Decreased Activity of Inducible Nitric Oxide Synthase Type 2 and Modulation of the Expression of Glutathione S-Transferase α, bcl-2, and Metallothioneins during the Differentiation of CaCo-2 Cells[1]

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Abstract

Reactive oxygen species modulate the cell growth of a wide variety of mammalian cells. To determine whether oxidative metabolism is altered during the differentiation process, we studied the expression of pro- and antioxidant proteins in proliferating and differentiated CaCo-2 cells, a human colon adenocarcinoma cell line. Nitric oxide synthase type 2 (iNOS) produces nitric oxide (NO). Depending on its rate of synthesis, NO may either promote cellular and DNA damage or reduce the ability of other free radicals to induce cell injury. Using Western and Northern blot analysis and arginine conversion assay, we demonstrate that the expression of iNOS decreases when cells undergo differentiation. This biological event entails a diminished production of NO metabolites and correlates with the loss of activation of soluble guanylate cyclase activity. In differentiated cells, a 2-fold down-regulation of the nuclear factor κB activity was observed, suggesting that nuclear factor κB could be one of the *iNOS* gene regulatory factors in the CaCo-2 model. In parallel, we studied the expression of other antioxidant proteins including glutathione S-transferase α (GSTα), bcl-2, and the metallothioneins (MTs). We show that the protein levels of GSTα and MT increase during the differentiation of CaCo-2 cells, whereas bcl-2 levels decrease. Our investigation indicates that the expression of iNOS, GSTα, bcl-2, and MT is associated with the enterocytic differentiation. The shift in the expression of specific antioxidant genes during CaCo-2 cell differentiation may occur to avoid alterations in the cell redox potential.

Introduction

Free radicals are involved in the control of cell growth and differentiation. At physiological levels, they promote mitogenic responses in several mammalian cell types (1), whereas at higher concentrations, they induce cell damage and apoptosis (2). Furthermore, the constitutive production of H_2O_2 (3), superoxide (O_2^-), and hydroxyl radical (·OH; Ref. 4) in several human tumor cell lines suggests that oxidative stress may play a role in cell dedifferentiation. Altogether, these observations support the notion that the balance between radical production and antioxidant defenses may be a determinant in cell growth, apoptosis, and differentiation.

NO[3] is a pleiotropic messenger (5) involved in endothelium-dependent vascular relaxation, neuromodulation, inhibition of platelet aggregation, immunological reactions (6), and iron homeostasis (7). It is produced by the metabolism of L-arginine, which is catalyzed by a family of enzymes, the nitric oxide synthases (8). iNOS, the inducible isoform, the expression of which is cytokine dependent, has been detected in normal enterocytes (9), but its function in this epithelium is not known. A higher level of iNOS activity has been found in human colon cancers as compared to peritumoral tissues,[4] and the fact that this enzyme is constitutively expressed in several human colon adenocarcinoma cell lines (10, 11) suggests that it has a role in the dedifferentiation of colon cells. NO may disturb cell growth by regulating the activity of cellular targets involved in signal transduction, ATP and oxygen production, and iron sequestration. Indeed, NO activates the sGC (5), inhibits (iron-cluster) enzymes including aconitase and mitochondrial complex I and II enzymes (6), and enhances the DNA binding of transcription factors connected to the iron homeostasis (7). Its dual chemical properties as a pro- or antioxidant compound (12–16) may also account for a role of NO in the modulation of cell proliferation via its effects on cell redox potential. NO has the potential to reduce the intracellular level of H_2O_2, because it inhibits the NADPH oxidase (14) and interacts with the su-

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[3] The abbreviations used are: NO, nitric oxide; BH4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; cGMP, cyclic GMP; DEA/NO, diethylamine/NO; DDPIV, dipeptidyl peptidase IV; sGC, soluble guanylate cyclase; GSTα, glutathione S-transferase α; iNOS, inducible nitric oxide synthase; MT, metallothionein; mU, milliunit(s); NFκB, nuclear factor kappa B; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene difluoride.
[4] S. Ambs, E. Felley-Bosco, W. P. Bennett, P. G. Shields, and C. C. Harris, Increased nitric oxide synthase activity in human tissues, manuscript in preparation.

peroxide anion yielding peroxynitrite ($ONOO^-$; Ref. 15), which is transformed rapidly into nitrate at a physiological pH. It can also control the synthesis of the highly reactive ·OH radical, arising from the interaction between H_2O_2 and ferrous complexes (Fenton reaction), by either chelating ferrous ions (16) or increasing iron release (7).

bcl-2 may also regulate cell differentiation by modulating the redox state of the cell. bcl-2 has been most studied as an antiapoptotic protein (17, 18), but it is also a key modulator of intracellular levels of oxygen species and has been shown to decrease lipid peroxidation (19). The biochemical mechanism of the antioxidant activity of bcl-2 remains to be determined, although the hypothesis has emerged that the localization of bcl-2 to the membranes of mitochondria, endoplasmic reticulum, and nucleus near the sites of superoxide generation allows a direct interaction between bcl-2 and O_2^-, leading to the removal of oxygen radicals.

GSTα is also an important component of cellular oxidant defenses (20). Its expression is redox regulated and seems to be correlated with the production of oxygen radicals (21, 22). GSTα reduces the concentration of fatty acid hydroperoxides (20), but in contrast to glutathione peroxidase, it cannot metabolize H_2O_2. Therefore, it may intervene at a second step to rescue the cell from lipid peroxidation-induced damages.

MTs are cysteine-rich, metal-binding proteins that are involved in cell protection against toxic metals (23). In addition, MTs inhibit the degradation of DNA by hydroxyl radicals (24), and an inhibition of MT expression has been shown to increase the cell sensitivity to oxidative injury (25). Indeed, MT expression is associated with oxidative stress (26, 27) and increases in the presence of an elevated level of free radicals produced during hypoxia (26), or in response to H_2O_2 (27).

Using Western and Northern blot experiments and enzymatic assays, we studied the variation of the expression of "sensor" oxidant proteins during CaCo-2 cell differentiation. In this report, we demonstrated that the genes encoding iNOS and bcl-2 were specifically switched off during the enterocytic differentiation, whereas the expression of *GST*α and *MT* genes were increased. This indicated that different redox-sensitive proteins were requisitioned depending on the proliferation or differentiation status of the cell, and that they might be linked to the oxygen radical production.

Results

CaCo-2: A Model of Intestinal Enterocytic Differentiation. CaCo-2 cells differentiate spontaneously to enterocyte-like cells when they are cultured for 20 days after confluency. This means that they form polarized monolayers sealed by tight junctions and display a well-developed apical brush border membrane expressing specific hydrolases (28). Also, CaCo-2 cells exhibit enzymatic and structural features that have been detected in intestinal epithelial cells that migrate from crypts to villi. We have studied two differentiation markers, the expression of which is associated with the brush border. The serine protease DDPIV is involved in the hydrolysis of X-pro-containing peptides and participates in the final step of the intestinal peptide reabsorption (29). Villin, an actin-binding protein, plays an important role in the maintenance of the brush-border cytoskeleton integrity (30). Using enzymatic assay and immunoblotting experiments, we observed that the expression of villin and DDPIV was 4.5- and 10-fold higher, respectively, in differentiated cells as compared to growing cells (Fig. 1, *A* and *B*, respectively), and these increases were similar to previously published data (28, 29).

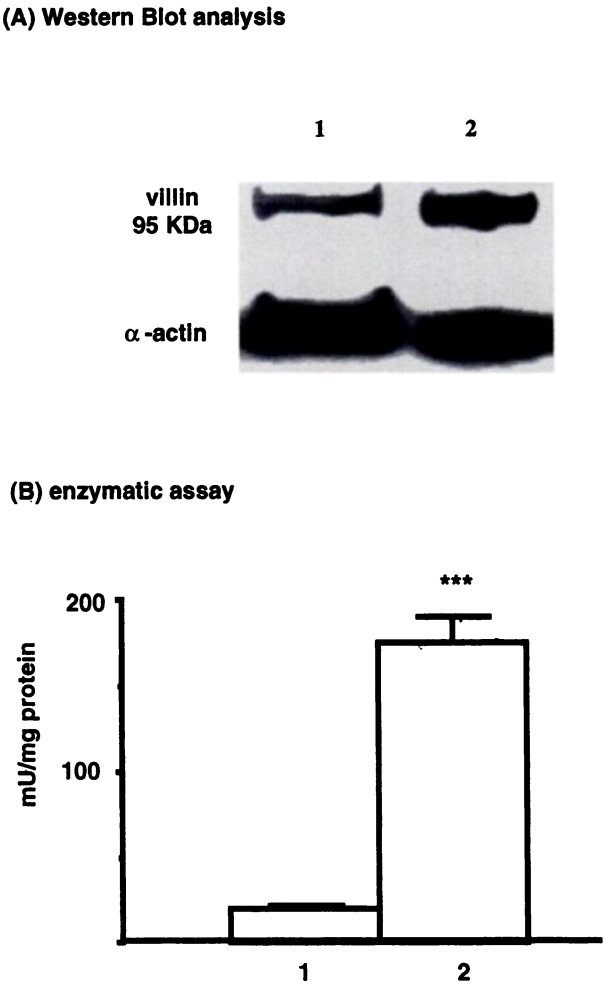

Fig. 1. Enzymatic and structural features of CaCo-2 cell differentiation. *A*, Western blot analysis of villin expression. Fifty-μg cell extracts of proliferating (*1*) or differentiated (*2*) CaCo-2 cells were subjected to electrophoresis on 10% SDS-PAGE polyacrylamide gel and transferred onto PVDF blotting filters. PVDF membranes were incubated with villin or actin antisera diluted 1:500 and 1:5,000, respectively, and with antimouse and antirabbit horseradish peroxidase conjugates, respectively (both diluted 1:10,000). Detection of the immune complex was performed by autoradiography. This is a representative experiment of three independent experiments. (*B*) DDPIV activity in either proliferating (17.4 ± 3.5 mU/mg protein) or differentiated (172.2 ± 20.7 mU/mg protein) CaCo-2 cells. Specific enzyme activities are expressed as mU/mg of protein. One unit is defined as the activity that hydrolyzes 1 μmol of substrate/min at 37°C. Data are means (*bars*, SD) representing duplicate assays of three independent experiments.

Modulation of *iNOS* Gene Expression during CaCo-2 Cell Differentiation. We have quantified the iNOS activity in CaCo-2 cells cultured 7 (growing cells) or 20 days (differentiated monolayer) by carrying out Western and Northern blot

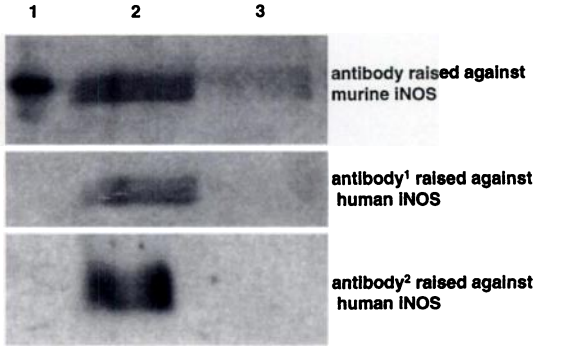

Fig. 2. Modulation of iNOS protein expression depending on CaCo-2 cell differentiation. Cell extracts were homogenized in a 50 mM HEPES buffer and centrifuged as described in "Materials and Methods." The S100,000 × *g* cytosolic fraction (2.5-mg cell extracts) from differently aged CaCo-2 cells were purified on a 2′-5′-ADP Sepharose column, precipitated with acetone, and mixed with Laemmli buffer. The samples were subjected to SDS-PAGE (7.5%), and proteins were transferred on PVDF membranes. Membranes were incubated with monoclonal antibodies directed against murine iNOS (dilution, 1:500) or with polyclonal antisera raised toward human iNOS (dilution, 1:100 for antibody[4] (Santa Cruz Biotechnology), and dilution 1:40,000 for antibody[5] (gift of Dr. Mumford, Merck Research Laboratories), and then with antimouse or antirabbit horseradish peroxidase conjugates (diluted 1:10,000). The immune complexes were revealed by autoradiography. Cell extracts from cytokine-induced mouse macrophages were used as a positive control (*1*); purified cytosolic extracts from CaCo-2 cells aged 7 days (*2*) or 21 days (*3*). These are representative experiments of three performed independently.

analysis, radioenzymatic assays, and determination of NO metabolites in the conditioned medium. To assess the specificity of the signal obtained in the Western blot analysis, we used three antibodies, one raised toward rodent iNOS but recognizing also the corresponding human protein, and two directed against the human iNOS cells, which do not cross-react with the murine iNOS isoform. The iNOS protein is detected at M_r 130,000, indicating that this enzyme is constitutively expressed by proliferating cells (Fig. 2). However, the signal disappears in differentiated cells, showing that a reduction in iNOS expression is correlated with the differentiation status of the CaCo-2 cell line. This observation was confirmed by radioenzymatic assays, which measured the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline. Lysates from proliferating cells were found to metabolize L-arginine at the rate of 8.7 ± 6.0 pmol citrulline formed/min · mg protein, whereas in cells cultured 20 days, this metabolic pathway was undetectable (Table 1). The activity found in growing cells corresponds to that described for other human colon cell lines (10, 11). In parallel, the amount of NO was quantified as the accumulation of NO_2^- and NO_3^- in the medium. Proliferating CaCo-2 cells had levels 2-fold higher ($P < 0.001$) as compared to that measured for the differentiated cells (Table 1). In addition, the iNOS mRNA detected as a 4.4-kb transcript was 3-fold down-expressed in differentiated cells *versus* proliferating cells (Fig. 3), supporting the hypothesis that the *iNOS* gene may be regulated at the transcriptional level in CaCo-2 cells. To investigate whether the NO decrease corresponded to a similar variation in the NO-dependent sGC activity, we performed measures of cGMP in differently aged CaCo-2 cells in the presence or absence of 5 μM DEA/NO (31), a well-described NO donor.

Table 1 Specific iNOS activity and measures of NO metabolite levels during CaCo-2 cell differentiation

The conversion of L-arginine into L-citrulline and the determination of nitrites and nitrates were performed as described in methods. Results are expressed as: pmoles of citrulline formed/min · mg protein, mean ± SD (n = 3). nanomoles NO_2^-/48 h/mg protein, mean ± SD (n = 3).

	Proliferating cells	Differentiated cells
Specific activity (pmol citrulline formed/ min · mg protein)	8.7 ± 6.0	ND[a]
NO_2^- and NO_3^- levels (nmol NO_2^-/mg protein)	8.7 ± 2.0	3.6 ± 0.3[b]

[a] ND, not detectable.
[b] $P < 0.001$ (differentiated *versus* proliferating cells).

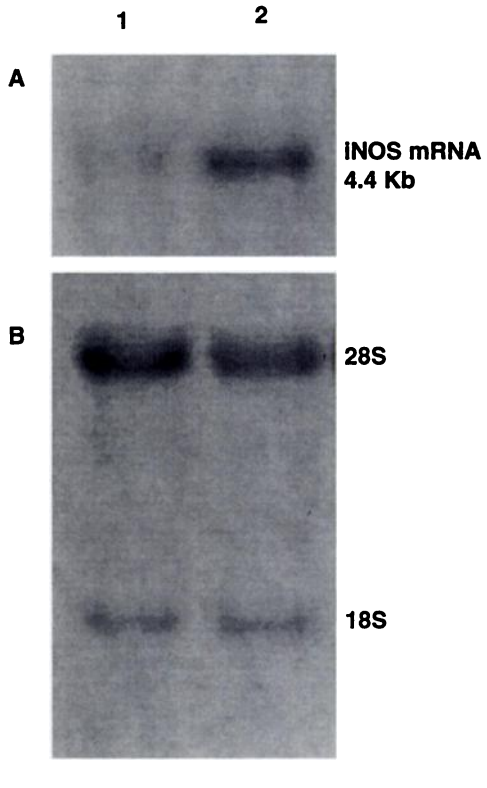

Fig. 3. iNOS mRNA expression in differentiated (1) and proliferating (2) CaCo-2 cells. RNA was extracted from differentiated or proliferating cell cultures, and 10 μg were loaded onto agarose gel and transferred. The membrane was then probed with the 4.1-kb human iNOS cDNA as described in "Materials and Methods." *A*, Northern blot analysis of iNOS mRNA. RNA was extracted from differentiated and proliferating CaCo-2 cells. *B*, ethidium bromide staining agarose gel. The 28S and 18S RNA are shown to account for loading.

Table 2 shows that the basal cGMP level in growing cells was very low but increased significantly ($P < 0.05$) after DEA/NO induced stimulation of sGC. In contrast, exposure of differentiated cells to DEA/NO did not increase cGMP levels, consistent with a loss of NO-dependent sGC activity. The higher amount of cGMP produced in differentiated cells in the absence of DEA/NO and detectable iNOS activity might be due to an increase in the particulate guanylate cyclase

Table 2 Variation in the cGMP levels during CaCo-2 cell differentiation: NO-dependent activation of the sGC in differently aged cells. CaCo-2 cells aged 5 or 21 days were incubated with 5 μM DEA/NO and 0.5 mM isobutylmethylxanthine. cGMP level was determined by RIA. Values (fmol cGMP/mg protein) are expressed as means ± SD (n = 4).

	Proliferating cells	Differentiated cells
Unstimulated	3.89 ± 6.3	31.1 ± 4.2
DEA/NO	17.53 ± 12.3[a]	32.2 ± 5.1

[a] $P < 0.05$ DEA/NO (treated versus control proliferating cells).

activity, which is known to be expressed in CaCo-2 monolayers (32). To identify the component(s) implicated in the decline of iNOS expression, we analyzed the expression of the suppressor gene *p53*, which down-regulates iNOS (33), and that of NFκB, which induces iNOS in rodent and human cells (34, 35). Western blot analysis indicated that the p53 level is identical in proliferating and differentiated cells (Fig. 4*A*). To determine the level of NFκB activation, CaCo-2 cells were stably transfected with NFκB-binding sites linked to the *luciferase* reporter gene and were cultured for either 7 or 21 days. As presented in Fig. 4*B*, a 2-fold diminution ($P < 0.01$) in the basal luciferase activity was observed in the differentiated cells, corroborating the fact that a decrease of NFκB basal activity is associated with the decline of iNOS expression. As a control, the transfected cells were incubated in the presence of PMA, an inducer of protein kinase C well known to activate NFκB. Total luciferase activity was similar when proliferating and differentiated cells were treated by PMA, showing that the basal NFκB activity, but not the NFκB protein level, decreases during differentiation.

Enhanced Expression of GSTα and MT and Decreased Level of bcl-2 during the CaCo-2 Cell Differentiation. The presence of GSTα, MT, and bcl-2 was characterized in crude extracts of proliferating and differentiated CaCo-2 cells using Western blot analysis. As shown in Fig. 5, the proteins GSTα, MT, and bcl-2 were detected as single bands at M_r 27,000, 8,000, and 25,000, respectively. The expression of GSTα and MT was higher in the older cells (a 4-fold increase was measured for the GSTα protein), whereas that of bcl-2 was 3-fold lower. This indicates that the regulation of antioxidant proteins depends on the status of enterocytic cell differentiation.

Discussion

The sustained level of radical production detected in human colon tumor cells (3, 4) suggests that oxidant stress promotes tumor cell proliferation. However, this implies the existence of a critical balance between the production of H_2O_2 and the level of specific antioxidant defenses to prevent DNA damage and lipid peroxidation induced by free radicals. We were interested to know whether a modification in the growth of cancer cells would be associated with altered expression of redox-sensitive proteins. To this end, we analyzed the expression of pro- and antioxidant capacities of intestinal CaCo-2 cells during their enterocyte-like differentiation.

This report shows for the first time that the expression of iNOS changes during the intestinal cell differentiation, being

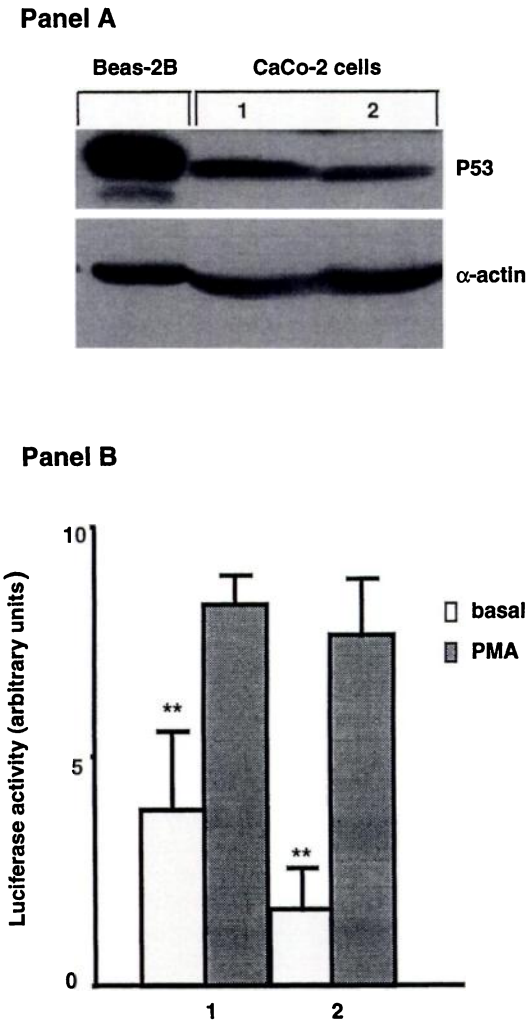

Fig. 4. p53 expression and NFκB activity in proliferating and differentiated CaCo-2 cells. *A*, Western blot analysis of p53 during CaCo-2 cell differentiation. Five hundred μg of CaCo-2 cell extracts from either proliferating (*1*) or differentiated (*2*) cells were homogenized in HEPES buffer as described in "Materials and Methods," loaded onto a 10% polyacrylamide gel, and transferred. Blotted membranes were incubated with p53 or actin antisera diluted 1:10,000 and 1:5,000, respectively, and then with antirabbit horseradish peroxidase conjugate diluted 1:10,000. Eighty μg of Beas-2B cell extracts were loaded in parallel as a positive control. *B*, NFκB-dependent luciferase activity during CaCo-2 cell differentiation. NFκB3-luc transfected cells from day 7 (*1*) or day 21 (*2*) were exposed to 200 nM PMA for 8 h, and cell extracts were collected in lysis buffer. Luciferase activity was determined, normalized to protein content, and expressed as an arbitrary unit. Values for proliferating cells cultured in the absence or in the presence of PMA correspond to 3.84 ± 1.66 and 8.27 ± 0.65, respectively. Values for differentiated cells cultured in the absence or in the presence of PMA correspond to 1.67 ± 0.90 and 7.61 ± 1.24, respectively. Data are expressed as means ± SD. $^{**}P < 0.01$ unstimulated differentiated *versus* unstimulated proliferating cells. Statistical comparisons were performed using the Student's paired t test.

higher in proliferating cells and lower in postconfluent cells, which have stopped growing. The parallel diminution of the iNOS mRNA raises the possibility that this biological process may include a decrease in the iNOS transcriptional level. The p53 protein, which can down-regulate iNOS transcription

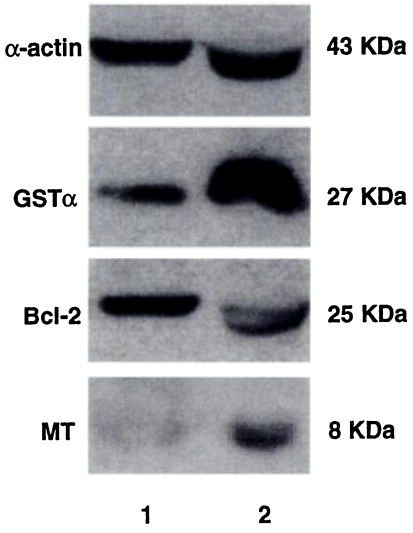

Fig. 5. Western blot analysis of bcl-2, GSTα, and MT in differently aged CaCo-2 cells. Fifty μg of proliferating (*1*) or differentiated (*2*) cell extracts were loaded onto 10% SDS-PAGE polyacrylamide gel (for the detection of bcl-2 and GSTα) or onto 15% SDS-PAGE polyacrylamide gel (for the detection of MT) and transferred onto PVDF membranes. Blotted membranes were incubated with antisera directed against human bcl-2 and GSTα diluted 1:40 and 1:2,000, respectively, and MT antibody was diluted 1:100. Antimouse horseradish peroxidase IgG was used for the detection of bcl-2 and antirabbit horseradish peroxidase conjugates for the revelation of GSTα, MT and actin. Both conjugates were diluted 1:10,000. This is a representative experiment of two performed independently.

(33), seems not to be involved in this event, given that its expression level did not change during differentiation. But we cannot totally exclude a regulatory effect of p53, because this protein is mutated in CaCo-2 cells[2], and it is not known whether this mutation changes the DNA-binding ability of p53. On the other side, the concomitant decrease of the basal NFκB activation argues for a role of NFκB in the regulation of iNOS activity in the CaCo-2 cells, as has been found in other human cells (11, 35). One might also speculate that the substrate itself, L-arginine, controls iNOS transcription, because the L-arginine y^+ transport is also lower in differentiated CaCo-2 cells (36). Supporting this idea is the observation that the other L-arginine metabolic pathway constituted by the ornithine decarboxylase enzyme decreases in older CaCo-2 cells (37).

What is the biological significance of the progressive decline in NO production during the cell differentiation? Several reports provide evidence that low but persistent levels of NO are associated with tumor progression. Jenkins *et al.* (38) observed that the injection of nude mice with the iNOS-transfected DLD-1 human colon cell line resulted in tumors with a higher growth rate and vascularization, compared to tumors formed by the parental cell line. iNOS activity in human breast cancer was found to be directly correlated with the state of the malignancy (39). Taken together, these observations emphasize the potential roles of NO in tumor growth. One of the mechanisms by which low-level NO synthesis elicits biological effects is through the activation of sGC (5), for the production of cGMP. Our data demonstrate the presence of NO-activated sGC in intestinal cells in response to exogenous NO and show that the diminution of iNOS expression is associated with a reduced activity of sGC in differentiated CaCo-2 cells. cGMP has been associated with growth-inhibitory effects on vascular smooth muscle cells (40), but whether cGMP has similar function in the epithelia is not known. Although NO-induced sGC stimulation has already been observed in bronchial epithelial cells (31), no correlation was found between cGMP synthesis and cell proliferation. NO controls iron availability (7), and this regulatory effect may constitute a physiological mechanism in the modulation of CaCo-2 cell proliferation, because iron starvation has been shown to limit cell replication (6). Iron release also involves targets sensitive to low NO concentrations (7, 41) corresponding to the level of iNOS-specific activity detected in growing CaCo-2 cells. Although we did not measure the intracellular iron concentration in differently aged CaCo-2 cells, this NO-regulated pathway is functional in CaCo-2 cells as demonstrated by Sanchez *et al.* (41), who observed an increase in iron absorption in CaCo-2 cells treated with 10 μM of the NO donor *S*-nitroso-*N*-acetylpenicillamine. Finally, NO might promote the tumor cell growth by preventing the cell death due to the deleterious effects of the other oxidants present in significant levels in human tumor cell lines (3, 4). Indeed, NO counteracts the toxicity mediated by the superoxide anion, hydrogen peroxide, and alkyl hydroperoxides (15, 16), and the mechanism of this effect has been proposed to be the scavenging of either O_2^- (15) or ferrous complexes (16) inhibiting the formation of the highly toxic radicals H_2O_2 and ·OH. In the intestine, low levels of NO have been shown to decrease injury triggered by lipopolysaccharide and H_2O_2 (42). Whether NO maintains a prooxidant state thought to promote growth responses (1) and mutagenesis (2), or functions as an antioxidant that abates the radical-mediated apoptosis of cancer cells and increases their survival is unknown. Our data show a decline in iNOS expression during the CaCo-2 cell differentiation, suggesting but not proving a role for NO in cell growth responses.

Concurrent with the decline in iNOS expression, bcl-2 levels decreased in the differentiated cells. A gradient of bcl-2 expression has already been described along the crypt-villus axis of the intestinal epithelium (17). The intense rate of cell proliferation in the crypt is accompanied by the generation of oxygen compounds. Therefore, high levels of bcl-2 could play a role in the survival of intestinal stem cells and perhaps in cancer cells that constitutively produce radicals. The coexpression of *bcl-2* and *iNOS* genes in proliferating CaCo-2 cells supports the hypothesis that they both act to inhibit the damage induced by oxygen species, thus facilitating tumor growth. Furthermore, iNOS and bcl-2 expression could be related; *i.e.,* NO may control the expression of bcl-2, as already observed in lymphocytes (43).

By contrast, the expression of GSTα was induced 4-fold during CaCo-2 cell differentiation. Similar results were reported by Zucco *et al.* (44), who demonstrated that glutathione peroxidase selenium-independent activity increases in CaCo-2 cells between 7 and 21 days of culture. The GSTα expression is controlled by antioxidant response elements consisting of activator protein 1-like binding sites in the 5′-flanking region of the *GST Ya* gene (21, 22). In the differ-

entiated human HepG2 cell line, the activator protein 1-mediated transcription of GSTα is induced by the production of ·OH radicals (22). Therefore, it is likely that the GSTα expression is also redox-regulated during CaCo-2 cell differentiation. The redox induction of GSTα constitutes a part of the cell responses to oxidative stress and may be triggered by the emergence of novel radical oxygen species.

Similar to the GSTα, the MT expression, although undetectable in proliferating cells, was largely induced in postconfluent CaCo-2 cells. The presence of a hypoxia-responsive element in the human *MT-II* gene (26) and that of H_2O_2-inducible antioxidant response element in the mouse MT-1 promoter (27) have been reported. However, the regulation of human MT expression is not really understood. It appears that the function (45, 46) and the regulation (26, 27) of MT are associated closely with the cell oxidative stress. MT protein is able to prevent the hydroxyl-mediated DNA fragmentation (24) and to inhibit the cell membrane peroxidation (45), although the mechanism of these actions is not clear. The presence of thiolate clusters and Zn^{2+} elements in MT proteins, which together act against lipid peroxidation, has been thought to account for the protective function of MT during cell injury (45, 46).

In conclusion, the present data showed a shift in specific "sensor" oxidant genes during the CaCo-2 cell differentiation. The level of iNOS and bcl-2 proteins were higher in proliferating cells, whereas in differentiated cells, these proteins were replaced by an enhanced expression of GSTα enzymes and MTs. These substitutions display an adaptive response of the cell and may be linked to the redox potential, which is then a critical determinant in cell proliferation, differentiation, and apoptosis.

Materials and Methods

Antibodies. Antibodies against mouse iNOS were purchased from Transduction Laboratories (Affiniti, Nottingham, England). Human iNOS antiserum[4] was obtained from Dr. A. G. Glaser (Santa Cruz Biotechnology, Basel, Switzerland), and antibody[5] was a kind gift of Dr. R. A. Mumford (Merck Research Laboratories, Rahway, NJ). Antibodies directed against human bcl-2 and p53 were obtained from Oncogene Science (Basel, Switzerland) and Signet Laboratories (Alexis Corp., Laufelfringen, Switzerland), respectively. Rabbit polyclonal antisera against human GSTα (47, 48) and α-actin are generous gifts from Dr. H. Mukhtar (University Hospitals of Cleveland, Cleveland, OH) and Dr. Y. C. Awasthi (University of Texas Medical Branch, Galveston, TX), and from Dr. G. Gabbiani (Department of Pathology, Geneva University, Geneva, Switzerland), respectively. Polyclonal MT antibodies have been developed in our laboratory (23). Antirabbit and antimouse horseradish peroxidase conjugates were obtained from Amersham (Zurich, Switzerland) and Bio-Rad (Glattbrugg, Switzerland), respectively. Protein concentration was determined by the bicinchonic acid reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard.

Cell Culture. CaCo-2 clone 1 cells (29) were isolated from the parental human colon adenocarcinoma CaCo-2 cell line (28) and were cultured during 20 passages (passages 50–70) under 9% CO_2 atmosphere at 37°C in DMEM (Hyclone, Integra Biosciences IBI, Wallisellen, Switzerland) supplemented with 10% (v/v) heat-inactivated (55°C for 30 min) fetal bovine serum (Life Technologies, Inc., Basel, Switzerland), 2 mM L-glutamine (Integra Biosciences), 1% nonessential amino acids (Seromed, Fakola, Basel, Switzerland), and 100 μg/ml of penicillin and streptomycin (Life Technologies. Inc.). CaCo-2 cells were shown to be mycoplasma-free using a mycoplasma detection kit (Boehringer Mannheim, Rotkreuz, Switzerland). Culture medium was changed every 2 days. *In vitro*, cells differentiate after 3 weeks postconfluency, and this biological process can be quantified by an increased synthesis of villin and DDPIV (Fig. 1).

Measurement of DDPIV Activity. Cells (10^6) were seeded in a 10-cm^2 culture dish and cultured for 7 or 21 days. Cells were rinsed and collected in 500 μl of ice-cold PBS with a rubber policeman. After rapid sonication at 4°C, 40 μl of the whole-cell homogenate (diluted 1:10) was incubated in a solution containing 120 μl of 0.01 mM Tris buffer (pH 7.6) and 40 μl of 1.4 mg/ml of the substrate H-gly-pro-*p*-nitroanilide (BACHEM, Inc., Bubendorf, Switzerland). The absorbance of the hydrolyzed substrate was measured every 5 min for 30 min at 405 nm using a microtiter plate reader (ELX808, Bio-Tek Instruments, Inc., Wilrijk, Belgium). Specific enzyme activities are expressed as mU/mg of protein. One unit is defined as the activity that hydrolyzes 1 μmol of substrate/min at 37°C. Mean ± SD represents duplicate assays of three independent experiments.

Measurement of NO_2^-/NO_3^-. Total nitrite was quantified using the Griess reagent after reduction of nitrate into nitrite as adapted from Pollock *et al.* (49). Briefly, 10^5–10^6 cells were seeded in a 12-well culture dish and cultured for 5 or 21 days. The culture medium was replaced 48 h before the collection of the supernatants, which were centrifuged for 5 min at 5000 rpm to remove particles. Samples were incubated for 15 min at 37°C with 40 μM NADPH (Fluka, Buchs, Switzerland), 1 μM flavine adenine dinucleotide (Sigma Chemical Co., Buchs, Switzerland), and 0.2 units/ml of nitrate reductase (Boehringer Mannheim). One mM sulfanilamide reagent (Sigma Chemical Co.), 1.4 N HCl, and 1 mM *N*-(1-naphthyl)ethylenediamine (Sigma Chemical Co.) were added sequentially to the incubation. NO_2^- concentration was determined by the change in absorbance at 540 nm, which was compared to a standard curve of $NaNO_2$ diluted in DMEM. The recovery of NO_3^- was 95%. Results are expressed as nmol of NO_2^-/mg protein, and are the mean ± SD of duplicate determinations of three separate experiments.

Transfection. The NFκB3-luc plasmid containing three NFκB consensus sequences cloned in front of the luciferase gene reporter was provided kindly by Dr. B. Sugden (McArdle Laboratory, Madison, WI; Ref. 50). CaCo-2 cells were transfected with a DNA mixture containing 6 μg NFκB3-luc vector and 600 ng hygromycin expression vector, using Lipofectin (Life Technologies, Inc.) according to the manufacturer recommendations. Forty-eight h after transfection, cells were trypsinized and cultured in the presence of 180 μg/ml hygromycin (Boehringer Mannheim) until a stable NFκB3-luc-transfected population was established. The transfected cells were seeded in a 12-well culture dish at a density of 10^5 cells/well and cultured either 7 or 21 days. Two wells per condition were then exposed to 200 nM PMA (Sigma Chemical Co.) for 8 h. After this time, cell extracts were collected in either 200 μl of 1× lysis buffer [1.25 mM Tris (pH 7.8) with H_3PO_4, 10 mM CDTA, 10 mM DTT, 50% glycerol, and 5% Triton X-100] for the 7-day-old cells, or in 500 μl of 1× lysis buffer for the 21-day-old cells. Luciferase assays were carried out according to the manufacturer instructions (Promega, Catalys SA, Wallisellen, Switzerland) using a luminometer (LKB Wallac, Lucerne, Switzerland). The values represent the mean ± SD of triplicate assays of two independent experiments.

Preparation of Cellular Extracts. For the detection of villin, bcl-2, MT, GSTα, and iNOS proteins, cells were collected in PBS and centrifuged for 5 min at 5000 rpm. The pellet was homogenized in a 50 mM HEPES buffer (pH 7.4) containing 10% glycerol, 100 μM DTT (Fluka), 5 μg/ml leupeptin (Sigma Chemical Co.), 1 μg/ml antipain (Sigma Chemical Co.), 1 μg/ml pepstatin (Sigma Chemical Co.), 5 μg/ml aprotinin (Sigma Chemical Co.), 1 mM sodium vanadate (Merck, Basel, Switzerland) and 200 μM phenylmethylsulfonyl fluoride (Boehringer Mannheim). After three freezing/thawing cycles, the homogenate was centrifuged at 4°C for 30 min at 100,000 × *g*, and the supernatant was collected. For the detection of p53, cells were scraped in 40 mM HEPES buffer (pH 7.2) containing 100 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM EDTA (Fluka), 0.5% NP40 (Fluka), and 2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged (75,000 rpm at 4°C for 15 min), and the supernatant was collected.

iNOS Purification. iNOS purification was carried out using a modification of a previously described protocol (51) and was set up using EC219 rat endothelial cells stimulated with IFN-γ and lipopolysaccharide (52). The S100,000 × *g* cytosolic fractions (2.5-mg proteins) were loaded on chromatography columns containing 2′-5′ ADP Sepharose 4B gel (Phar-

[5] P. Shaw, personal communication.

macia AG, Dubendorf, Switzerland), which had been equilibrated with a buffer A containing 10 mM K_2HPO_4 (pH 7.4), 100 mM NaCl, 10% glycerol, 0.5 mM L-arginine, 1 μM BH4 (Alexis Corp.), and 3 mM DTT. The columns were washed with buffer A containing 300 mM NaCl and then rinsed with a buffer B [10 mM K_2HPO_4 (pH 7.4), 100 mM NaCl, 0.5 mM L-arginine, 1 μM BH4, and 3 mM DTT]. iNOS was eluted with buffer B, containing 10 mM NADPH and 0.5 μM instead of 1 μM BH4. The NADPH-eluted fractions were then precipitated with 5 volumes of acetone and dried, and the pellets were homogenized in Laemmli buffer. The yield based on chromatography was 40%.

SDS-PAGE and Western Blot Analysis. Fifty μg of protein (for the detection of bcl-2, villin, actin, and GSTα) or 500 μg of protein (for the detection of p53) were mixed with Laemmli buffer and were loaded on 10% SDS-PAGE polyacrylamide gel (53). For the detection of MT, 50 μg of protein were mixed with a buffer consisting of 300 mM sucrose, 0.35% SDS, and 0.6 mM $CdCl_2$, and loaded on 15% SDS-PAGE polyacrylamide gel (23). The iNOS samples were loaded on 7.5% SDS-PAGE polyacrylamide gel. The separated proteins were transferred onto nylon filters (Dupont De Nemours, Regensdorf, Switzerland), according the procedure of Towbin *et al.* (54). Blotted membranes were incubated 2 h in the presence of washing buffer [150 mM NaCl, 10 mM Tris-HCl (pH 8), and 0.05% Tween 20] containing appropriate antisera. Antibodies raised against mouse iNOS were diluted 1:500, and those directed against human iNOS were used at a dilution of 1:100 (for Santa Cruz Biotechnology antibodies) or 1:40,000 (for antibodies from Dr. R. A. Mumford), respectively. Antibodies directed against human bcl-2, villin (30), GSTα (47, 48), p53, and MT (23) were diluted 1:40, 1:500, 1:2,000, 1:10,000, and 1:100, respectively. Human actin antiserum diluted 1:5,000 was used as an internal loading control. For the detection of p53, cell extracts from Beas-2B cells (a nontumorigenic human bronchial epithelial cell line; Ref. 55) were used as positive controls. After four washes, filters were incubated for 1 h in washing buffer containing either antirabbit or antimouse horseradish peroxidase conjugates diluted 1:10,000. Detection of the immune complex was performed using the luminol enhancer solution supersignal TM CL-horseradish peroxidase (Pierce, Lausanne, Switzerland). Cell extracts from cytokine-induced mouse macrophages (Affiniti) were used as a positive control. Induction factors were measured using a scanning densitometer (2202 Ultrascan, LKB Bromma). Three experiments were performed and results are from one representative experiment.

iNOS Activity Assay. The iNOS activity assay was performed as reported previously (31) using a cation-exchange column to separate radiolabeled citrulline from arginine. Briefly, 100 μl of cell homogenate were mixed with 150,000 cpm L-[^{3}H]arginine (59 Ci/mmol; Amersham Corp.), 50 μM arginine, 2 mM NADPH, 5 μM flavine adenine dinucleotide, 5 μM BH4, and 1 mM EGTA, and incubated for 30 min at 37°C in the presence or in the absence of N^G-methyl-L-arginine (Alexis Corp.) to determine the specific L-arginine metabolism due to iNOS. The incubation was applied to a Dowex AG 50WX-8 (Bio-Rad, Richmond, CA) resin column and L-[^{3}H]citrulline was eluted with cold L-citrulline and measured by scintillation counting. The results were expressed as specific activities, pmol of citrulline formed/min · mg protein. Mean ± SD (n = 3) represents three measures performed separately.

Determination of cGMP Concentration. CaCo-2 cells were seeded in a 12-well culture dish at a density of 10^5 cells/well. They were grown either 5 or 21 days and incubated 10 min with 5 μM of the NO donor DEA/NO (Ref. 31; a generous gift from L. Keefer, National Cancer Institute, Frederick, MD), in the presence of 0.5 mM isobutylmethylxanthine (Calbiochem, Lucerne, Switzerland). The supernatant was then withdrawn and the cells were lysed with 500 μl of cold ethanol containing HCl 10 mM and frozen at −70°C. After evaporation under vacuum, the pellet was reconstituted in 250 μl of 50 mM sodium acetate buffer (pH 4), acetylated for 10 min with acetic anhydride/triethylamine (2:1, 5 μl per 100 μl buffer), and the cGMP determination was made by RIA using anti-cGMP antibodies from Meloy Laboratories (Springfield, VA). The sensitivity of the RIA is 1 fmol of cGMP. Results represent the mean ± SD (n = 4).

Northern Blot Analysis. Extraction of RNA using guanidinium thiocyanate and Northern blot analysis were performed as already described (31) using the human hepatocyte iNOS cDNA (56). Levels of iNOS RNA were measured by scanning and directly compared, because the RNA loading indicated by the rRNAs 18S and 28S onto the agarose gel was identical. The results are representative of two independent experiments.

Statistical Analysis. Data represent means ± SD and were compared using the Student's t test. A probability value <0.05 was considered a significant difference.

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