Free Rad. Res., Vol 34, pp. 57-68
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Iron-Induced Oxidative Damage in Colon Carcinoma (Caco-2) Cells

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Accepted for publication by Prof. B. Halliwell

(Received 25 April 2000; In revised form 14 June 2000)

Intestinal epithelial cells have an active apical iron uptake system that is involved in the regulated absorption of iron. By the action of this system, intestinal cells acquire increasing amounts of iron with time. Since intracellular reactive iron is a source of free radicals and a possible cause of colon carcinoma, this study analyzed the oxidative damages generated by iron accumulation in Caco-2 cells. Cells cultured with increasing concentrations of iron increased both total intracellular iron and the reactive iron pool, despite an active IRE/IRP system, which regulates intracellular iron levels. Increasing concentrations of iron resulted in increased protein oxidative damage, as shown by the immunoreactivity for 4-hydroxy-2-nonenal-modified proteins, and markedly induced DNA oxidation determined by 8-hydroxy-2'-deoxyguanidine production. Iron also impaired cell viability, resulting in increased cell death after 6 days of culture. In summary, iron accumulation by intestinal Caco-2 cells correlated with oxidative damage to proteins and DNA. Oxidative damage finally resulted in loss of cell viability. The Fe-induced oxidative damage observed may be relevant in understanding the cascade of events associated with iron-mediated colon carcinogenesis.

Keywords: ferritin, IRP, oxidative damage, HNE, 8-hydroxy-guanidine, cell death

INTRODUCTION

Iron was first implicated in carcinogenesis in autopsy studies of individuals with hemochromatosis, a disorder of iron metabolism characterized by increased body iron absorption [1–3]. With time, evidence accumulated establishing that iron is both toxic and carcinogenic [reviewed in [4]. Recent trials using iron chelators against a number of aggressive cancers have reported some arrest in tumor growth [5,6], an indication that iron can have a neoplastic action, although they could merely be sequestering iron from ribonucleotide reductase, an enzyme essential for cell proliferation. The intimate mechanisms by which iron induces carcinogenesis are still not understood, but evidence is building-up indicating that iron toxicity is mediated by its capacity to produce free radicals [reviewed in [7]. Nevertheless, substantial opinions have been advanced stating that iron plays no major role in tissue damage or in carcinogenesis [8,9,10].

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High risk of colon cancer has been associated with a high iron diet and with increased body iron stores [11–16]. Blakeborough et al. [11] and Babbs [12] conceived the stimulating hypothesis that oxidizing radicals, generated by extracellular iron present in the fecal material of the colon, were a cause of colorectal cancer. However, other studies have failed to demonstrate a correlation between an iron-rich diet with such a risk [17,18], raising the need of additional evidence establishing a link between colon cancer and iron.

Excess intracellular iron is also a cause of oxidative injury [19], hence, the mechanisms that regulate intracellular iron levels are relevant for the understanding of iron toxicity. Cellular iron homeostasis is governed by the activity of iron regulatory proteins (IRP1 and IRP2; IRPs), cytosolic proteins that bind to structural elements named iron-responsive elements (IREs). These IREs are present in the untranslated region of the mRNAs of the major proteins that regulate cellular iron homeostasis: the transferrin receptor, involved in plasma-to-cell iron transport, and the iron-storage protein ferritin [20]. The activities of both IRP1 and IRP2 respond to changes in cellular Fe, but through different mechanisms. Low levels of intracellular Fe cause IRP1 to bind to, and stabilize, transferrin receptor (TfR) mRNA, and to bind to ferritin mRNA diminishing its translation [21–22], while IRP2 is always active to bind to IREs, but its mass is down-regulated through Fe-induced oxidative damage, ubiquitination and proteasome degradation [23]. Overexpression of a mutant IRP1 constitutively active in binding to IRE produces cells that express high levels of TfR despite Fe repletion [24]. Furthermore, overexpression of IRE in Caco-2 cells, a model of intestinal epithelia of colonic origin, results in obliteration of IRP activity, low levels of TfR and high levels of ferritin [25].

Intestinal cells have an active IRE/IRP system [26,27]. In particular, Caco-2 cells regulate in a concerted way IRP1 activity, apical Fe uptake activity, ferritin levels and transferrin receptor density [28]. These cells have basal levels of IRP2 activity, apical iron uptake and TfRs that are not down regulated by high iron concentration [28]. Moreover, the presence in intestinal brush border membranes of a constitutively expressed isoform of iron transporter DMT1 (isoform I) [29] assures basal levels of apical iron uptake in intestinal cells. If intestinal cells indeed have basal levels of iron uptake, then in time they should acquire increasing amounts of iron, despite having an active IRE/IRP system. With this consideration in mind this study was undertaken to test the hypothesis that intestinal cells acquire in time increasing amounts of iron, and that this process ends up in iron-mediated oxidative damage.

MATERIALS AND METHODS

Reagents

Anti-human ferritin antibody was from DAKO (Carpinteria, CA). Fetal bovine serum, culture medium, desferrioxamine, DTPA, NTA, protease inhibitors, culture media, buffers and salts were purchased from Sigma Chem. Co., St. Louis, MO. Calcein-AM, 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (DCFDA) and 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) were from Molecular Probes (Eugene, OR). Salicylaldehide isonicotinoyl hydrazone (SIH) was the kind gift of Dr. Prem Ponka, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, Montreal, Canada. ^{59}Fe in the ferric chloride form was from New England Nuclear (Boston, MA). Culture plasticware and Transwell bicameral inserts were from Costar (Cambridge, MA). To eliminate contaminant Fe, all buffer solutions were filtered through Chelex-100 (Sigma).

Cell extracts

Caco-2 cells, from the American Type Culture Collection (# HTB37, Rockville, MD), were cul-

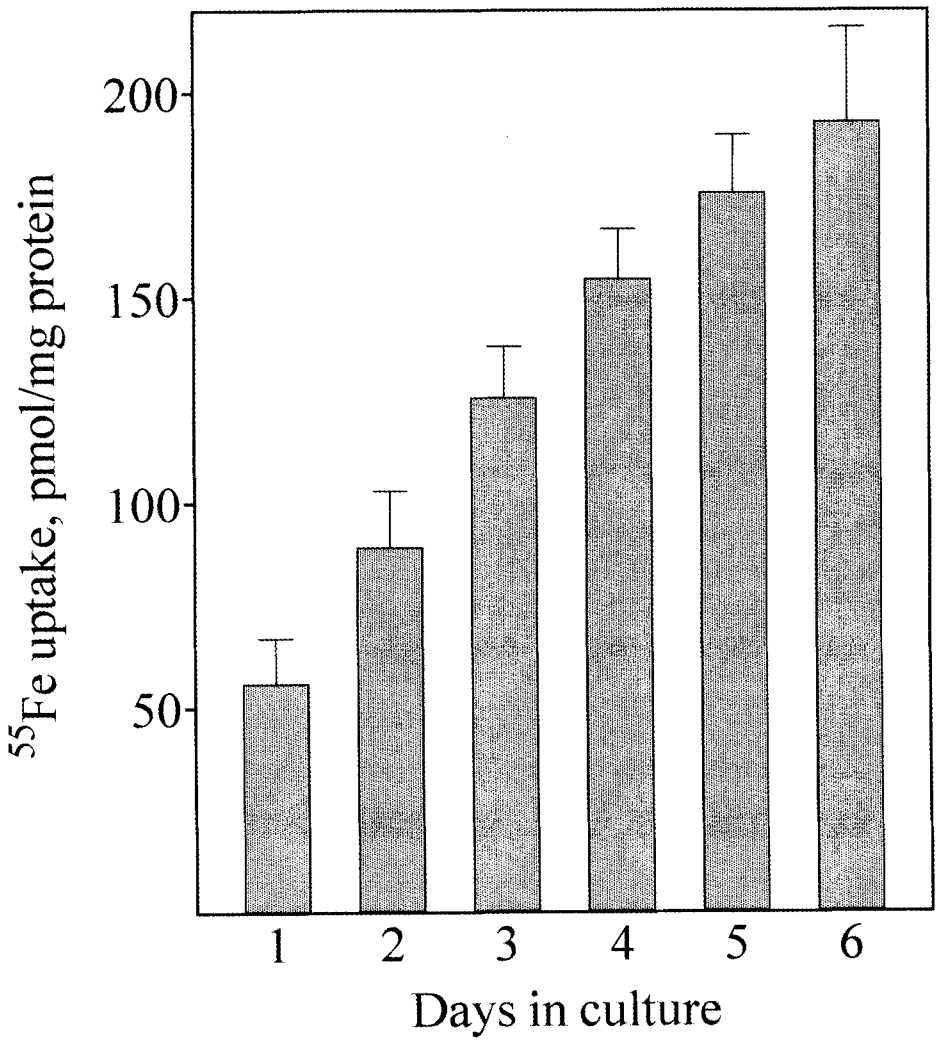


FIGURE 1 Kinetics of iron uptake by Caco-2 cells. Shown is ^{55}Fe uptake as a function of time by Caco-2 cells cultured in the presence of 10 μM ^{55}Fe. No arrest in ^{55}Fe uptake was observed during the period of the experiment. Values represent the mean ± SD of three independent experiments

tured in DMEM supplemented with 10% fetal bovine serum. Culture medium was changed every 2–3 days. Cells were trypsinized and re-plated once a week. To prepare cell extracts, cells were treated with lysis buffer (50 μL per 1×10^6 cells of 10 mM HEPES, pH 7.5, 3 mM $MgCl_3$, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 5% glycerol, 1 mM dithiothreitol, 0.5% Triton X-100). The mixture was incubated for 15 min on ice and sedimented for 10 min at 10,000 × g. The supernatant was stored at –70 °C and aliquots were used for the determination of ferritin and the immunodetection of HNE-modified proteins.

Incubation of Caco-2 cells with ^{55}Fe and determination of cellular levels of ^{55}Fe and *ferritin*

Caco-2 cells, seeded at 1×10^5 cells in 2 cm^2wells, were allowed to grown for 1 day in DMEM-10% fetal bovine serum. The medium was then changed to low Fe Iscove medium (GIBCO) and 10% fetal bovine serum previously depleted of iron ([Fe] < 0.3 μmol/L) [28], supplemented with variable amounts (1–50 μM) of Fe^{3+} as the complex $^{55}FeCl_3$-sodium nitrilotriacetate (^{55}Fe-NTA, 1:2 molar ratio). The cell-associated ^{55}Fe was determined after 6 days of incubation. When time course of ^{55}Fe uptake was determined, the cells were incubated with 10 μM ^{55}Fe as above, and cell-associated ^{55}Fe was determined every day for 6 days. Intracellular levels of ferritin were determined in Caco-2 cell extracts containing different concentrations of Fe, using a sandwich enzyme-linked immunosorbent assay as described [29]. Polyclonal rabbit antihuman ferritin and peroxidase-labeled rabbit antihuman ferritin antibodies were used.

Determination of oxidative damage

Caco-2 cells grown in glass coverslips were incubated for 4–6 days with 1, 5 or 50 μM Fe in the culture media as described above. Evaluating HNE-modified proteins assessed oxidative damage to proteins. HNE is an α,β-unsaturated aldehyde product of the peroxidation of ω6-unsaturated fatty acids that form adducts with amino acid residues such as histidine, lysine and cysteine. HNE was detected with monoclonal antibody HNEJ-2, which recognizes HNE-histidyl adducts [30]. The antibody was developed with biotinated anti mouse IgG and a peroxidase-based Vectastin ABC kit (Vector Labs., Burlingame, CA). Oxidative damage to DNA was assessed with monoclonal antibody N45.1, which recognizes 8-hydroxy-2'-deoxiguanosine (8-OHdG), a highly specific product of DNA damage [31]. This antibody was also developed with the peroxidase-based Vectastin ABC kit (Vector Labs., Burlingame, CA). No peroxidase reaction product was observed if the primary antibodies were omitted or if it they were replaced by non-immune serum. Protein bands in HNE Western blotting were detected using an ECL kit (Amersham).

Cell viability was determined with DCFDA following the instructions of the manufacturer. DCFDA is a non-fluorescent molecule that upon reaction with reactive oxygen species (ROS) becomes the fluorescent compound DCF. In viable cells DCF fluorescence reflects ROS levels while in non-viable cells DCF fluorescence is lost [32]. Transmission and DCF fluorescence images were obtained in a Zeiss MP40 confocal microscope. Cell viability was quantified by the MTT assay following the instructions of the manufacturer. This assay determines the mitochondrial-dependent formation of a colored product [33].

Measurement of the reactive iron pool

The intracellular labile or reactive iron pool of Caco-2 cells was determined as described [25,34]. Briefly, Caco-2 cells were grown on cover slips for 2 days in DMEM, 10% FBS followed by incubation for 5 days in low Fe Iscove medium supplemented with either 1 μM, 10 μM or 50 μM Fe^{3+} as the complex $^{55}FeCl_3$- sodium nitrilotriacetate. Calcein-AM (0.5 μM, Molecular Probes, Eugene, OR), was then loaded into the cells for 5 min at 37°C. After washing the fraction of calcein that was not internalized, the cells were transferred to a cuvette containing 3 ml of MOPS saline (20 mM MOPS-OH, 150 mM NaCl, 1.8 mM $CaCl_2$, 5 mM glucose, pH 7.4) and 5 μl of anti-calcein antibody (the kind gift of Dr. Z.I. Cabantchik). After determination of the basal calcein fluorescence (excitation 488 nm, emission 517 nm), the fluorescence of the calcein-Fe complex was dequenched by addition of 100 μM SIH. The increase in fluorescence thus obtained was directly proportional to the iron labile pool.

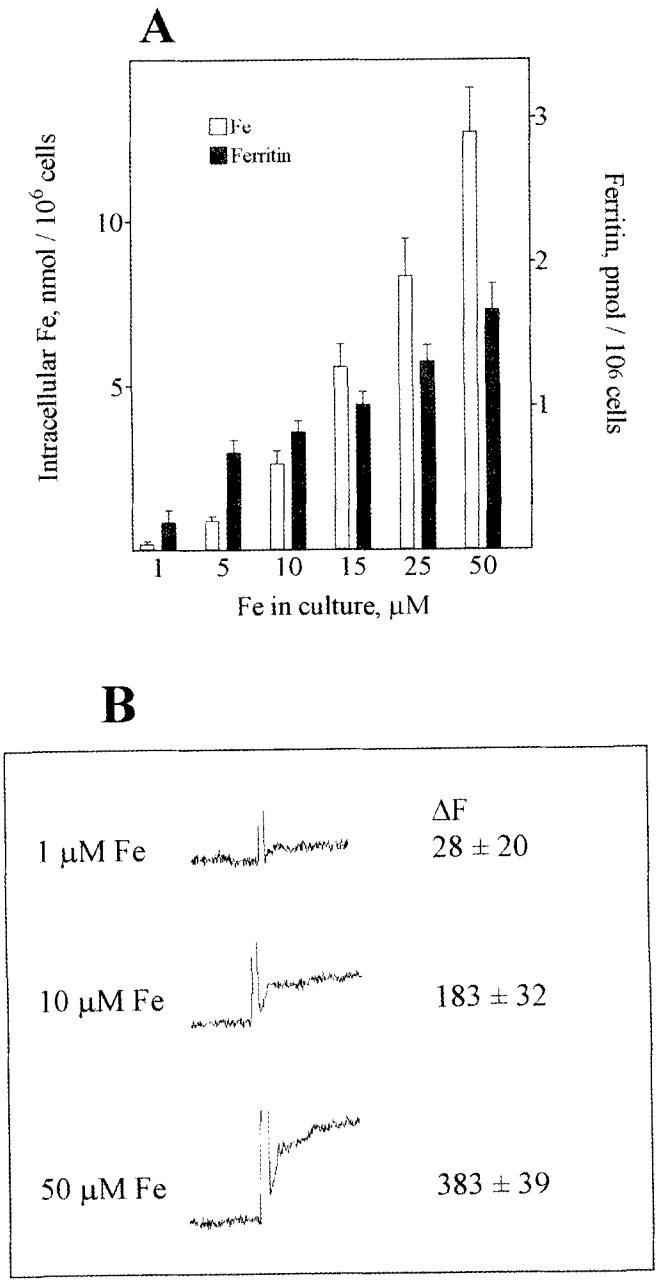


FIGURE 2 Iron, ferritin and labile iron pool in Caco-2 cells as a function of extracellular iron. ***A***. Caco-2 cells were incubated for 6 days with varied concentrations of ^{55}Fe in the culture medium. Extracts of cells were analyzed for cell-associated ^{55}Fe and for ferritin content as a function of the extracellular ^{55}Fe concentration. ***B***. The labile iron pool was measured determining the fluorescence of calcein in Caco-2 cells cultured for 5 days with 1, 10 or 50 μM Fe. SIH is a membrane-permeant Fe chelator that takes Fe from the calcein-Fe chelate thus increasing calcein fluorescence. Thus, the level of the cellular labile Fe pool is directly proportional to the increase in SIH-induced calcein fluorescence. The right column shows mean ± SD of fluorescence changes (ΔF), for three determinations. Shown are representative traces

Data analysis

Variables were tested in triplicate, and experiments were repeated at least 2 times. Variability between experiments was <15%. One-way ANOVA was used to test for differences in means, and a post-hoc *t* test was used for comparisons. Differences were considered significant if $P < 0.05$.

RESULTS

We first tested the characteristics of iron uptake by Caco-2 cells cultured for extended periods of time in a medium containing 10 μM ^{55}Fe (Figure 1). The cells acquired increasing amounts of iron with time. No shut-off of iron uptake was observed after 6 days of culture, although a decrease in the rate of ^{55}Fe uptake was evident. Caco-2 cells also acquired increasing amounts of iron when incubated with increasing concentrations of iron in the culture media (empty bars, Figure 2-A). Cell ferritin levels increased as well, (filled bars, Figure 2-A), an indication that the IRE/IRP system was responding to variations of intracellular iron levels [18,19] It is noteworthy that in a mol to mol basis intracellular iron increased more than intracellular ferritin, and that the molar ratio iron/ferritin surpassed the maximal iron binding capacity of ferritin (4,500 atoms of iron per molecule of ferritin) at about 10–15 μM Fe. Importantly, increasing concentrations of iron in the culture media increased the reactive iron pool, determined by calcein fluorescence quenching (Figure 2-B). Thus, higher concentrations of iron in the extracellular medium resulted in higher levels of intracellular iron and a higher reactive iron pool, despite an active IRE/IRP system.

Iron is potentially toxic to cells because it generates reactive oxygen species. Therefore, high levels of intracellular iron may cause oxidative stress. With this in mind we searched for oxidative damage in high iron cells. To test for protein damage, we measured the formation of HNE adducts. The reaction of Caco-2 proteins with the monoclonal antibody HNEJ-2 revealed that increasing concentrations of iron in the culture media induced increasing amounts of HNE adducts (Figure 3). Immunostaining of similar cell cultures also revealed higher amounts of HNE adducts in cells cultured with 50 μM Fe, when compared to cells cultured with 5 μM Fe (Figure 4).

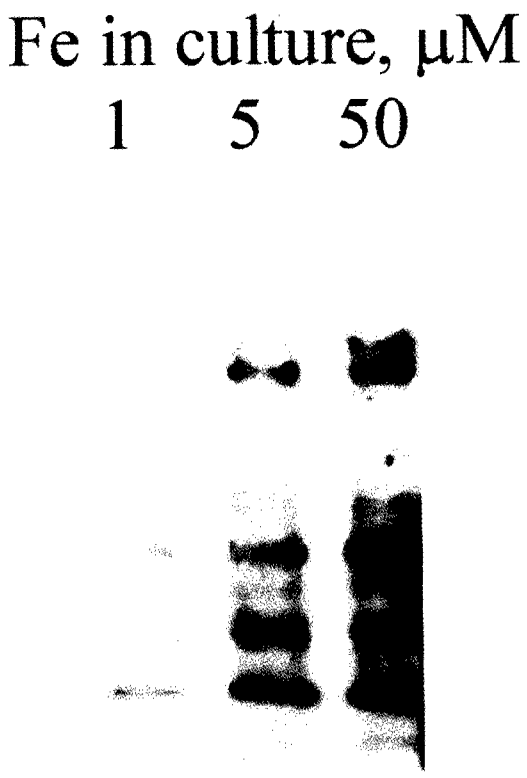


FIGURE 3 Detection of HNE-protein adducts. Extract from Caco-2 cells cultured for 4 days in 1, 10 or 50 μM Fe were separated by SDS-gel electrophoresis. The protein bands were blotted into nitrocellulose and the HNE-protein adducts detected with monoclonal antibody HNEJ-2

Fe-mediated oxidative damage to DNA was assessed with monoclonal antibody N45.1, which recognizes 8-OHdG, a highly specific product of DNA damage. Immunostaining of Caco-2 cells cultured for 5 days with high (50 μM) iron revealed marked oxidative damage to DNA (Figure 5-B) while no damage was evident in cells cultured with 5 μM Fe (Figure 5-A).

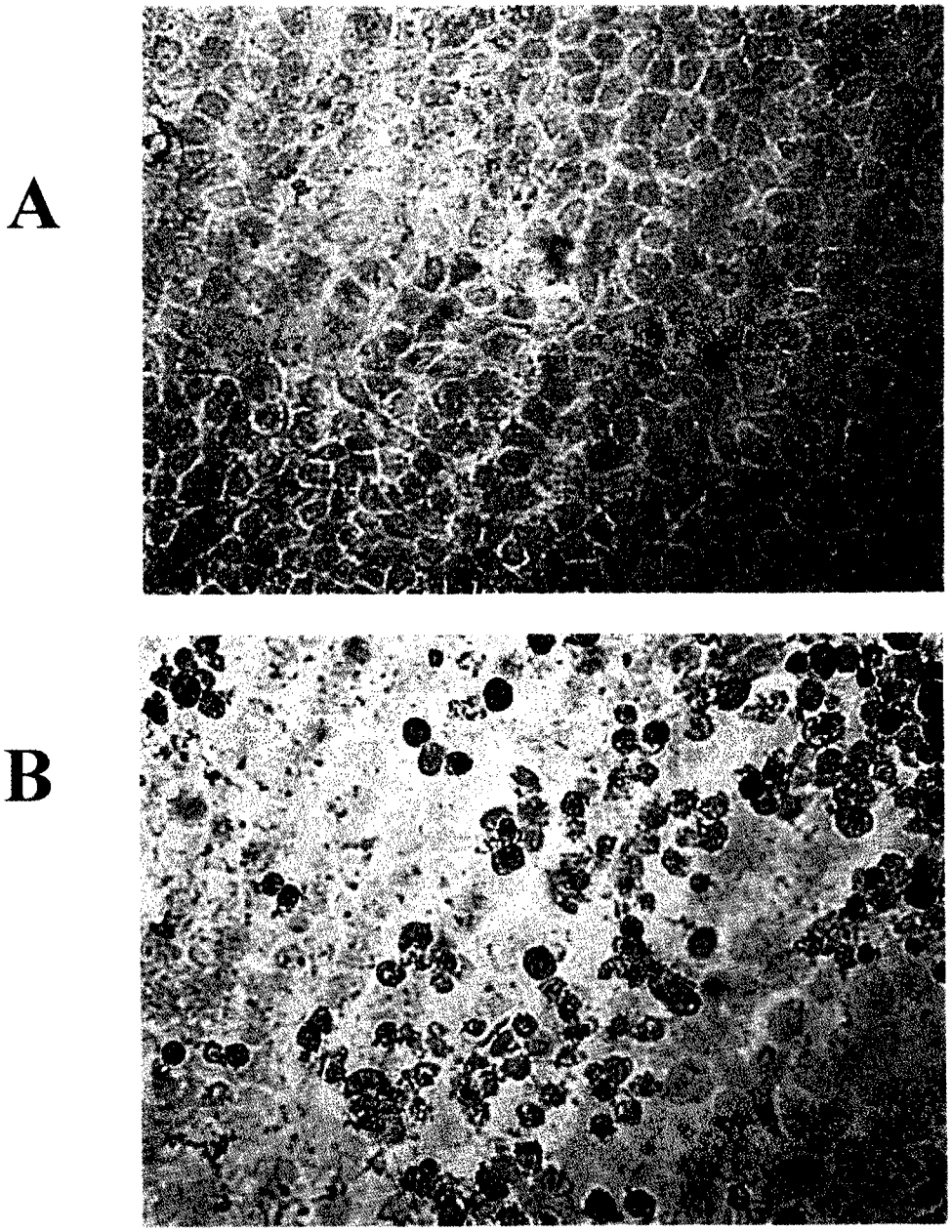
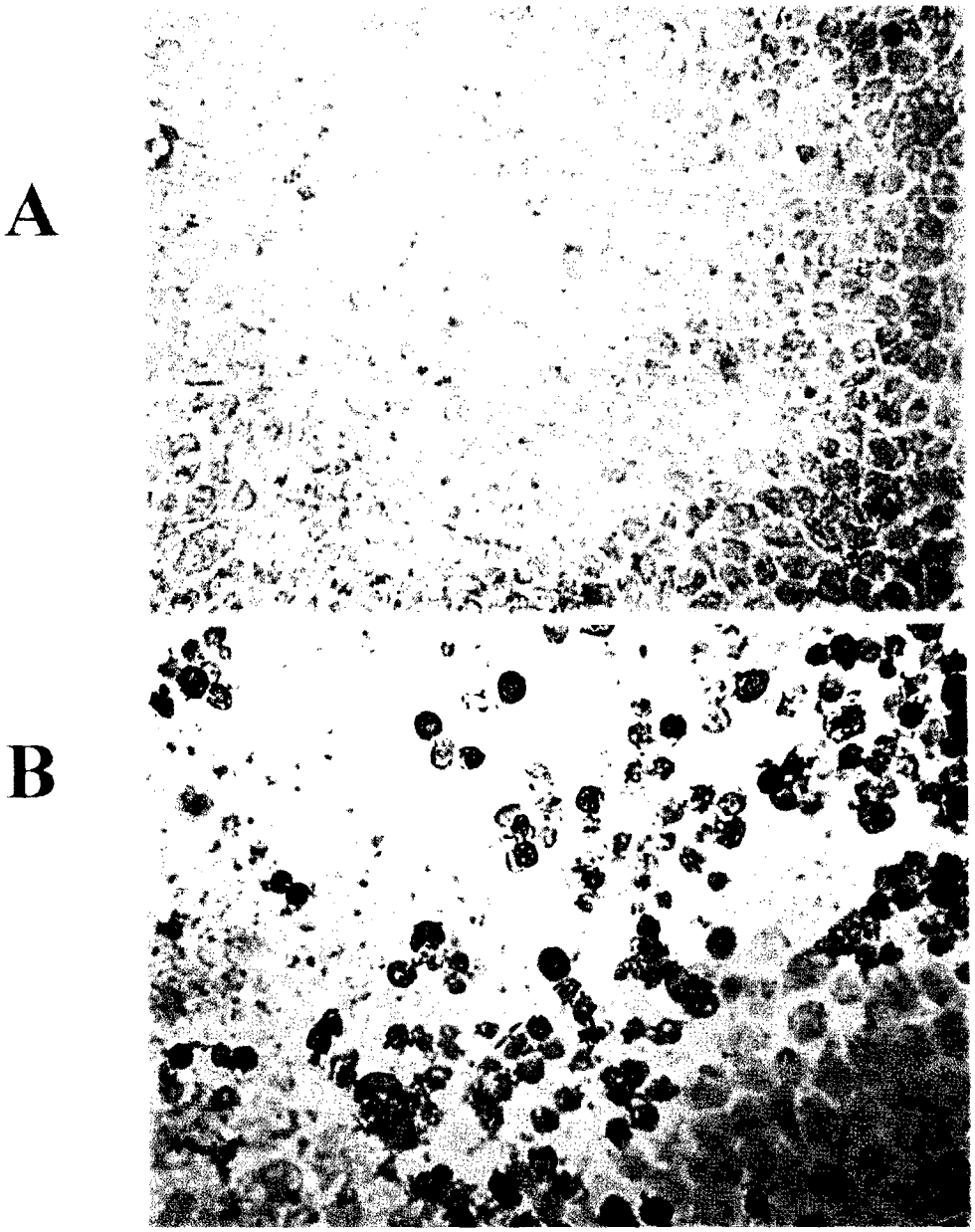


FIGURE 4 Cell immunostaining of HNE adducts. Caco-2 cells grown for 4 days either in 5 μM Fe (**A**) or 50 μM Fe (**B**) media were stained for HNE adducts. Control, 5 μM Fe, cells (**A**) presented little damage as shown by scarce dark peroxidase staining, while about 10 % of the cells incubated with 50 μM Fe (**B**) were markedly HNE-positive (See Color Plate VIII at the back of this issue)



Color Plate VIII (See page 63, Figure 4) Cell immunostaining of HNE adducts. Caco-2 cells grown for 4 days either in 5 μM Fe (**A**) or 50 μM Fe (**B**) media were stained for HNE adducts. Control, 5 μM Fe, cells (**A**) presented little damage as shown by scarce dark peroxidase staining, while about 10 % of the cells incubated with 50 μM Fe (**B**) were markedly HNE-positive

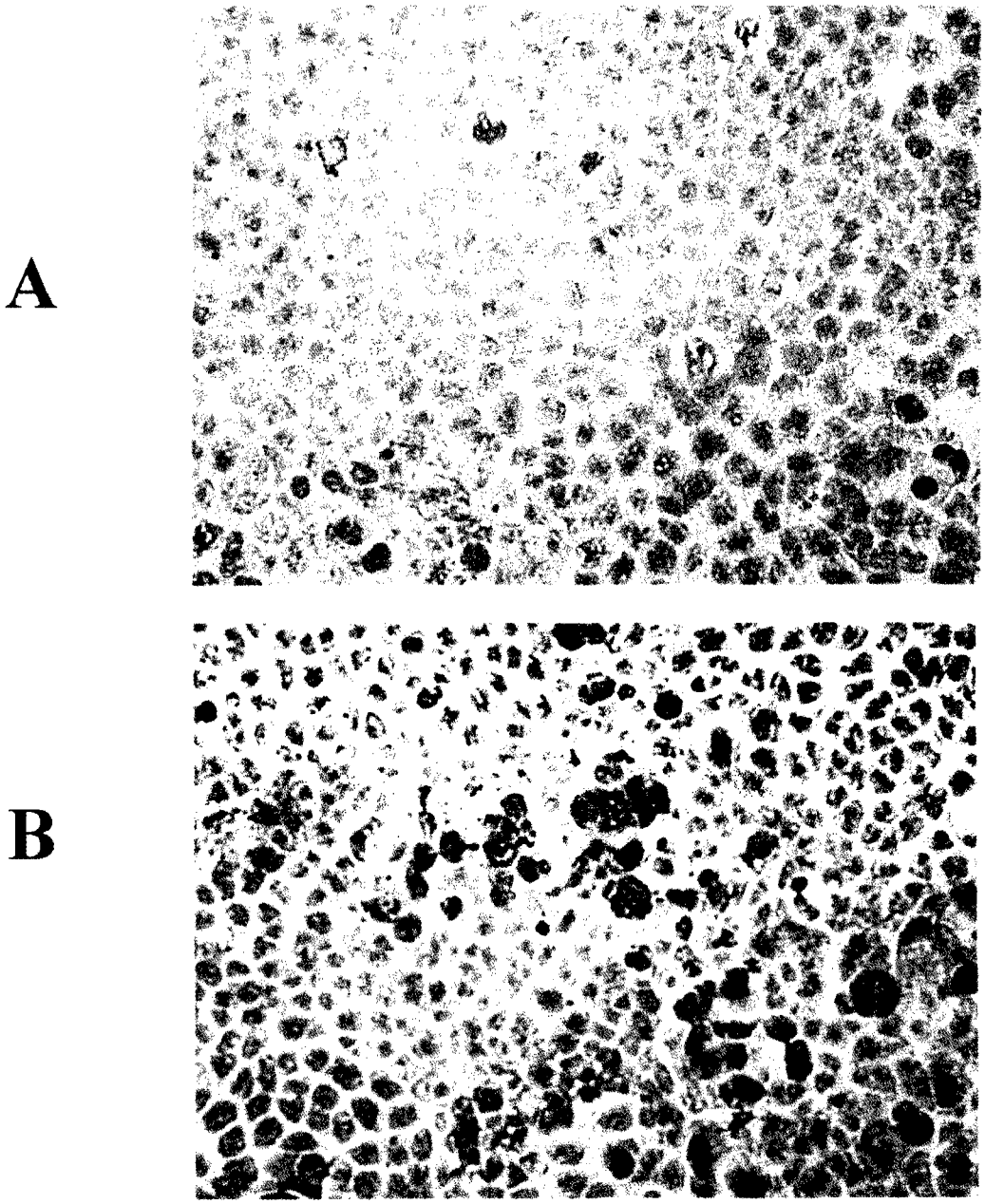
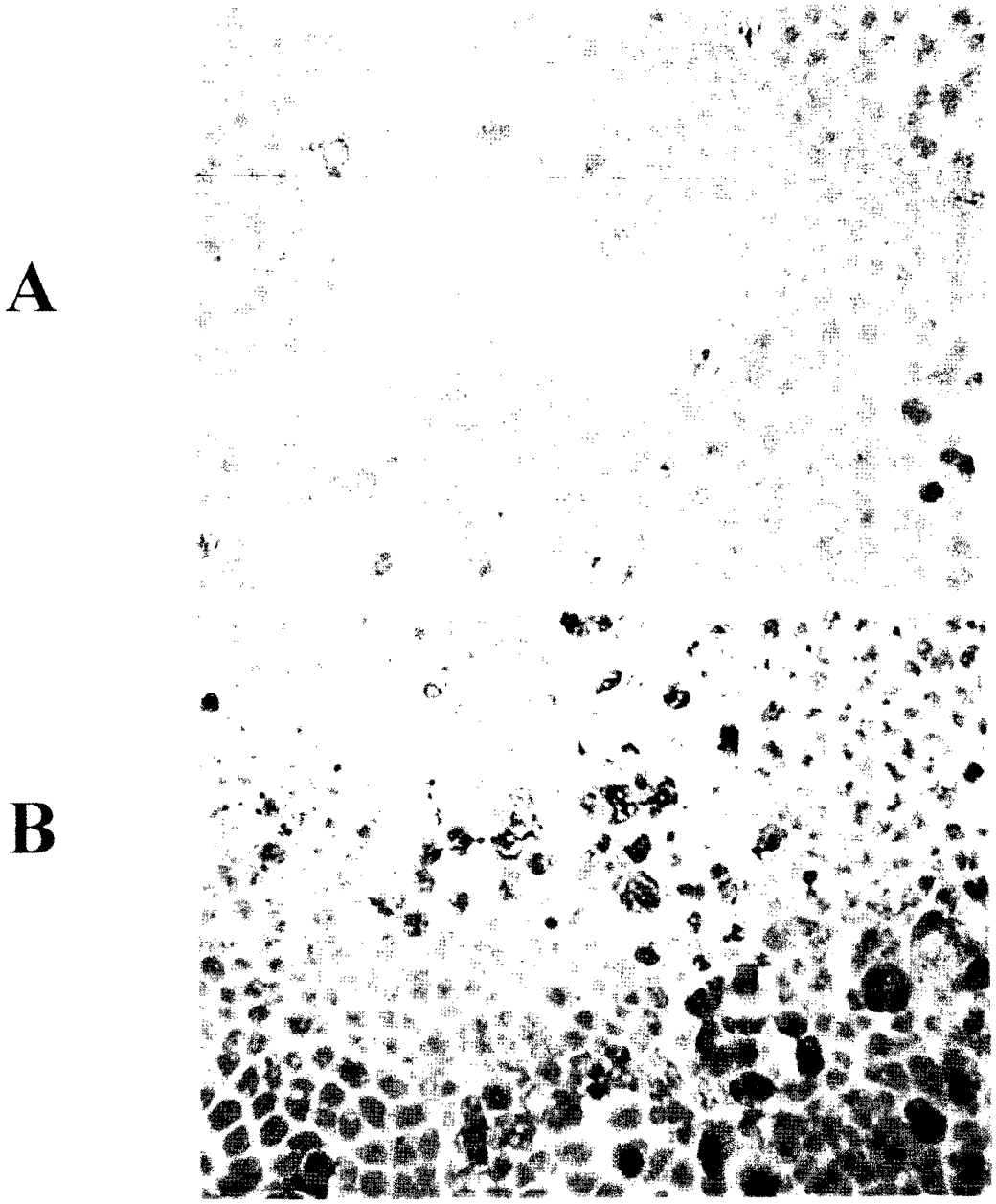


FIGURE 5 Iron-induced DNA damage. Caco-2 cells grown for 4 days either in 5 μM Fe (**A**) or 50 μM Fe (**B**) media were immunostained for 8-OhdG to detect for DNA damage. Cells incubated with 50 μM Fe (**B**) were markedly 8-OHdG-positive as compared to cells incubated in 5 μM Fe (**A**) (See Color Plate IX at the back of this issue)

Cell viability was tested utilizing DCFDA. Cells grown for 6 days in 1 μM Fe or in 5 μM Fe showed a normal morphology, with cell limits clearly delineated by the probe fluorescence



Color Plate IX (See page 64, Figure 5) Iron-induced DNA damage. Caco-2 cells grown for 4 days either in 5 μM Fe (**A**) or 50 μM Fe (**B**) media were immunostained for 8-OhdG to detect for DNA damage. Cells incubated with 50 μM Fe (**B**) were markedly 8-OHdG-positive as compared to cells incubated in 5 μM Fe (**A**)

(Figure 6-A). DCF fluorescence in cells grown in 5 μM Fe was consistently stronger than that of cells grown in 1 μM Fe (Figure 6-A), an indication of a larger production of reactive oxygen species under the 5 μM situation. Cells grown under high (50 μM) Fe showed foci of cell disintegration, with evident loss of cell border, a clear indication of plasma membrane oxidative damage (Figure 6-A). These foci represented about 5 % and 10 % of the cells in the culture after 5 or 6 days of culture, respectively. Determination of cell viability by the MTT assay revealed a small viability loss in the 50–100 μM Fe range (Figure 6-B). When compared to the control (5 μM Fe) situation, viabilities of 91.2 ± 3.2 % and 84.4 ± 7.3 % were found for the 50 μM and 100 μM Fe, respectively. These differences were significant, with $P < 0.05$ and $P < 0.01$, respectively. Hence, Caco-2 cells exposed to high, but physiological concentrations of iron, evidenced extensive oxidative damage in proteins and DNA, which in time resulted in structural cell damage.

DISCUSSION

Using Caco-2 cells as a model of intestinal epithelia, we found that despite an active IRE/IRP system, intracellular iron accumulation did not preclude further iron uptake by the cells. This behavior resulted in an undeterred cellular iron accumulation. Although the above finding may not be applied to all cell types, we have found similar responses using K562 cells and N2A neuroblastoma cells (unpublished results). Correspondingly, Caco-2 cells grown in a medium with a high but physiological content of iron presented oxidative damage to proteins and to DNA, an indication that cell iron accumulation, and the accompanying increase in the labile iron pool, were the direct cause of the oxidative damage. Iron-mediated oxidative damage was accumulative, and with time generated loss of cell integrity and cell death.

Analysis of the IRE/IRP system indicates that it responds optimally to a low iron challenge, which leads to the activation of IRPs. On the contrary, under high iron conditions, a low level of TfRs is expected because of basal translation of TfR mRNA. In intestinal Caco-2 cells, this basal translation is compounded by an IRP-2 activity that is unresponsive to Fe overload, producing basal levels of both apical Fe uptake and basolateral Tf-associated iron uptake [28], and by the expression of a non-IRE form of the apical iron transporter DMT1 [29]. Similarly, the expression in hepatocytes of TfR2, a TfR homologue without IRE motifs in its mRNA [35], allows for the continued uptake of iron even under iron overload conditions. Thus, some cell types have mechanisms that escape cellular iron homeostasis, a conduct that results in high intracellular iron levels. The findings presented in this work fit the concept of antagonist pleiotropy [36,37], which establishes that evolution will select genes favoring conditions of early development and entrance in reproductive age, as is the case of an ample intracellular iron supply, in detriment of other effects late in life, as it could be the iron-mediated production of reactive oxygen species.

In summary, intestinal Caco-2 cells accumulated iron in a time and concentration-dependent manner. Iron accumulation by Caco-2 cells correlated with oxidative damage to proteins and DNA, finally resulting in loss of cell viability. These results underscore the importance of Fe in the generation of oxidative damage and may bear relevance to understand the development of colon cancer.

Acknowledgements

This work was supported by project P99-031 of the Millennium Institute for Advanced Studies in Cell Biology and Biotechnology, Santiago, Chile, and by a Cátedra Presidencial en Ciencia to MTN. Shigeru Okada is a nominee of Exchange Fellowship Program sponsored by Japan Society for the Promotion of Science.

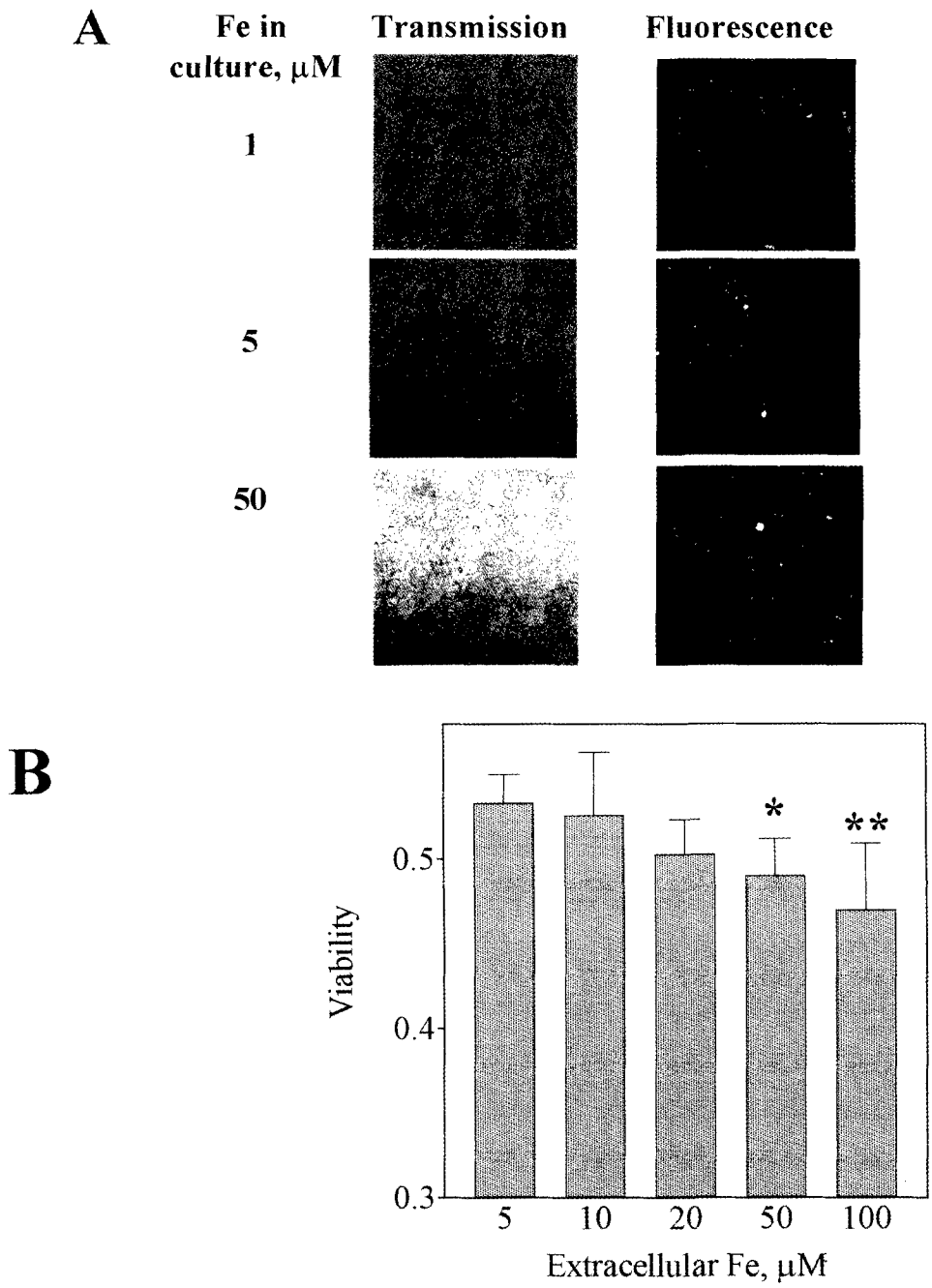


FIGURE 6 **A**. Cell viability of iron-treated cells. Caco-2 cells cultured for 6 days either in 1 μM, 5 μM or 50 μM Fe medium were assayed for viability with DCFDA. Shown are transmission microscopy (Transmission) and fluorescence microscopy (Fluorescence) images. No evidence of cell damage was observed in 1 μM and 5 μM Fe cells (**A**) while 50 μM Fe cells showed focuses of cell disintegration, with evident loss of cell border. **B**. Cell cultured for 6 days in medium containing 5 μM, 10 μM, 20 μM, 50 μM or 100 μM Fe were assayed for viability by the MTT assay. *, significantly different ($P < 0.05$) to the 5 μM Fe condition; **, significantly different ($P < 0.01$) to the 5 μM Fe condition

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