Differential effects of ferritin, calcium, zinc, and gallic acid on in vitro proliferation of human glioblastoma cells and normal astrocytes

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In vitro, when using low concentrations of ferritin (ng/ml) or $CaCl_2$ (μg/ml), multiplication of a human, 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU)-resistant glioblastoma cell line (U251) is enhanced 1.5 to 2 times more actively than multiplication of a normal astrocyte line (CRL 1656). Ferritin and Ca^{2+} ions exhibit a marked effect on DNA isolated from these cells: glioblastoma DNA relaxation is strongly increased (as evidenced by increased 260 nm ultraviolet absorbance), being from 5 to 6 times that of astrocyte DNA, which remains only slightly affected. Under identical experimental conditions, Zn^{2+} and gallium ions selectively inhibit glioblastoma cell multiplication but at the same concentrations do not inhibit astrocyte multiplication. Ultraviolet absorbance measurements demonstrate that both of these agents condense relaxed glioblastoma DNA in vitro. Zn^{2+} or gallium ions added to culture medium containing stimulatory concentrations of ferritin or Ca^{2+} ions selectively and strongly inhibit enhancement of glioblastoma cell multiplication by these mitogens while not affecting normal multiplication of astrocytes. (J Lab Clin Med 1994;123:547-55)

Abbreviations: BCNU = 1,3-*bis*(2-chloroethyl)-1nitrosourea; EDTA = ethylenediaminetetracetic acid; UV = ultraviolet

For many years our central line of work has been the study of the differential response of cancer and normal cell DNAs to various substances and the search for molecules that act selectively on either cancer or normal DNA.[1,2] Fifteen years ago we provided evidence of the essential difference that characterizes cancer DNA, namely that its secondary structure is always more relaxed than that of normal DNA. Whereas the latter's strands only separate locally and temporarily for replication and for gene expression, cancer DNA contains multiple and extensive areas in which, because of H-bond breakage, strand separation is permanent.[3,4] Cancer DNA is then destabilized.

Based on comparison of numerous cancer DNAs and normal DNAs from corresponding tissues, experimental support for this finding includes the following: (1) 260 nm UV absorbance is consistently higher for cancer DNA (hyperchromicity); (2) cancer DNA exhibits increased in vitro synthesis; (3) a clear, positive correlation exists between cancer DNA hyperchromicity, its increased in vitro replication, and enhanced in vitro and in vivo malignant cell multiplication.[2]

Moreover, destabilization, caused by H-bond breakage leading to chain opening, makes cancer DNA highly receptive to numerous agents, both exogenous and endogenous, that experience no difficulty in binding to isolated strand areas.

We showed that among those agents, some—the carcinogens—increase cancer DNA destabilization, whereas others have the unique property of restabilizing cancer DNA by binding to its relaxed strands and bringing them back into their normal position. These

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opposite activities are most clearly evidenced by the changes that occur in DNA UV absorbance, which carcinogens increase and restabilizing molecules decrease. On the other hand, normal DNAs are but slightly affected by both types of agents, because the latter experience much more difficulty in binding to nonrelaxed, "closed" chains.[4,5] Nevertheless, persistence or high doses of carcinogenic agents (or both) may overcome the normal cell's ability to repair its DNA and may lead to malignant transformation.

After demonstrating the carcinogenic activity of cancer markers such as ferritin[6] and the restabilizing properties of a number of plant-derived alkaloids, we turned our attention to trace metals, starting our investigations with iron, calcium, zinc, and gallium, whose reported intervention in cell multiplication processes is well documented.[7] Indeed, trace elements (discovered at the beginning of this century by French biochemist Gabriel Bertrand,[8] who pointed out their central role in metabolism) have lately received increasing attention. Trace amounts of metals such as iron (notably in its protein-bound storage form, ferritin), calcium, and zinc are present and play active parts in all body tissues. Among its numerous assignments, iron is required for cell growth and multiplication[9]; it was found to accelerate in vitro malignant cell multiplication,[10-12] whereas tumor growth decreased in iron-deficient mice. Iron is required by ribonucleotide reductase.[11] Besides, ferritin and iron induce a drastic accumulation of Ca^{2+} in Ehrlich carcinoma cells, accompanied by a modification of calcium homeostasis.[13] Conversely, Ca^{2+} ions are involved in cellular iron uptake.[14] They also accumulate in various cancer cells and contribute to resistance to therapeutic drugs such as nitrosourea.[15] In vitro, not only ferritin but also Ca^{2+} ions were shown to increase cancer cell multiplication.[16,17]

In contrast, Zn^{2+} and gallium ions antagonize some of the biologic activities of iron and especially of ferritin.[18-20] The presence of zinc is required for DNA synthesis[21] and relates to gene expression (zinc fingers). Gallium was recently shown to compete with iron at a site central to the activity of ribonucleotide reductase, an enzyme vital to DNA synthesis,[22] and gallium-induced cytotoxicity may be suppressed by iron.[23] Gallium nitrate is preferentially taken up by certain tumors.[25,26,27]

The presence of iron, ferritin, and calcium in brain cells[28-30] raises the question of their respective roles in brain tumor development. In this article, we compare the effects of ferritin and three metal ions on the in vitro multiplication of two human brain cell lines: the malignant, BCNU-resistant glioblastoma U251 and the normal astrocyte CRL 1656. We show that ferritin and calcium chloride have more pronounced mitogenic activity in the cancer cells than in the normal cells. In contrast, zinc ions and gallic acid selectively inhibit cancer cell multiplication and counteract the effects of ferritin and calcium. Emphasis is laid on the DNA destabilizing and restabilizing effects of tested substances, and therapeutic implications are suggested.

METHODS

Chemicals. Chemicals used were obtained from the following sources: $CaCl_2$, grade A, from Prolabo, Lyon, France; ferritin (human liver), from ICN Biochemicals, Cleveland, Ohio; BCNU, gift of Professor Labat, CHU, Brest, France; trypsin, RPMI 1640 culture medium, fetal calf serum, and glutamine, from Gibco, Grand Island, N.Y.; other chemicals were from Prolabo, France.

Isolation of DNAs. DNAs from glioblastoma (U251) and normal astrocyte (CRL 1656) cell lines were isolated and purified by the method described elsewhere.[2,4] Purity of DNAs was checked by conventional methods.[24] The amount of DNAs was determined by using UV absorbance at 260 nm (260/280 = 2.05). After incubation with 0.1N KOH, DNAs exhibited a 45% to 52% hyperchromicity.

DNA UV absorbance measurement during experiments. DNA was dissolved in a 0.01 mol/L Tris-HCl buffer solution (pH 7.50). Increasing concentrations of substances under test were dissolved in 10 μl of the same buffer and added both to the blank and DNA solutions. Mixture was gently agitated at room temperature, and its 260 nm UV absorbance was determined.

Cell lines and culture techniques. The established human glioblastoma cell line, U251, was obtained from the Swedish Cell Collection, Uppsala, Sweden, and the normal astrocyte line, CRL 1656 Mpf (cell repository line), was from the American Type Culture Collection, Rockville, Md. Cells were grown at 37° in an incubator. Stock cultures were maintained by continuous passage in RPMI 1650 medium containing 10% fetal calf serum. Culture medium was changed 3 days after seeding and subsequently twice a week. Absence of mycoplasma and bacteria was checked every 3 months. For experiments, each cell type was subcultured in 6-well tissue culture plates (9.8 cm^2 wells) starting from a 4×10^4 inoculum. Cell viability was determined by using Trypan blue dye; viability was 99% to 100% at the start of experiments.

Growth inhibition and stimulation. Increasing concentrations of filtration-sterilized tested substances (ferritin, $CaCl_2$, $ZnCl_2$, and gallic acid) were added to the cell suspensions. After a 48-hour incubation, 0.05 ml/well of trypsin plus 0.05% EDTA were added and, after a 5-minute incubation, cells were detached from the culture plates. Trypsinization was stopped by using 4 ml RPMI 1640/well. Cells were then counted with a Coulter counter. All experiments were performed three times, and each in triplicate.

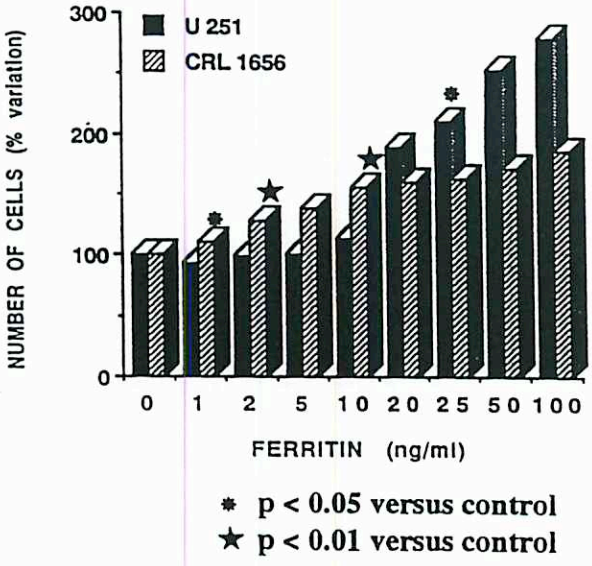

Fig. 1. Stimulating effect of ferritin on in vitro proliferation of normal astrocyte and BCNU-resistant glioblastoma cell lines. Astrocyte (CRL 1656) and glioblastoma (U251) cell lines were grown in the absence and presence of different concentrations of ferritin for 48 hours at 37° C. The results are expressed as percent increase (mean values of three separate experiments). CRL 1656:SD ± 14.45; U251: SD ± 13.12. *p* values determined by Student *t* test.

RESULTS

Effect of ferritin on glioblastoma and normal astrocyte cell line multiplication. Fig. 1 shows the dose-dependent, differential effects of low (ng/ml) ferritin concentrations. Multiplication of glioblastoma cells started to increase with about 20 ng/ml ferritin and then augmented steeply, reaching an almost threefold maximum value with 100 ng/ml ferritin. Astrocyte multiplication was first enhanced by only 2 to 5 ng/ml ferritin but increased less markedly, up to a maximum of less than twofold with 100 ng/ml ferritin. A difference in multiplication enhancement between the two cell lines was already discernible after only 24 hours, that is, long before confluence was reached (48 hours) and measurement performed.

Cell trypsinization before plating apparently did not remove ferritin receptors from either cancer or normal cells, because ferritin persistently remained actively mitogenic, more so than free iron and transferrin. When tested under the same conditions, free iron, though it did stimulate cell multiplication, exerted no differential effect on glioblastoma cells and astrocytes (Table I), and transferrin did not stimulate cell multiplication at all.

Effect of Ca^{2+} ions on glioblastoma and astrocyte cell line multiplication. As may be seen from Fig. 2, stimulation of multiplication started in both cell lines with

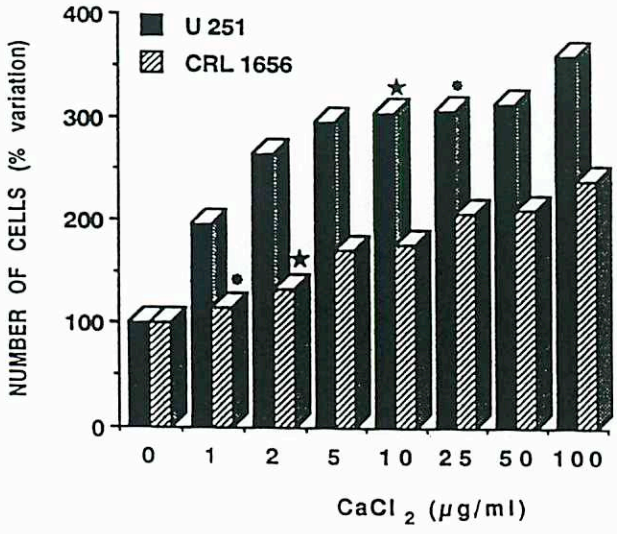

Fig. 2. Stimulating effect of $CaCl_2$ on in vitro proliferation of normal astrocyte and BCNU-resistant glioblastoma cell lines. Astrocyte (CRL 1656) and BCNU-resistant glioblastoma (U251) cell lines were grown in the absence and presence of different concentrations of $CaCl_2$ for 48 hours at 37° C. The results are expressed as percent increase (mean values of three separate experiments). CRL 1656: SD ± 3.74; U251: SD ± 16.69. *p* values determined by Student *t* test.

Table I. Astrocyte (CRL 1656) and glioblastoma (U251) cell lines grown for 48 hours in the absence and presence of $FeCl_3$

	$FeCl_3$ (μg/ml)	Number of cells (%)
CRL 1656	0	100
	40	162 ± 3.4
U 251	0	100
	40	148 ± 8.25

Average values of three independent experiments. For experimental conditions, see Methods

$CaCl_2$ concentrations of 1 to 2 μg/ml; but with increasing calcium ion concentrations, glioblastoma cells were induced to multiply at about a maximum of two times more actively than normal astrocytes.

In vitro effect of ferritin and of Ca^{2+} ions on UV absorbance of glioblastoma and astrocyte DNAs. UV absorbance at 260 nm of glioblastoma and astrocyte DNAs was measured in the presence and absence of ferritin and of Ca^{2+} ions. Fig. 3 indicates that both ferritin (ng/ml) and Ca^{2+} (μg/ml) induce a dose-dependent increase of UV absorbance, which is high for glioblas-

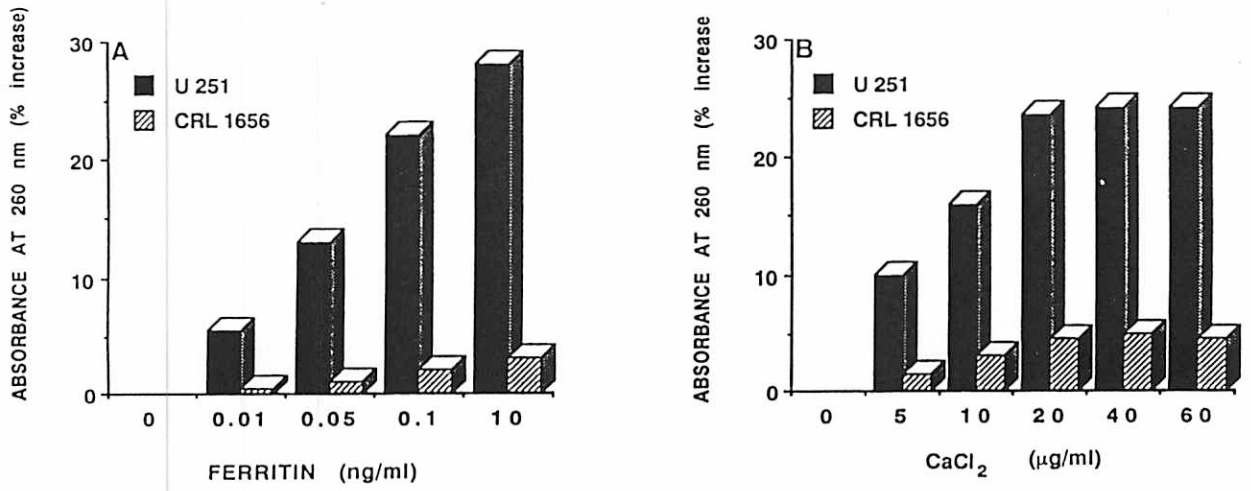

Fig. 3. Effects of ferritin and $CaCl_2$ on UV in vitro absorbance of normal astrocyte (CRL 1656) and glioblastoma (U251) DNA. For experimental conditions, see Methods.

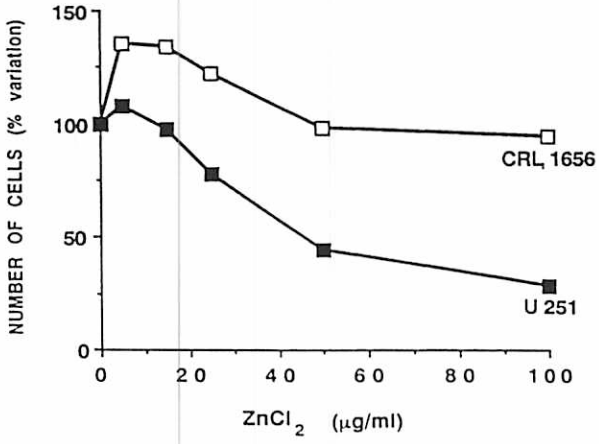

Fig. 4. Selective in vitro inhibition by $ZnCl_2$ of the multiplication of BCNU-resistant glioblastoma cell line. BCNU-resistant glioblastoma and normal astrocyte cell lines were grown in the absence or presence of various concentrations of $ZnCl_2$ for 48 hours at 37° C. The results are expressed as percent decrease of the number of cells (mean values of three separate experiments). CRL 1656: SD ± 10.2; U251: SD ± 4.179.

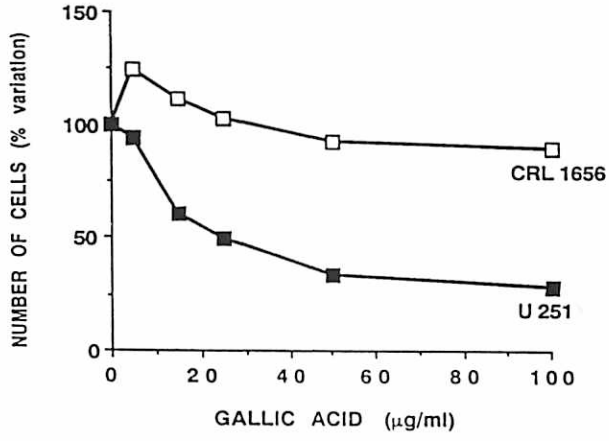

Fig. 5. Selective in vitro inhibition by gallic acid of the multiplication of BCNU-resistant glioblastoma cell line. BCNU-resistant glioblastoma and normal cell lines were grown in the absence and presence of various concentrations of gallic acid for 48 hours at 37° C. The results are expressed as percent decrease of the number of cells (mean values of three separate experiments). CRL 1656: SD ± 3.6; U251: SD ± 9.26.

toma DNA, with maximum values of 30% for 10 ng/ml ferritin and 25% for 60 µg/ml $CaCl_2$, but slight for astrocyte DNA, exhibiting a maximum of about 5% with both agents.

Effect of Zn^{2+} ions and of gallic acid on in vitro multiplication of glioblastoma and astrocyte cell lines. Fig. 4 shows that Zn^{2+} ions induce an active decrease of glioblastoma cell multiplication, which starts with 25 µg/ml $ZnCl_2$ and reaches 75% with 100 µg/ml. Astrocyte multiplication first increases by about 30% with low Zn^{2+} concentrations (1 to 25 µg/ml $ZnCl_2$), then is not significantly modified by higher concentrations. Thus Zn^{2+} ions exert a differential effect on glioblastoma cells and astrocytes, checking multiplication of malignant cells, but not of normal cells.

We chose to use gallic acid as a source of gallium ions because it is less toxic than the more commonly used gallium nitrate. Fig. 5 shows that the decrease in glioblastoma cell multiplication starts with 10 µg/ml of gallic acid and reaches 75% with 100 µg/ml. Astrocyte multiplication increases by about 25% to 30% with low gallic acid concentrations, but above 10 µg/ml neither

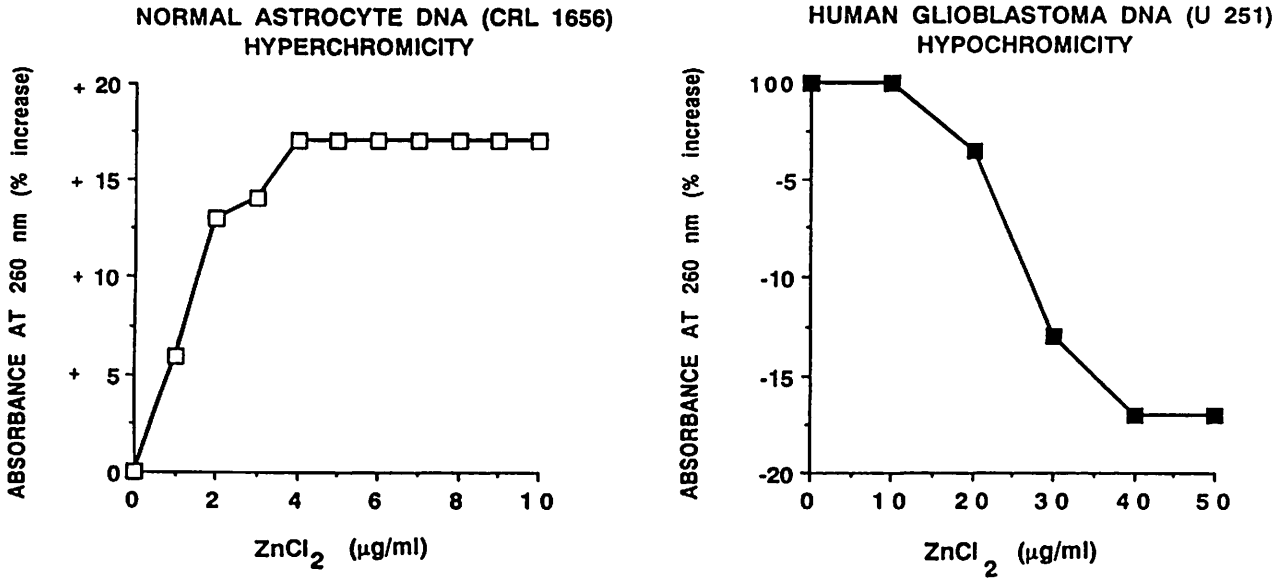

Fig. 6. Effect of $ZnCl_2$ on in vitro UV absorbance of glioblastoma (U251) and normal astrocyte (CRL 1656) DNAs. For experimental conditions, see Methods.

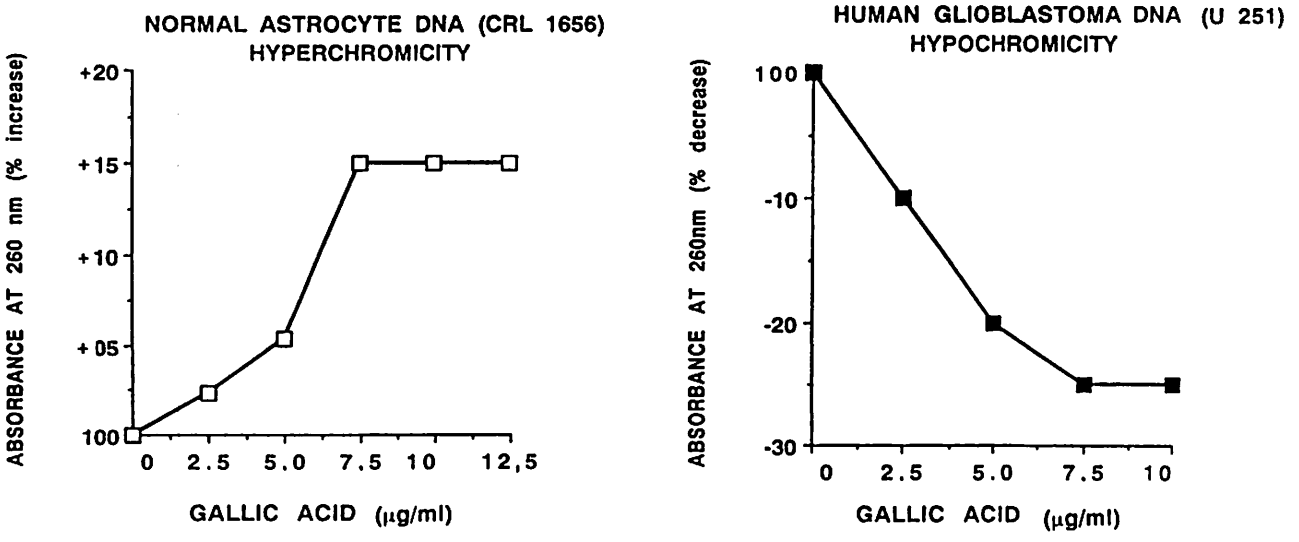

Fig. 7. Effect of gallic acid on in vitro UV absorbance of glioblastoma (U251) and normal astrocyte (CRL 1656) DNAs. For experimental conditions, see Methods.

stimulation nor inhibition is observed. Gallium ions thus also exert a differential effect on malignant and normal cells, inhibiting only the former.

In vitro effect of Zn^{2+} ions and gallic acid on UV absorbance of glioblastoma and astrocyte DNAs. In the presence of increasing concentrations of each of these agents, glioblastoma DNA hyperchromicity gradually disappears, indicating that Zn^{2+} ions (Fig. 6) and gallium ions (Fig. 7) correct the uncontrolled H-bond breakage characteristic of cancer DNA and bring the separated strands back into their proper normal position. Both Zn^{2+} and gallium ions induce a slight increase of astrocyte DNA absorbance, which plateaus at less than 20% for about 4 µg/ml $ZnCl_2$ and about 6 µg/ml gallic acid.

Zn^{2+} ions and gallic acid inhibit the stimulatory effect of ferritin and Ca^{2+} on cell multiplication. When glioblastoma cells are grown on culture medium containing given amounts of ferritin, and increasing concentrations of Zn^{2+} ions (Fig. 8) or of gallic acid (Fig. 9)

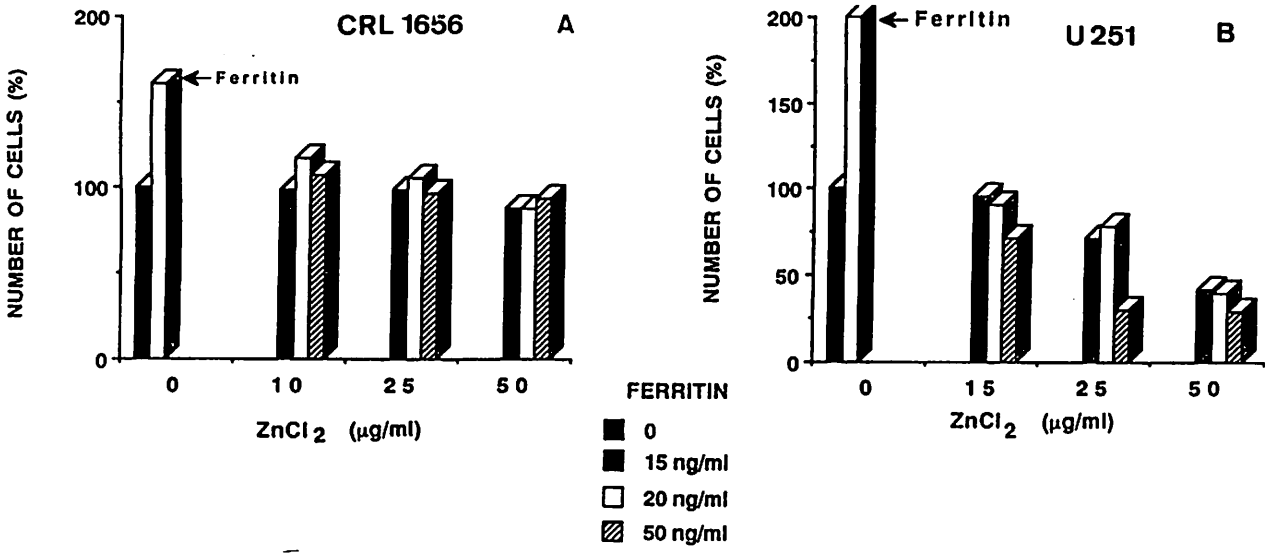

Fig. 8. Effect of $ZnCl_2$ on in vitro proliferation of human BCNU-resistant glioblastoma (U251) and normal astrocyte (CRL 1656) cell lines in the presence of ferritin. For experimental conditions see legend to Fig. 1.

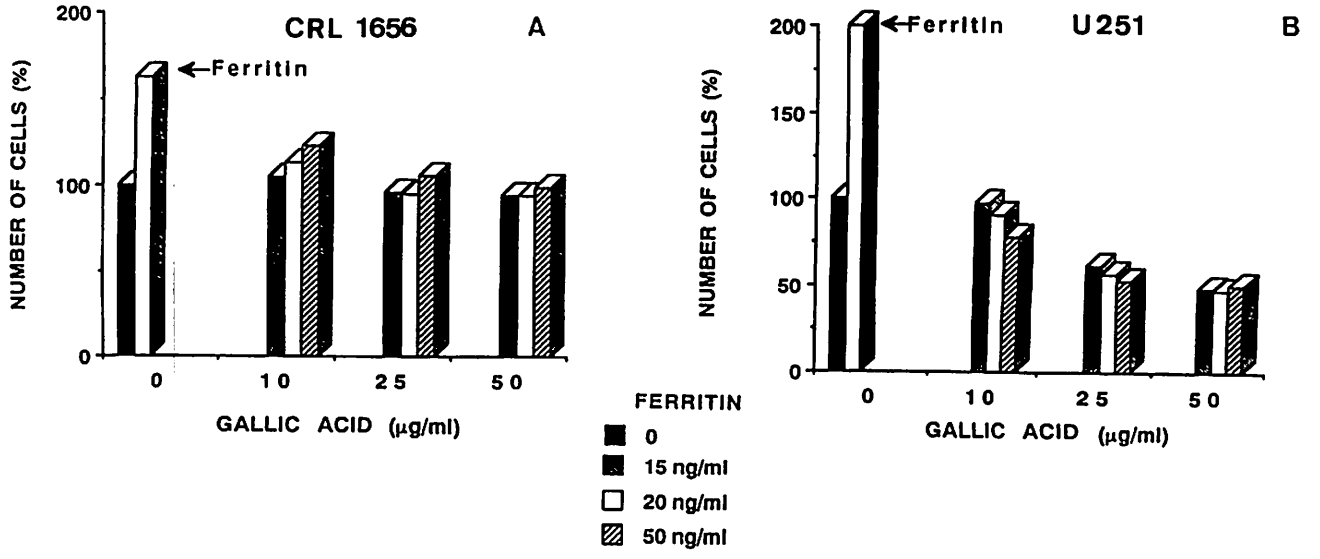

Fig. 9. Effect of gallic acid on in vitro proliferation of human BCNU-resistant (U251) and normal astrocyte (CRL 1656) cell lines in the presence of ferritin. For experimental conditions see legend to Fig. 1.

are added, not only is the stimulatory effect of ferritin abolished, but cell number is moreover reduced by up to 50%. In contrast, when astrocytes are grown under the same conditions, Zn^{2+} and gallic acid merely suppress the stimulatory effect of ferritin, without decreasing normal cell multiplication, which becomes similar to that of controls grown in the absence of any of the four agents used in these experiments. Corresponding results were obtained by using Ca^{2+} ions in place of ferritin (Fig. 10).

DISCUSSION

In this article we first demonstrated that two agents known to increase cancer cell multiplication,[16,17] ferritin (ng/ml) and $CaCl_2$ (µg/ml), exert differential mitogenic activities, in vitro, on malignant and normal brain cell lines. With the range of concentrations used for either of these agents, maximum increase of glioblastoma cell multiplication is on the average 1.5-fold that of astrocyte multiplication: maximum enhancement induced by 100 ng/ml ferritin is almost 300% for

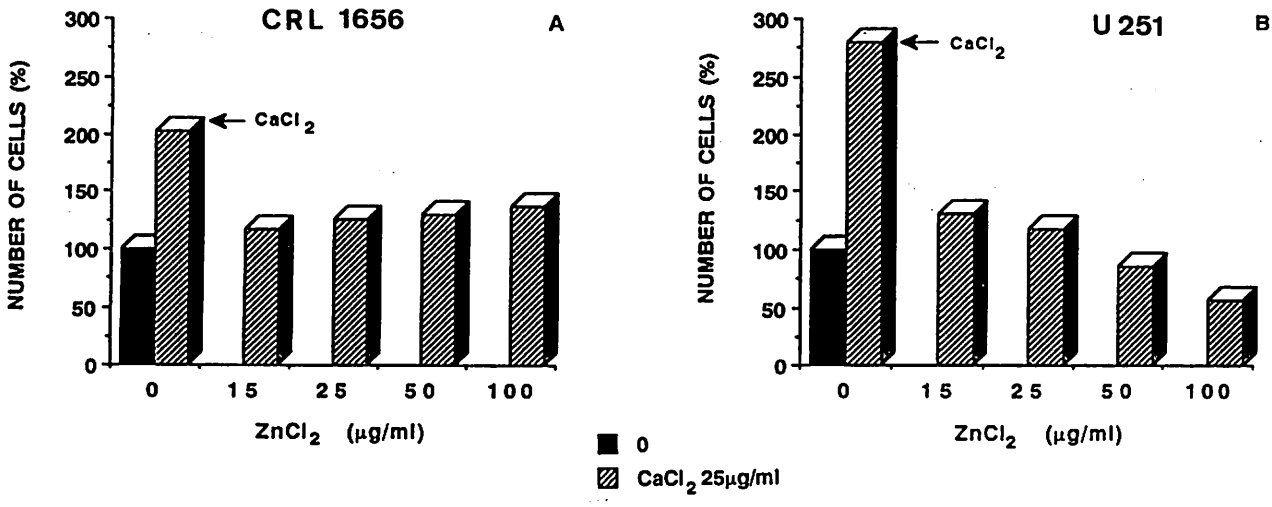

Fig. 10. Effect of $ZnCl_2$ on in vitro proliferation of human BCNU-resistant glioblastoma (U251) and normal astrocyte (CRL 1656) cell lines in the presence of $CaCl_2$. **A**, SD ± 3.74; **B**, SD ± 1.71. For experimental conditions, see legend to Fig. 1.

cancer cells and less than 200% for normal cells; corresponding values when using 100 μg/ml $CaCl_2$ are, respectively, nearly 400% and about 250% (Figs. 1 and 2).

Moreover, measurement of 260 nm UV absorbance of glioblastoma cell and astrocyte DNAs in the presence of either ferritin or $CaCl_2$ (i.e., Ca^{2+} ions) brings out a clear-cut difference between the activities of these mitogens on cancer and on normal DNAs: when using 10 ng/ml ferritin, increase of cancer DNA UV absorbance is six times that of normal DNA, and when using 10 μg/ml $CaCl_2$, a fivefold increase is seen (Fig. 3).

The increase of DNA UV absorbance is known to be due to H-bond breakage within the molecule. As more bonds are severed, the DNA molecule becomes destabilized. As explained above (see Introduction), we showed quite a long time ago that cancer DNA is always destabilized and that carcinogens further increase this destabilization by breaking more H-bonds. This is indeed what ferritin and calcium do to glioblastoma cell DNA. In addition, a positive correlation exists between cancer DNA destabilization and increased malignant cell multiplication. This may be explained by the fact that on isolated strand portions resulting from H-bond breakage, normally unavailable initiation sites for DNA replication and transcription become exposed, accounting for the exaggerated proliferation and aberrant gene expression exhibited by malignant cells. In the present case, glioblastoma cell multiplication undergoes a higher increase than do normal astrocyte cells in the presence of ferritin and Ca^{2+} ions. Yet there is a discrepancy between the fivefold to sixfold enhancement of glioblastoma DNA UV absorbance over that of astrocyte DNA and the 1.5-fold enhancement of malignant cell multiplication as compared with that of normal cells. Most probably some of the numerous regulation pathways and molecular interactions within the living cell serve to prevent the two mitogens from exerting as straightforward an action as on isolated DNAs. By contrast, we do not exclude the possibility that glioblastoma cells might carry more ferritin receptors than do astrocytes (maybe one of the novel properties induced by malignancy); by facilitating entry of the mitogen, this would, in the end, favor its activity at DNA level. Using the same experimental procedures as for the mitogens, we then investigated the activities of two trace metals, zinc and gallium, which have been reported to antagonize some of the biologic activities of iron (of which ferritin is a protein carrier). We found that both Zn^{2+} and gallium ions induce an active, dose-dependent decrease of glioblastoma cell multiplication: this decrease reaches 75% with 100 μg/ml of either $ZnCl_2$ or gallic acid (Figs. 4 and 5). In contrast, astrocyte multiplication first increases by about 30% with very low trace metal concentrations but is then no further affected by higher ones. Measurement of DNA UV absorbance at 260 nm yields results that follow a similar trend: both $ZnCl_2$ (40 μg/ml) and gallic acid (7.5 μg/ml) induce a maximum decrease of glioblastoma UV absorbance (respectively about 18% and 25%), whereas astrocyte DNA absorbance reaches a plateau after an increase of a little over 15%, induced by about 5 μg/ml of either $ZnCl_2$ or gallic acid (Figs. 6 and 7).

Zinc and gallium ions, like ferritin and calcium, exert a differential effect on malignant and normal cells.

But contrary to the effect of these mitogens, zinc and gallium ions check cancer cell multiplication, and the decrease in cancer DNA UV absorbance that these trace metals induce indicates that they behave as DNA restabilizers, correcting cancer DNA strand relaxation. In contrast, they neither inhibit normal cell multiplication nor decrease normal DNA UV absorbance. Specific curbing of malignant cell proliferation and of cancer DNA UV absorbance, coupled with lack of inhibitory activity on normal cells, is consistent with the behavior of cancer DNA restabilizing agents, of which we discovered a number, when using our in vitro Oncotest.[1,2] An outstanding property of these agents is their selectivity for cancer DNAs and cells.

To complete this series of experiments, we further showed that Zn^{2+} ions and gallic acid are able to counteract, in a dose-dependent way, the mitogenic effects of ferritin and of calcium on glioblastoma cells: not only is mitogen-induced stimulation of cancer cell multiplication inhibited, but in addition, there is a 50% decrease in the amount of malignant cells; mitogenic effects of ferritin and calcium on astrocytes are also suppressed, but after that, normal proliferation is not inhibited (Fig. 9). Ferritin, by increasing cancer DNA destabilization, facilitates access of the restabilizing ions to the relaxed DNA strands.[6] Obviously this may increase the therapeutic potentials of zinc and gallium.

The tumor-enhancing properties of iron and ferritin, and the process by which zinc and gallium may antagonize this activity, have given rise to divergent interpretations. At first sight, iron could have many opportunities of enhancing tumor cell multiplication. To name but a few, its central requirement during cell growth and development,[32-34] its stimulatory activity on enzymes[9] such as reverse transcriptase[35] or terminal deoxynucleotidyl transferase[36]—which may interfere with normal regulation of DNA activity—or its ability to turn the superoxide ion into highly toxic free radicals.[37] The antagonistic effect of gallium was, in part, interpreted by the ability of this metal to replace iron inside the M2 subunit of ribonucleotide reductase, an enzyme that supplies DNA building blocks[15]; gallium, a group III metal, has an ionic radius similar to that of iron.[38]

These interpretations are not relevant to our results. In our in vitro experiments, the trace metal ions were allowed to act directly on purified DNA from cancer and normal cells. The potent effect observed on 260 nm UV absorbance of cancer DNA, contrasting with the slight effect on absorbance of normal DNA, and reflected in corresponding in vitro cell multiplication, speaks in favor of a straightforward action mediated by binding of the metal ions to the relaxed strands of cancer DNA. We already observed such direct binding when using the plant alkaloid serpentine, an efficacious DNA restabilizer. A UV absorbance peak, intermediate between those of the cancer DNA and serpentine, pointed to the formation of a serpentine-DNA complex.[4] Furthermore, experiments (data not shown) regarding an eventual competition between the mitogens ferritin and calcium, and the restabilizers zinc and gallium point to the latter having a common binding site on cancer DNA that is different from the common binding site of the former.

Other molecules already bound to DNA might mediate binding and activity of the destabilizing and restabilizing metal ions we utilized. Cancer DNAs carry more peptides than do normal DNAs. We found that even highly purified Ehrlich ascitic tumor DNA contains small amounts of peptides, notably cysteine, which is seemingly absent from normal DNAs (unpublished results). As previously stated, it must be stressed that the destabilized cancer DNA is easily accessible to many molecules; some of them may influence or even direct the DNA binding of the metal ions we tested and thus contribute to their selective properties. We might add that at the cell regulation level, channels that mediate metal ion entry may differ as to their amount or nature (or both) in cancer and in normal cells. Further investigation of these various points will follow.

We are now pursuing our experiments with the view of including restabilizing metal ions into our anticancer strategy,[31] which uses selective DNA restabilizing agents. In consideration of results reported in this article, it must already be stressed that intake of iron and calcium by cancer patients, as well as their plasma ferritin levels, should be closely monitored. These factors should be taken into account for improvement of individual patient therapy.

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