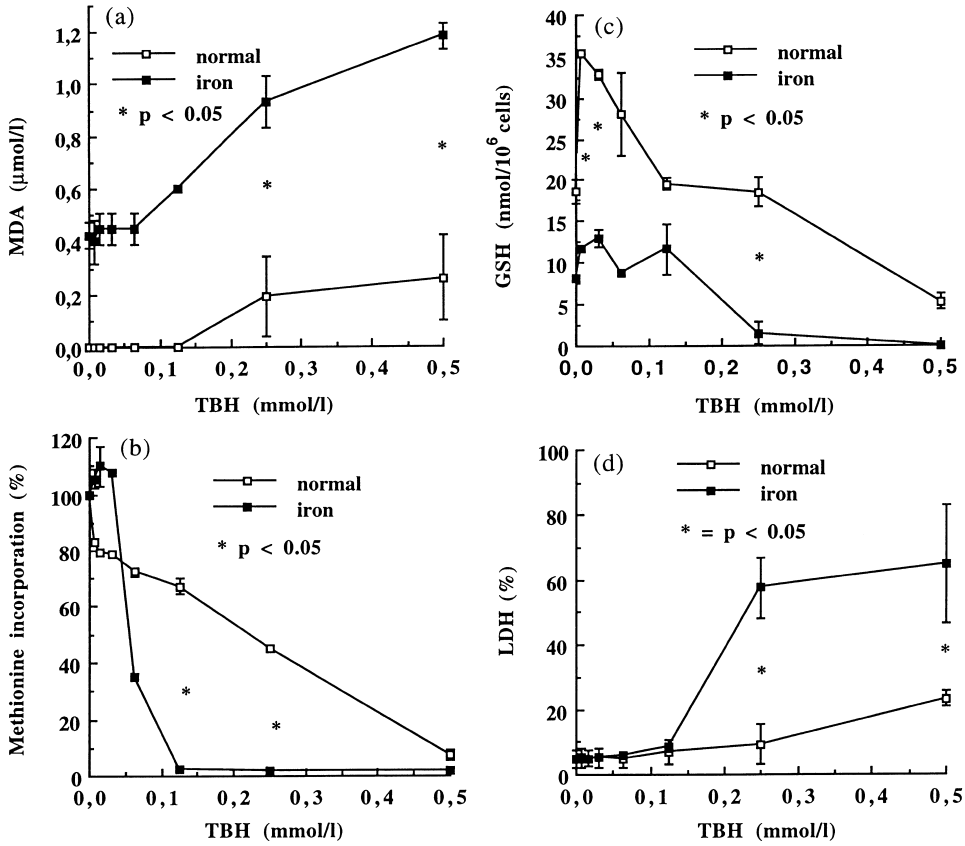

Fig. 4. The effects of *tert*-butyl hydroperoxide (TBH) on cellular lipid peroxidation, protein synthesis, glutathione concentrations and viability. Hepatocytes from normal and iron loaded rats were isolated and plated on collagen. Cells were then exposed to 0–0.5 mM TBH for 5 h. Samples from cell culture media were collected, and concentrations of malondialdehyde (MDA, μM) were measured as described in Section 2 (A). The cells were incubated with TBH in a methionine free medium supplemented with [^{35}S] methionine (B) to assess the rate of protein synthesis. Cell homogenates were prepared from the different culture conditions for analyses of cellular glutathione (GSH) contents, and glutathione was measured and expressed as nmol/10^6 cells (C). As an indicator of cell viability, the activities of lactate dehydrogenase (LDH) were analyzed in samples from cell culture media (D). The activities are expressed as per cent, where 100% represent the activities in cells completely lysed by Triton X. The data represent mean ± SD from three separate experiments, with two cell culture dishes at each point.

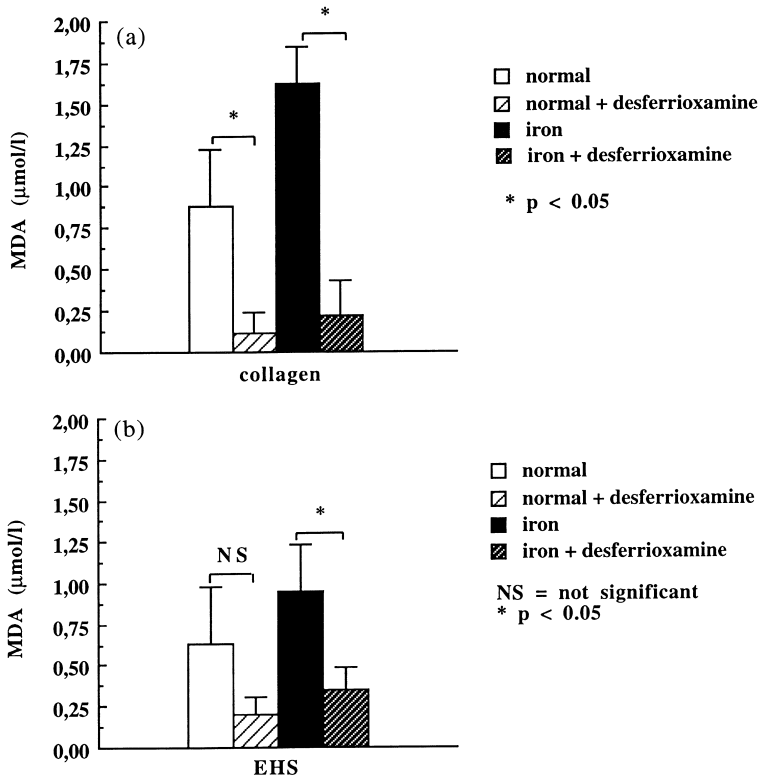

Fig. 5. The effect of iron chelation on lipid peroxidation in cells exposed to *tert*-butyl hydroperoxide (TBH). Hepatocytes from normal and iron loaded rats were isolated and plated on collagen (A) and EHS (B), respectively. Cells were then exposed to 0.5 mM TBH for 5 h, with or without the presence of 1 mM desferrioxamine. Samples from cell culture media were collected, and the concentrations (μM) of malon dialdehyde (MDA) were analyzed as stated in Section 2. The data represent mean ± SD from three separate experiments, with two cell culture dishes at each point.

ted on collagen and exposed to TBH, both in normal and iron loaded cell cultures. The addition of desferrioxamine caused a significant reduction of MDA concentrations only in iron loaded cells when cells were plated on EHS and exposed to the hydroperoxide.

Table 1 shows data from iron measurements in hepatocytes. There were no significant losses of

Table 1
Iron concentrations in iron loaded hepatocytes during incubation. Results expressed as μg Fe/mg protein. Mean (range). Unloaded hepatocytes showed a mean iron concentration of 0.35 μg Fe/mg protein (data not shown)

Time (h)	Controls	TBH	TBH + D
0	1.9 (1.6–2.1)	1.9 (1.5–2.3)	1.9 (1.8–1.9)
5.0	1.6 (0.8–2.6)	2.2 (1.6–2.5)	1.8 (1.2–2.3)

iron during the time of incubation, and the addition of desferrioxamine did not affect the total amount of intracellular iron.

4. Discussion

The effects of the extracellular matrix on hepatocytes in culture have been studied earlier. It was shown, that the phenotype of hepatocytes plated on EHS differs from that of hepatocytes plated on collagen [15].

Protection from reactive oxygen species is of importance in order to maintain normal cell functions when cells are exposed to oxidative stress. TBH is mainly metabolized by GSH peroxidase, but may also be converted via other pathways [7]. It has been shown that cells can be protected against the effects of TBH if scavenging mechanisms are present [14, 16]. In a study by Shertzer et al., high concentrations of reduced GSH in the cytoplasm of mouse hepatocytes were shown to give increased resistance to iron and TBH [7]. In the present study, GSH concentrations were higher in cells plated on EHS than cells plated on collagen, and the damaging effects of the hydroperoxide were reduced. The difference in the response to the hydroperoxide was not significant in normal hepatocytes on collagen or EHS; however, the difference was significant in iron loaded cells. This difference could be the result of protection from harmful effects of iron by the higher GSH contents in the cells. This effect appears to agree with the findings by Shertzer in experiments with mutant mouse hepatocytes [7].

Mn-SOD and ferritin are other factors of importance in the cellular defence against oxidative stress and the effects of iron. Loss of Mn-SOD and ferritin gene expression due to a change of matrix from EHS to collagen could not explain the susceptibility of hepatocytes on collagen, since mRNA levels of both Mn-SOD and H-ferritin were similar in the two groups in the present investigation.

TBH is known to cause oxidative stress in cells, and lipid peroxidation of subcellular membranes, leading to impaired cell function [17]. The Fenton and Haber–Weiss reactions – where iron plays an important role – are of great significance in oxidative stress [18]. Indeed, free radical formation, and inhibited cellular function as measured by protein synthesis capacity [19], are seen with iron overload, both when hepatocyte cultures are incubated with iron and when iron is supplemented to the animal.

It was obvious that iron loaded hepatocytes plated on collagen, had a higher rate of lipid peroxidation (measured as MDA) when exposed to oxidative stress by the hydroperoxide than with normal hepatocytes. We found an increase in GSH concentrations in cells exposed to low doses of TBH. These findings are consistent with earlier observations [6] that GSH synthesis can be stimulated by a low grade of oxidative stress. The cellular GSH concentrations were reduced with increasing concentrations of TBH, and the reduction was seen at lower TBH concentrations in iron loaded cells than in normal cells. The impairment of cell function was higher in iron loaded cells when exposed to the hydroperoxide as was the cell damage measured as LDH activities in cell culture medium. It is interesting to note that protein synthesis is inhibited at lower TBH concentrations in iron loaded cells, indicating a possible target for iron toxicity. The exact mechanism for this inhibition is not known, but mitochondrial enzymes are known to be affected during iron overload [7].

An efficient way of protecting the cells – noted in this study – was by the addition of a chelator. Desferrioxamine is known to prevent the effects of iron loading; this is achieved not by elimination of iron from the cell, but rather by sequestration of the chelatable iron in the cytoplasm [9, 19].

In conclusion, the results of this study show that hepatocytes on EHS contain more GSH than hepatocytes on collagen. Plating of iron overloaded hepatocytes on collagen leads to a significant increase in the susceptibility to hydroperoxide. This susceptibility can be diminished by binding of the chelatable intracellular iron pool.

The findings seem to suggest that heavily iron loaded cells surrounded by collagen type I in a cirrhotic liver may have lowered ability to maintain a normal cell phenotype and function, and – as a consequence – be more susceptible to oxidative stress.

The study provides an in vitro model that may be useful for further investigations of the mechanisms whereby the extracellular matrix affects the resistance to oxidative stress in normal and iron loaded hepatocytes.

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