Free Rad. Res., Vol. 34, pp. 221-235
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Iron Induces Bcl-2 Expression in Human Dermal Microvascular Endothelial Cells

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Accepted for publication by Prof. B. Halliwell

(Received 8 June 2000; In revised form 22 July 2000)

Iron is suspected to be involved in the induction and/or progression of various human tumors. The present study was designed to investigate the effects of iron on endothelial cells, keeping in mind that the homeostasis of microvessels plays a critical role in neo-angiogenesis. Applying a model of human dermal microvascular endothelial cell terminal differentiation and death induced by serum deprivation, we found that iron salts (iron chloride and ferric nitrilotriacetate) provided a survival advantage to endothelial cells. Using immunohistochemistry and Western Blot analysis, we found that the extended cellular life span induced by iron was paralleled by an increase of Bcl-2 protein expression. Taken together, these observations suggest that iron may give a survival advantage to endothelial cells and represent a novel mechanism through which iron may contribute to tumorigenesis.

Keywords: bcl-2, cancer, human dermal microvascular endothelial cells, iron, tumor

INTRODUCTION

Iron, a major catalyst in the formation of hydroxyl radicals, is thought to be involved in the development of cancers in humans[1,2]. Injections of iron complexes have been observed to result in sarcomas at the sites of deposition[3,4]. There is also a positive correlation between body iron levels and cancer risk[5]. Patients with hemochromatosis, a genetic disease characterized by iron overload show a markedly enhanced susceptibility to primary liver cancer and various other malignancies[2,6]. The pathogenic role of iron in cancer development and/or progression remains unclear. At least four schematic carcinogenic pathways have been described. First, iron may promote the formation of mutagenic hydroxyl radicals. Second, iron excess diminishes host defenses through inhibition of the activity of macrophages and lymphocytes[2,7]. Third, iron can enhance host cell production of viral nucleic acids[8] which, in turn, may be involved in the development of some human cancers. Fourth, iron can directly promote the growth of some cancer cells[9,10], probably through its role in the activation of ribonucleotide reductase, a key-enzyme in DNA

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synthesis, responsible for the reduction of ribonucleotides to deoxyribonucleotides[1,2].

One of the major processes involved in the development of tumour growth is the 'switch' to an angiogenic phenotype, involving a change in the equilibrium between negative and positive regulators of the growth of microvessels[11]. Endothelial cells, because of their unique and strategic position in the lining of blood vessels, are a target for circulating iron. In this report, we investigated whether iron might affect the balance between rates of endothelial cell growth and cell loss and might modify their Bcl-2/Bax ratio which is a key element in the control of apoptotic cell death[12]. Elevated levels of Bcl-2 protein can block or delay apoptosis induced by a wide variety of stimuli and insults, including serum deprivation[13–15]. The survival advantage conferred by Bcl-2 may lead to subsequent acquisition of genetic changes resulting ultimately in tumor progression[16–18].

MATERIAL AND METHODS

Compounds

Iron chloride ($FeCl_3$) was obtained from Fluka (Buchs, Switzerland). Ferric nitrilotriacetate (FeNTA), N-acetylcysteine (NAC), interleukin-1β (IL-1β) were from Sigma (Bornem, Belgium). Basic fibroblast growth factor (bFGF) was from ICN (Aurora, OH). Desferrioxamine (DFO) was purchased as its commercially available mesylate salt. Bax MoAb (G206–1276) was from Pharmingen (San Diego, CA); Bax polyclonal antibody (I-19) from Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-2 MoAb (100) from Oncogene Research Products (Cambridge, MA) and proliferating cell nuclear antigen (PCNA) MoAb (PC10) from Boehringer Mannheim (Germany). Normal immunoglobin fractions and CD71 MoAb (anti-transferrin receptor) (M734) were from Dako (Glostrup, Denmark).

Cell Culture

Human dermal microvascular endothelial cells (HDMEC) were cultured by a method similar to that reported by Kluger et al. (1997)[19]. HDMEC were isolated from normal adult breast skin obtained as discarded tissue from reduction mammoplasties (Department of Plastic Surgery, Erasme University Hospital). Informed consent was obtained from the patients. Fresh skin was stretched flat and sectioned horizontally. After a 80-min incubation in dispase II 2 mg/ml (Boehringer, Brussels, Belgium) at room temperature, the epidermis was peeled off and cells from both sides of the underlying epidermis were gently scraped into RPMI (Gibco) and filtered through a 70-μM nylon mesh. The filtrate, containing single cells, was washed once in minimum essential medium D-Val and plated onto tissue culture plastic pre-coated with the cell culture medium (C-22020, PromoCell, Heidelberg, Germany) supplemented with 10% FCS (Gibco). HDMEC were allowed to attach approximately 4 h before gentle aspiration (to remove cell debris) and addition of fresh media. The cells were used at passage 5–12. FACS analysis revealed that > 95% of the cell population was positive for endoglin (Pharmingen). Immunostaining with a specific anti-human fibroblast antibody (Dianova, Hamburg, Germany)[20] was negative.

Induction of Apoptotic Cell Death by Serum Deprivation

Briefly, exponentially growing HDMEC were allowed to proliferate in complete growth medium until they reached 85–90% confluence. Apoptosis was induced in subconfluent cell cultures by serum deprivation as described by Hébert et al. (1994)[21]. After having removed the FCS containing medium, serum-free RPMI (with HEPES and glutamine (Biowhittaker, Verviers, Belgium) supplemented with 1% non-essential amino-acids was placed onto the monolayer cultures. Since RPMI contains only traces of iron

(manufacturer's data and personal assay using ion coupled plasma optical emission spectroscopy torche axiale (Thermo-optek) showing an iron concentration < 10 μg/ml (lower limit of detection of the system), this procedure withdrew the iron from the culture medium.

Iron Treatment

$FeCl_3$ prepared in RPMI containing HEPES (concentrations as indicated) was added either to HDMEC cultured with 10% FCS or to HDMEC that had been exposed to overnight serum deprivation. Using a PHM82 standard pH meter (Van der Heyden, Brussels, Belgium), we found that this treatment did not change the pH of the culture medium. The cells cultured with FCS were further cultured with serum and the serum-deprived cells were further cultured in the absence of serum. At various time points, viable cell numbers were estimated by the trypan blue exclusion test. Cells in individual monolayer cultures were harvested and made into single-cell suspensions by trypsinisation. The cell suspensions were then incubated for 3 min with trypan blue dye. The cells remaining negative to the blue dye staining after trypan blue exclusion were counted as viable cells. The ability of the cells to survive and further divide was determined by the retention of their colony-forming ability upon return to serum-supplemented conditions. Cells remaining adherent to the plate were harvested at various time intervals after the serum withdrawal and equal volumes from control and iron-exposed cultures were re-suspended in complete growth medium containing 10% FCS. Plating efficiency was determined at various time intervals as the total number of cells per dish and as the number of colonies ≥ 4 cells per 10 high power fields.

Determination of Iron Incorporation

Iron uptake was determined on cytospin preparation using the method of Lillie (1965)[22]. Briefly, cytospin preparations were placed in a potassium ferrocyanide solution for 1 hr and washed in 1% aqueous glacial acetic acid. The preparations were then placed in a basic fuchsin solution for 10 min and rinsed in distilled water. Finally, they were dehydrated in 95% absolute alcohol and cleared in xylene, 2 changes each. Iron taken up appeared as dark blue particles in pink-colored cells.

Immunohistochemistry

The cells were trypsinised 24 hours after the addition of iron and made into single-cell suspensions. Immunohistochemistry was carried out on cytospins. Briefly, subconfluent cell cultures were harvested and made into single-cell suspension by trypsinisation. For Bax (I-19), the cytospin preparations were fixed in Bouin for 15 min and then incubated in 3% (v/v) H_2O_2 in PBS for 30 min at room temperature. The samples were incubated in normal goat serum for 30 min to block nonspecific interactions, and then anti-Bax antibody was applied at the dilution of 2 μg/ml overnight at 4°C. Normal rabbit immunoglobin fractions at the same concentration as the primary antibodies served as negative controls. Immunohistochemical staining was achieved with the PK-4001 Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin. For Bcl-2 MoAb, two different fixation procedures were used: 1) the cells were fixed in formol for 1 hr at room temperature and incubated in 3% (v/v) H_2O_2 in PBS for 30 min. They were then microwaved at 250 Watts for 2 × 5 min in a citrate buffer, pH 6 and then blocked with normal goat serum 1/20 for 30 min; 2) they were fixed in absolute methanol for 10 min at room temperature, incubated in 3% (v/v) H_2O_2 in PBS for 30 min, and then blocked with the normal goat serum. The cytospins were then immersed for 10 min in PBS and incubated in 3% (v/v) H_2O_2 in PBS for 30 min before immunocytochemical staining with 5 μg/ml Bcl-2. For stable

PCNA, the samples were fixed in absolute methanol for 10 min and permeabilized with Triton-X100 (0.1% in PBS), rinsed twice with PBS, and incubated in 3% (v/v) H_2O_2 in PBS for 30 min before immunocytochemical staining (IP-10, 5 μg/ml). For total PCNA, there were fixed in 1% paraformaldehyde in PBS (pH 7.2), containing 6.6 μg of lysolecithin, followed by immersion for 10 min in absolute methanol at ice temperature and permeabilization with 0.1 % NP-40 in PBS on ice, as previously described[23]. The formed immune complexes were detected with Envision™+/HRP (Dako). Peroxidase activity was developed with 3,3'-diaminobenzidine tetrahydrochloride (0.012 % in PBS) and H_2O_2 (0.1 %) and the cells were counterstained with haematoxylin.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Desoxyuridinetriphosphate (dUTP) Nick End Labelling (TUNEL) Assay

TdT assay for strand breaks was performed as previously reported[24]. Briefly, the formalin-fixed cytospin preparations were incubated with 20 μg/ml proteinase K (Sigma) for 15 min at room temperature in order to strip the nuclei from proteins. The samples were then washed four times in double-distilled water for 2 min. Endogenous peroxidase was inactivated by covering the slides with 2% (v/v) H_2O_2 in PBS for 30 min at room temperature. The preparations were rinsed with double-distilled water, and immersed in terminal TdT labelling buffer (30 mM Trisma base, pH 7.2, 140 mM sodium cacodylate, 1mM cobalt chloride). TdT (0.3 e.u./μl). dATP and biotinylated dUTP in TdT buffer were then added to cover the cytospin preparations in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to terminating buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The slides were then rinsed with double-distilled water, covered with a 2% aqueous solution of bovine serum albumin for 10 min at room temperature, rinsed in double-distilled water and immersed in PBS for 5 min. Peroxidase activity was developed with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2.

Western Blot Analysis of Protein Expression

Confluent cell cultures were washed with complete PBS and lysed in SDS buffer (5 % β-mercaptoethanol, 10 % glycerol, 80 mM SDS, 60 mM Tris, pH 6.8). For each sample, a total quantity of 40 μg of protein was electrophoresed on a 12 % sodium dodecyl sulfate-polyacrylamide linear gradient slab gel, in Tris-buffered saline solution. The proteins were then electrophoretically transferred to nitro-cellulose sheets. The blots were subsequently incubated for 1 h in 1% fat-free milk in TBS and for 18 h either with Bcl-2 (Ab-1) MAb at a 2 μg/ml concentration in TBS (20 mM Tris, 125 mM NaCl) or with Bax (G206–1270) MAb at a 4 μg/ml concentration. After extensive washing in TBS, the immune complexes were detected with either a biotinylated anti-rat Ig (Bax) or an anti-mouse Ig (Bcl-2) (diluted 1:250 in TBS) (Amersham, Ltd), serving as binding bridge to biotin-streptavidin peroxydase preformed complexes used at the same dilution. Preformed complexes were detected by photographic recording of the chemiluminescence (ECL) emitted by a H_2O_2 reacting probe, using the Boehringer Mannheim's kit. Lysates from Kaposi's sarcoma (KS)-derived cells and from MCF-7 cells were used as positive controls for Bcl-2[25]. Lysates from NIH-3T3 fibroblasts served as positive control for Bax[26]. The immunoblots were scanned with a Microtek Phantom 4800 apparatus.

Statistical Analysis

The Student's t-test (2-tailed) was used to compare the different groups of data.

RESULTS

Survival Kinetics of HDMEC in Serum-Free Conditions

Previous studies indicated that cell death induced by serum withdrawal or by growth factors deprivation occurs through apoptosis[13,15,27]. Serum deprivation resulted in progressive contraction of the cell population, followed by production of blebs and detachment from the plate (Fig. 1A and 1B). By contrast to the adherent cells, the detached cells were positive to the trypan blue dye staining. Detached cells also appeared smaller than adherent cells. The morphological features of the two populations suggest that serum deprivation-induced apoptosis is a dynamic process, commencing with adherent cells and being completed following detachment (Fig. 2). This indicates that the cells die and detach, rather than die because they have detached. Cell death was first apparent 1 day after serum deprivation. By day 3, only 10 % of the initial HDMEC population survived. Six days after the serum withdrawal, only a minor proportion of the cells (0.005 ± 0.003%) remained adherent to the plate. Most of these cells had a spindle-shaped morphology very similar to that of spindle cells derived from Kaposi's sarcoma (Fig. 1C). This suggests that, while the majority of HDMEC rapidly died following serum deprivation, a small proportion could survive and gradually undergo morphological differentiation. By contrast, HDMEC cultured with FCS exhibited neither significant cell detachment nor significant morphological change. To assess whether the cells were still able to divide after serum deprivation, we studied the plating efficiency and established the growth curves upon return to serum-supplemented conditions. As long as the cell growth could be re-initiated, the cell colonies exhibited a typical cobblestone morphology. These cells were still positive for endoglin, which rules out the possibility of selection of non-endothelial cells following conditions of serum deprivation.

Effect of Iron on HDMEC Kinetics

Since iron may exert dual effects in terms of cell life and death, we first examined the effects of iron on the balance between endothelial cell survival, proliferation and death. Trypan blue exclusion demonstrates that iron did not significantly prevent the cell loss induced by serum deprivation (Fig. 3). TUNEL assay revealed that iron did not significantly prevented the serum deprivation-induced cell death (5.5 ± 2.2 *vs* 3.5 ± 1.2 % TUNEL-based apoptotic index after a 32 hours serum deprivation period in control and 20 μM iron-treated cells, respectively). Concentrations higher than 100 μM were readily cytotoxic. Since the trypan blue exclusion test and the TUNEL assay only account for cell viability and fail to discriminate between differentiated cells and cells keeping their proliferative potential, we performed plating efficiency experiments. Cell survival assays demonstrated that, when re-suspended in growth medium containing 10% FCS, the cells previously exposed to iron displayed a significant survival advantage as compared to the control cells (i.e., not treated with iron) (Fig. 4). Determination of the number of colonies ≥ 4 cells (number of cells having accomplished at least 2 mitoses) that could be observed 4 days after re-suspension in optimal growth conditions yielded similar results than cell counts (data not shown). The effective iron concentrations ranged between 10 and 25 μM. Similar results were observed with FeNTA (data not shown). No significant inhibition of terminal differentiation could be observed when $FeCl_3$ was pre-incubated with a stoechiometric amount of DFO. No survival advantage was evidenced when iron was added to HDMEC cultured with FCS (data not shown).

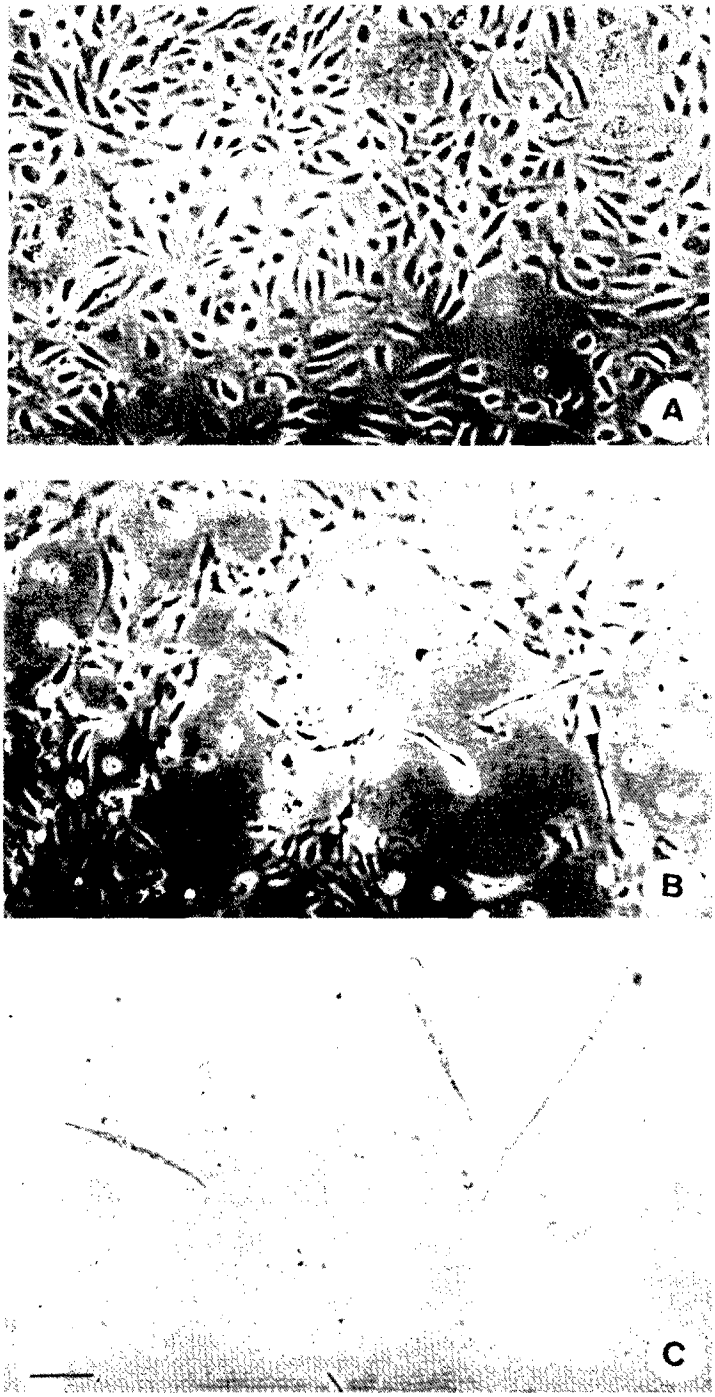


FIGURE 1 Morphological changes of HDMEC under serum deprivation conditions. A, phase-contrast photomicrograph of confluent monolayer HDMEC showing typical cobblestone morphology. B, same culture 2 days after serum withdrawal showing retraction of the cytoskeleton, rouding up of cells and detachment from the plate. C, same culture 6 days after serum withdrawal showing some cells exhibiting an elongated, spindle-shaped morphology. Bars: 100 μM

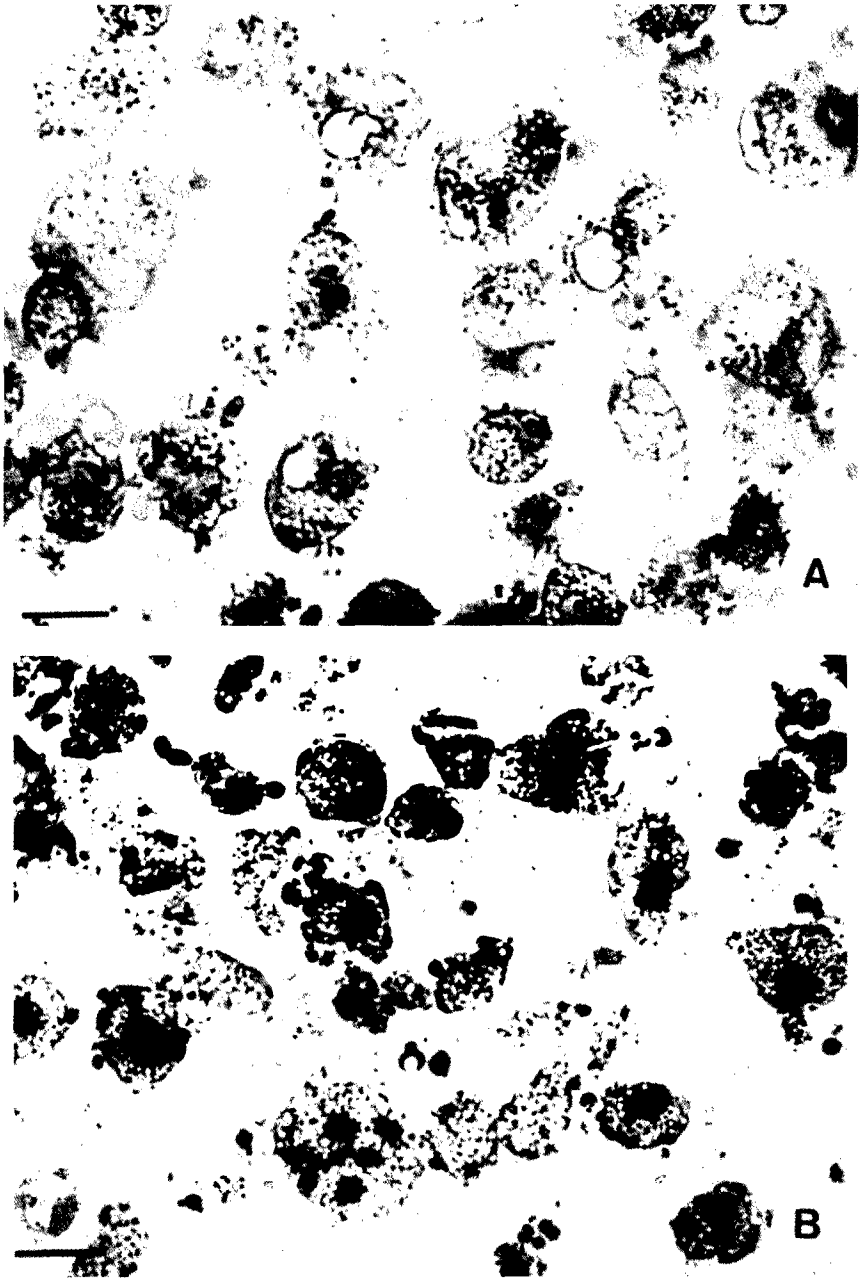


FIGURE 2 Induction of apoptosis in HDMEC cultured under serum-free conditions. Ultrastructure of trypsin-EDTA released adherent HDMEC (A) compared with detached floating cells (B). Some of the floating detached cells are highly vesiculated. Bars: 20 μM

Since iron displays mitogenic properties in various cell types, we next investigated whether it could increase the proportion of cells in the cell cycle in conditions of serum deprivation. Total PCNA index declined from 52 ± 19% at the start of the experiment to 6 ± 5% at 24 hours. Simi-

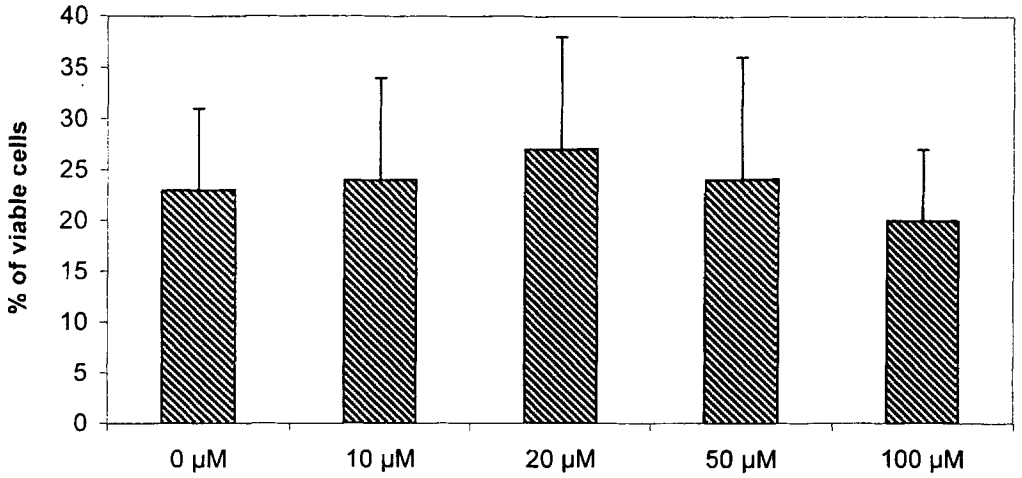


FIGURE 3 Effect of iron supplementation on endothelial cell loss induced by serum deprivation. After overnight serum deprivation, the cells were pulsed with $FeCl_3$ (concentrations as indicated) and were further cultured for 1 day in serum-free conditions. Results are expressed as means ± SE of 3 independent experiments performed in triplicate

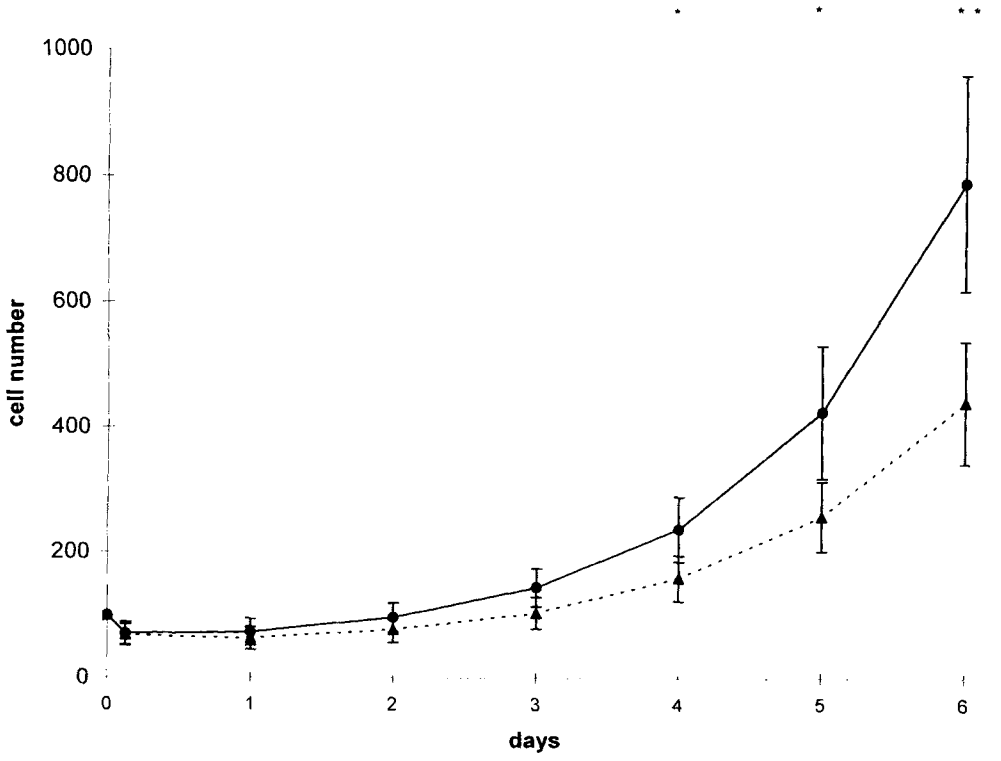


FIGURE 4 Growth of control and iron-treated cells upon return to serum-supplemented medium. HDMEC supplemented (•) or not (▲) with 20 µM iron were cultured in serum-free conditions for 4 days. The cells were counted at various time intervals after re-suspension in medium containing 10% FCS. $^{*}p < 0.05$; $^{**}p < 0.01$, significant difference between the growth of cells supplemented or not with iron. Results are expressed as means ± SE of 4 independent experiments

larly, serum-deprived HDMEC expressed low to undetectable levels of stable PCNA, which further indicates the synchronization and the G_0/G_1 growth arrest of the cells upon serum-free conditions[28,29]. The cell growth arrest was confirmed by the low number of positive cells (0 to 0.2%) after a 2 h pulse of BrdUrd (data not shown). As a whole, these data demonstrate that serum deprivation-induced HDMEC death is not a form of abortive mitosis. At any concentration studied (5 to 100 μM), iron did not significantly increase the number of PCNA-labelled cells nor the incorporation of BrdUrd (data not shown). This suggests that iron provides a survival advantage without eliciting HDMEC replication during serum deprivation. Analogously, iron did not significantly increase the PCNA index of HDMEC cultured with serum (data not shown).

To confirm that the serum deprivation, and hence the absence of unsaturated transferrin, did not prevent the cellular iron uptake, we used the method of Lillie (1965)[22]. This showed the presence of blue particles in the cytoplasm of iron-treated cells. By contrast, no apparent particles could be found in non iron-treated cells (Fig. 5).

Iron Increases Bcl-2 Expression in HDMEC

Bcl-2 is an oncoprotein known to be involved in cell survival upon growth factors deprivation conditions[29]. More particularly, Bcl-2 overexpression is known to protect endothelial cells from the effect of serum deprivation[15]. In a series of experiments we examined whether iron could induce Bcl-2 expression in HDMEC. After overnight serum deprivation, the cells were pulsed with $FeCl_3$ or FeNTA (concentrations as indicated) and were further cultured in serum-free conditions. Confirming previous studies[25], immunohistochemistry revealed low to undetectable levels of Bcl-2 protein in unstimulated HDMEC. Exposure of the cells to iron led to a significant induction of the protein (Fig. 6). Concentrations lower than 5 μM had a negligible effect (data not shown). The control isotypes were repeatedly negative, indicating that the observed immunohistochemical effect of iron was not due to a non-specific oxidation of the diaminobenzidine tetrahydrochloride. No induction of Bax protein could be noted (data not shown). No significant induction of Bcl-2 could be evidenced when iron was added to HDMEC cultured with FCS.

To further confirm the iron-induced expression of Bcl-2 in HDMEC, immunoblot analysis was performed. As depicted in Fig. 7, the Bcl-2 Moab detected a protein of approximately 24 kDa, corresponding to the size expected for Bcl-2. Immunoblotting confirmed that iron induced the expression of Bcl-2 in HDMEC. Interestingly, the levels of Bcl-2 in iron-stimulated HDMEC remained far lower than those observed in Kaposi's sarcoma (KS)-derived cells which are thought to represent malignant or activated endothelial cells[30–32]. This clearly suggests that iron alone is not sufficient to induce the phenotypic change of endothelial cells into malignant or activated endothelial cells. In addition, the progeny of the iron-exposed HDMEC did not display increased levels of Bcl-2 under iron-free culture conditions. This suggests that the expression of Bcl-2 is rather functional than mutational and requires the presence of iron in the culture medium. Bax MoAb (G206–1276) and polyclonal antibody (I-19) revealed undetectable to low protein levels at 22 KDa, respectively. In contrast to Bcl-2, no significant change in the Bax expression levels could be observed in HDMEC in response to iron (data not shown).

As iron may induce the formation of cytotoxic reactive oxygen intermediates, we tested the possibility that these radicals were involved in the iron-induced expression of Bcl-2 observed in HDMEC. In contrast to iron, H_2O_2 induced a dose-dependent cell loss that was paralleled by a concomitant increase of both Bcl-2 and Bax protein expression (Fig. 8). Up to 5mM concentra-

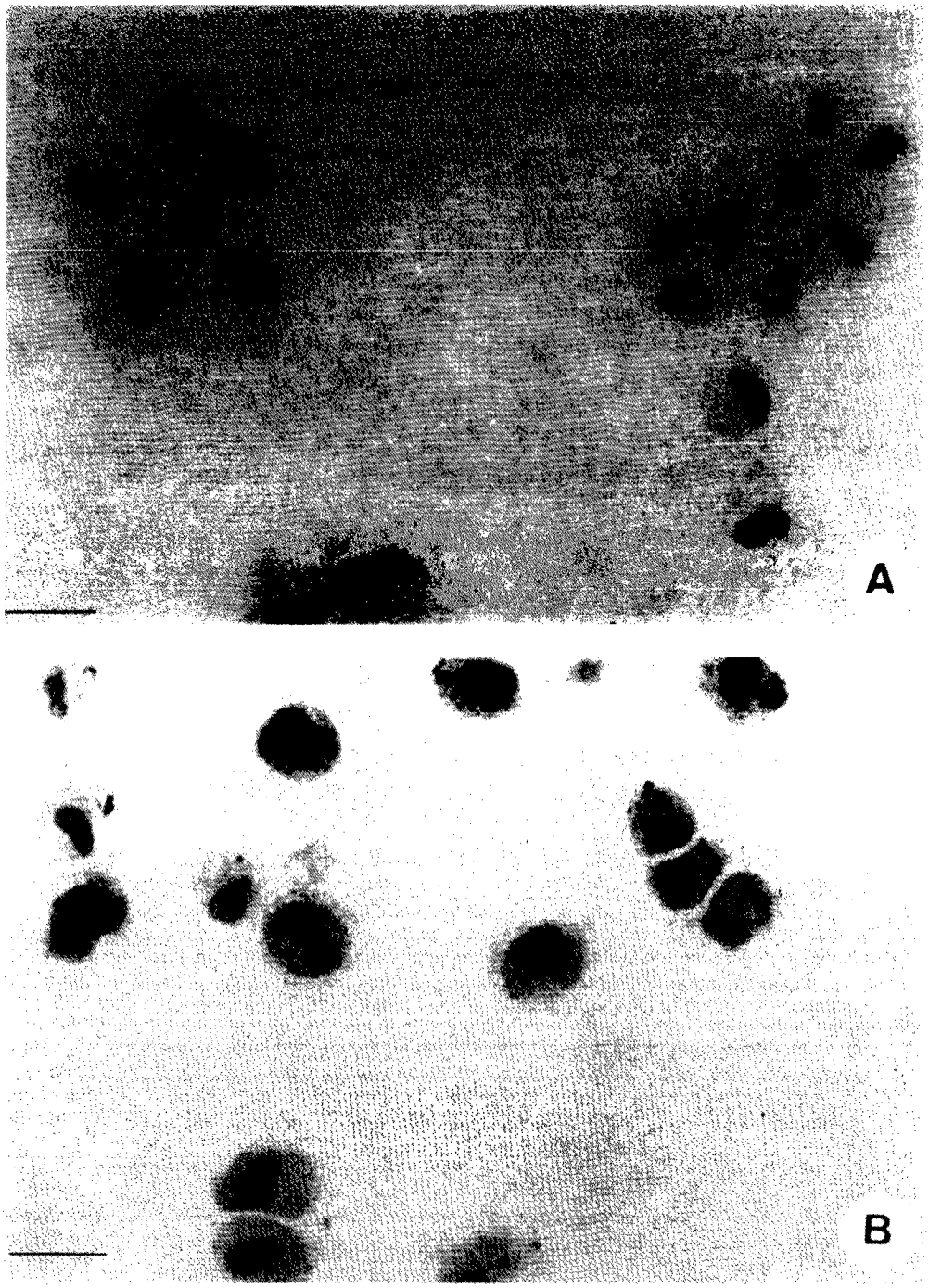
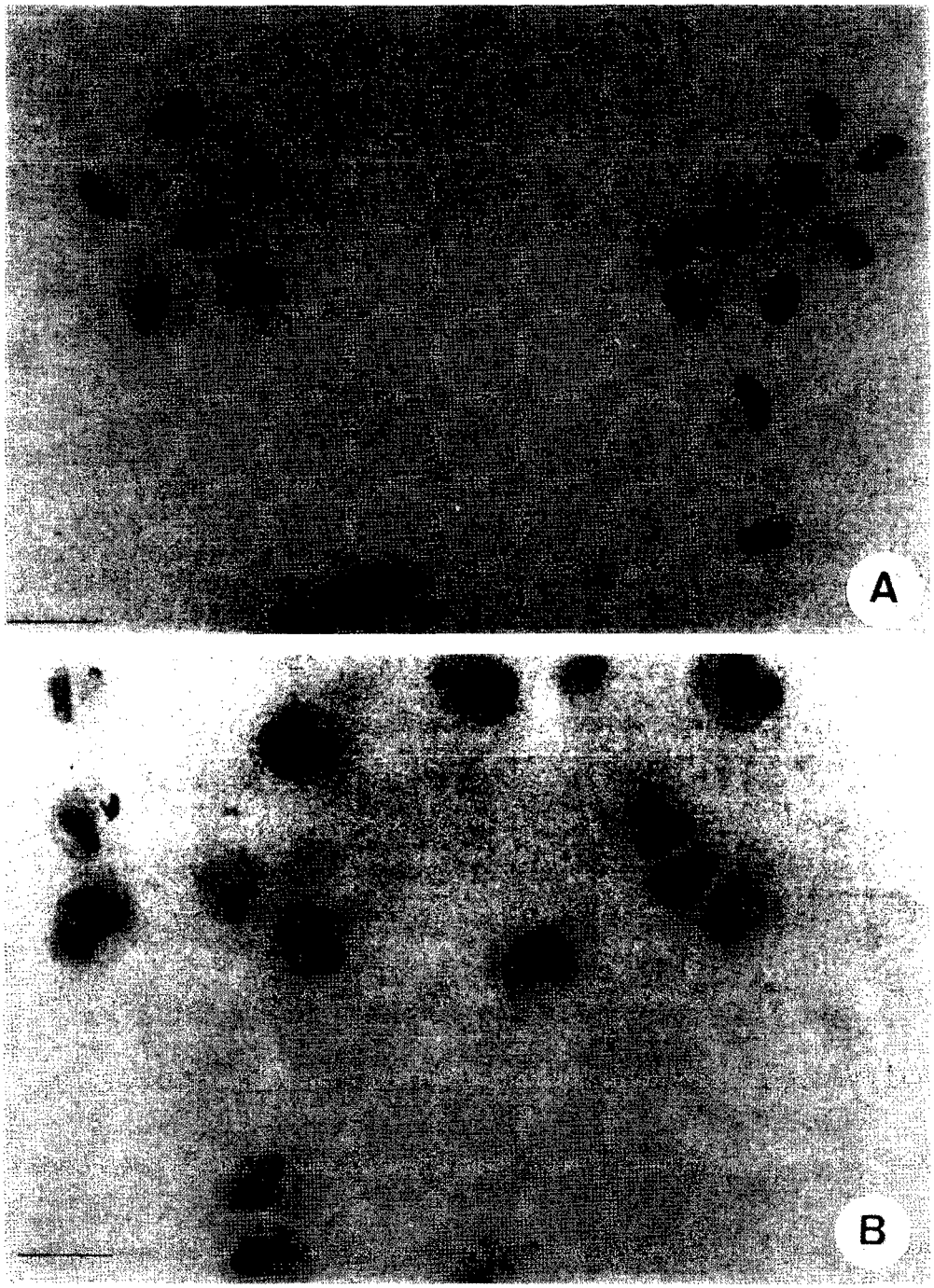


FIGURE 5 Staining of HDMEC cytospin preparations of HDMEC by the method of Lillie. A, no cytoplasmic granules can be demonstrated by this method in not iron-treated cells. B, after a 24 hour period treatment with 20 μM $FeCl_3$, the cellular uptake of iron was determined by the method of Lillie revealing the presence of dark prussian blue cytoplasmic granules. Bars: 40 μM (See Color Plate I at the back of this issue)



Color Plate I (See page 230, Figure 5) Staining of HDMEC cytospin preparations of HDMEC by the method of Lillie. A, no cytoplasmic granules can be demonstrated by this method in not iron-treated cells. B, after a 24 hour period treatment with 20 μM FeCl3, the cellular uptake of iron was determined by the method of Lillie revealing the presence of dark prussian blue cytoplasmic granules. Bars: 40 μM

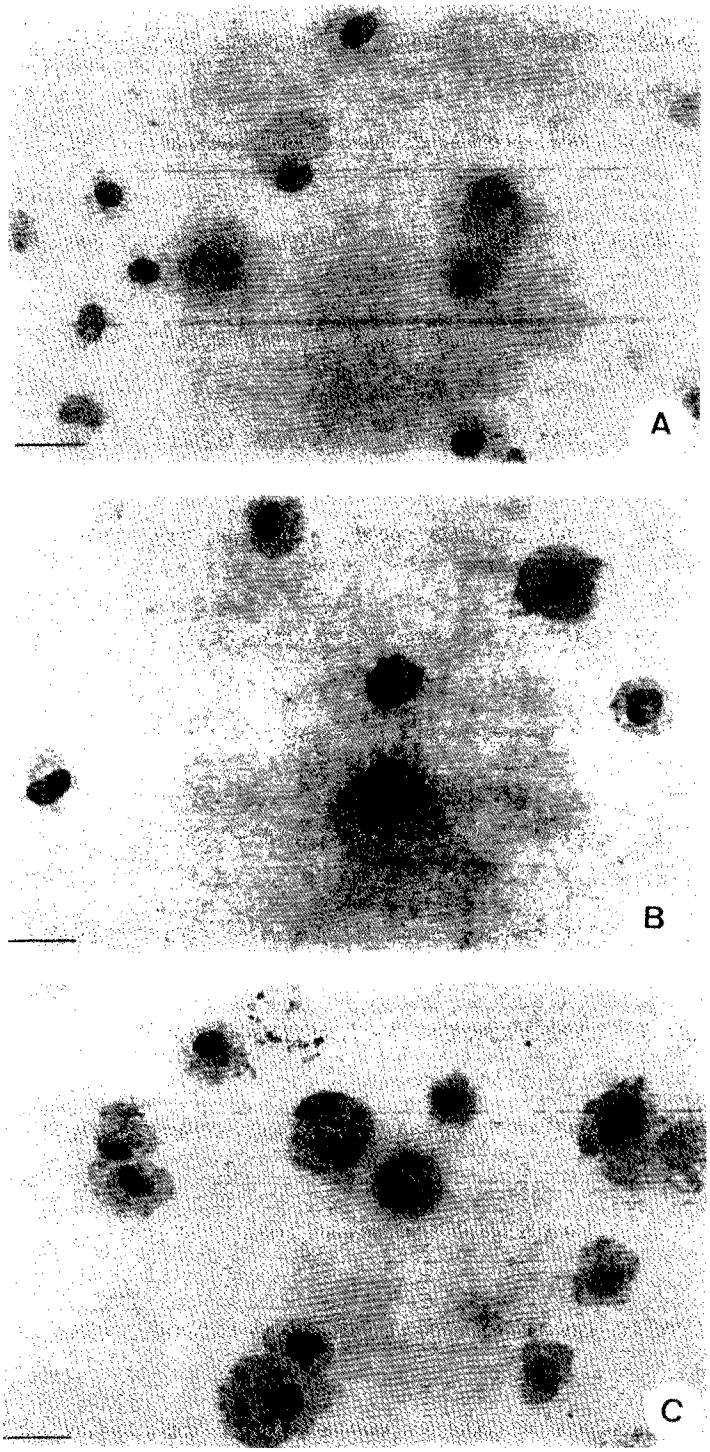
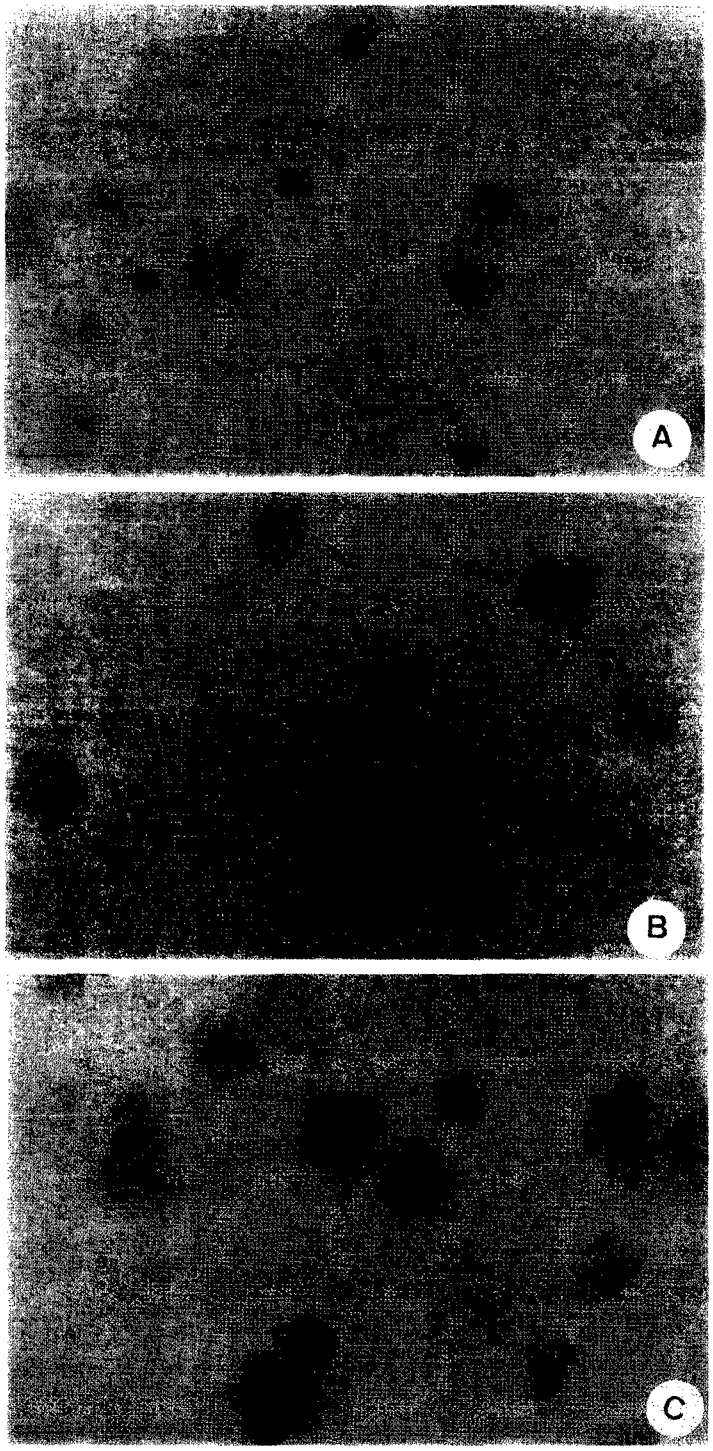


FIGURE 6 Immunostaining of HDMEC cytospin preparations with Bcl-2 (clone 100) MoAb. A, negative control; B, not iron-treated cells; C, 20 μM iron-treated cells. Bars: 40 μM (See Color Plate II at the back of this issue)



Color Plate II (See page 231, Figure 6) Immunostaining of HDMEC cytospin preparations with Bcl-2 (clone 100) MoAb. A, negative control; B, not iron-treated cells; C, 20 μM iron-treated cells. Bars: 40 μM

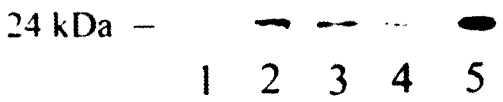


FIGURE 7 Immunoblot analysis of Bcl-2 (clone 100) MoAb. Cell lysates (40 μg of protein for each sample) were separated on a 10 to 17% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets. The proteins were immunoblotted with anti-Bcl-2 Moab. The immunoblots were scanned with a Microtek Phantom 4800 apparatus. (1), not iron-treated HDMEC; (2), iron 20 μM; (3), iron 100 μM; (4), iron 20 μM + DFO 20 μM; (5), KS-derived cells

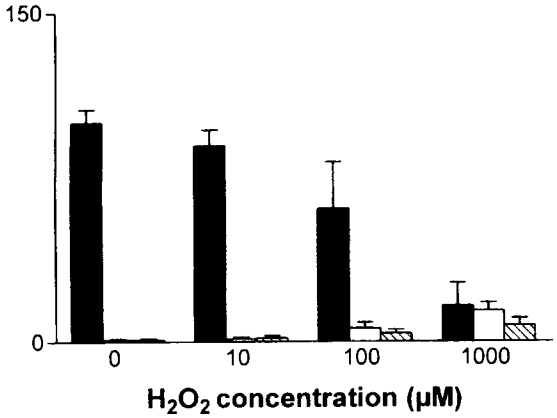


FIGURE 8 Effect of H_2O_2 on HDMEC cell viability (black columns), Bcl-2 expression (white columns) and Bax expression (hatched columns). Serum-deprived HDMEC were treated with various concentrations of H_2O_2. After 24 h, the cells were harvested by trypsinization. Number of viable cells was determined by trypan blue exclusion test and was expressed as a percentage of untreated cells. Bcl-2 and Bax indices were determined by immunohistochemistric analysis of cytospin preparations and were determined as the number of positive cells per 100 (Bcl-2) or 1000 (Bax) counted cells. Results are expressed as means ± SE of 3 independent experiments.

tions of NAC, a potent antioxidant, failed to suppress the iron-induced Bcl-2 expression. By contrast, significant inhibition of the iron-induced Bcl-2 expression was observed when $FeCl_3$ was pre-incubated with DFO.

Effect of Iron on Cell Morphology and Activation

Since inflammatory cytokines may alter cell kinetics and may induce the expression of Bcl-2 family members on endothelial cells[15], we investigated whether the effects of iron were accompanied by endothelial cell activation. $FeCl_3$ at concentrations below 100 μM induced no significant morphological change. FACS analysis revealed that iron did not induce (or increase) the expression of CD40, VCAM-1 and ICAM-1 on the surface of both serum-deprived and -replete HDMEC. Iron exposure did not restore the downregulation of CD71 expression induced by serum starvation. In view of the linkage between the density of transferrin receptors and the initiation of DNA synthesis[10,33], this further suggests that iron had no effect on the proliferation of the cells. To further investigate whether iron could lead to the activation of HDMEC, we compared the profile of cytokine secretion in IL-1β- versus iron-stimulated cells under conditions of serum starvation. Using commercially available ELISA kits (BioSource, Fleurus, Belgium), we found, in accordance with other studies[34], that the treatment of HDMEC with IL-1β induced the production of IL-6 and increased the release of IL-8. By contrast, no induction of IL-6 or IL-8 production could be evidenced in the iron-treated cultures (Table I).

TABLE I Cytokine production by HDMEC treated with IL-1β(150 pg/ml) or with iron (20 μM)

	Control	*IL-1β*	*Iron*
TNF-α[a]	< 1	< 1	< 1
IL-6[a]	25 ± 20	1140 ± 450	40 ± 35
IL-8[a]	5,200 ± 2900	17,800 ± 4900[b]	4,400 ± 2650
IL-12[a]	< 1	< 1	< 1
IFN-γ[c]	< 1	< 1	< 1

a. Values expressed in pg/ml.
b. $p < 0.02$ compared to control (mean of 3 experiments).
c. Values expressed in IU/ml.

DISCUSSION

Iron participates in several biological reactions and is essential to virtually all forms of life. Iron-containing proteins of the respiratory chain are involved in electron transport to provide the energy for cellular activities. Iron is also required for cell multiplication in view of its role in the activity of ribonucleotide reductase, a key-enzyme in DNA synthesis, responsible for the reduction of ribonucleotides to deoxyribonucleotides[10,35]. Iron is also suspected to be involved in the pathogenesis of various human tumors. The present study was designed to investigate the effects of iron on endothelial cells, keeping in mind that the homeostasis of microvessels plays a critical role in tumour development and progression[11].

Confirming previous studies, we found that serum withdrawal, and hence growth factors and iron deprivation causes growth arrest in the G_0/G_1 state and induces endothelial cell death[21,27]. In addition, serum deprivation gradually increases the proportion of cells unable to re-enter the cell cycle and to divide upon return to serum-supplemented conditions. The proportion of dead cells gradually increased, which may point to similarities in the molecular pathways of terminal differentiation and apoptosis[36]. Applying this model of endothelial cell terminal differentiation and death, we show here that exposure to iron confers a survival advantage to HDMEC. The survival advantage elicited by iron might be linked with previous studies showing that, under conditions of serum starvation, iron acts as a growth factor for various cell types. DNA replication indeed depends on iron-containing ribonucleotide reductase which catalyses an obligatory step in DNA synthesis[35]. However, analysis of BrdUrd incorporation and of PCNA and CD71 expression indicated that, in conditions of serum deprivation, the survival advantage of endothelial cells elicited by iron is clearly independent of any mitogenic effect. An alternative explanation is that iron, which is known to be involved in key enzyme systems catalyzing the electron-transfer and respiration reactions, inhibits the terminal differentiation induced by serum deprivation. This survival advantage was paralleled by a significant increase of the expression of Bcl-2 protein, which is known to interfere with apoptosis and differentiation[37,38]. There are few data on the induction of Bcl-2 on endothelial cells by exogenous agents. It has been shown that inflammatory cytokines and VEGF[15,39] increased the Bcl-2 expression in endothelial cells in a dose-dependent manner. However, the herein observed absence of effect of iron on inflammatory cytokine release and on cell activation surface markers suggests that iron did not exert its survival promoting effect through the release of inflammatory cytokines. Neither significant survival advantage nor significant Bcl-2 induction was evidenced when iron was added to HDMEC cultured with FCS, which might be explained by the presence of free or protein-bound iron in the culture medium.

Although Bcl-2 seems involved in cell protection during growth factors deprivation[29,39], the mechanism of this resistance remains unclear. More particularly, our results do not allow to answer whether Bcl-2 is directly involved in the survival advantage elicited by iron or whether Bcl-2 is simply induced to overcome the oxidative stress triggered by iron. One of the earliest mechanisms proposed for Bcl-2 was that it acts as an antioxidant that suppresses the formation or action of reactive oxygen species[40]. However, the persistence of the iron-induced Bcl-2 expression after co-incubation with NAC, a potent anti-oxidant, does not support the hypothesis that Bcl-2 acts as a protective counteracting force. Some studies have demonstrated that the cell death-protective activity of Bcl-2 was associated with diminished cell proliferation and prolongation of the G_1phase of the cell cycle, with subsequent facilitation of a prolonged metabolically dormant state from which cells recover with high efficiency[41]. These find-

ings do not seem relevant in the case of endothelial cell death induced by serum deprivation; the low levels of any form of PCNA as well as the lack of BrdUrd incorporation suggest that, upon serum starvation, the majority of the cells are dying while being already growth-arrested. Direct molecular experiments, such as microinjection of antisense cDNA might give more information regarding the relationship between iron and Bcl-2 expression.

In summary, we have found that iron can promote the growth potential of endothelial cells by a mechanism independent of any mitogenic effect. The extended cellular life span induced by iron was paralleled by an increase of Bcl-2 protein expression. Taken together, these observations suggest that iron may give a survival advantage to endothelial cells and hence, may alter the homeostasis of microvessels. This may highlight the tumorigenic effect of iron.

Acknowledgements

The authors thank Dr. S. Horman for helpful suggestions on the Western Blot analyses. This work was supported by the Erasme Foundation, Brussels, Belgium

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