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THE USE OF HYDROGEN PEROXIDE TO ENHANCE THE EFFICACY OF DOXORUBICIN HYDROCHLORIDE IN A MURINE BLADDER TUMOR CELL LINE

KEVIN R. LOUGHLIN, KELLEDY MANSON, DINO CRAGNALE, LISE WILSON, ROBERT A. BALL AND KENNETH R. BRIDGES

From the Division of Urology, Department of Surgery and Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Purpose: We determined whether the cytotoxicity of doxorubicin hydrochloride would be enhanced by adding hydrogen peroxide as a source of oxygen free radicals.

Materials and Methods: Mouse bladder tumor cells (MBT-2) were grown in RPMI 1640 medium and treated with various concentrations of doxorubicin hydrochloride for 2 hours. Protein content was assayed as a measure of cell growth. A similar set of experiments was done with cells exposed to hydrogen peroxide only and combined doxorubicin and hydrogen peroxide. Protein content was again assayed as a measure of cell growth. Cells were also assayed for glutathione peroxidase and malonyl dialdehyde, a product of lipid peroxidation, to determine the mechanism of cell damage. Furthermore, MBT-2 cells were incubated with 100 M. α-tocopherol, a free radical scavenger, before exposure to hydrogen peroxide to determine whether the effects of hydrogen peroxide could be reversed.

Results: We observed a dose dependent inhibition of MBT-2 cell growth after exposure to doxorubicin hydrochloride. Exposure to doxorubicin and hydrogen peroxide resulted in greater cell growth inhibition than exposure to either agent alone. The effects of hydrogen peroxide on cell proliferation were reversed by pre-incubation with α-tocopherol.

Conclusions: As a source of oxygen free radicals, hydrogen peroxide enhances the antiproliferative effect of doxorubicin hydrochloride on a mouse bladder tumor cell line. Thus, hydrogen peroxide may be a relatively inexpensive, nontoxic method of augmenting the cytotoxicity of doxorubicin hydrochloride. Further studies are warranted to determine whether these observations may have clinical application.

KEY WORDS: bladder; bladder neoplasms; hydrogen peroxide; doxorubicin; mice, inbred C3H

Various intravesical therapeutic agents have been used for treating superficial bladder cancer. Doxorubicin hydrochloride is a commonly used clinical chemotherapeutic agent. The cytotoxicity of doxorubicin hydrochloride is achieved at least partially through the generation of free radicals with subsequent DNA damage. Using the MBT-2 murine bladder cancer cell line we demonstrated a significant increase in the inhibition of cell growth achieved by doxorubicin hydrochloride with the addition of hydrogen peroxide as a source of oxygen free radicals. Malonyl dialdehyde assay implied that lipid peroxidation was not the primary mechanism by which hydrogen peroxide decreased the growth of these cells. Preincubation of MBT-2 cells with α-tocopherol vitamin E, a potent free radical scavenger, caused reversal of the growth inhibitory effects achieved by exposure to hydrogen peroxide.

Superficial bladder cancer accounts for approximately 75% to 85% of all newly diagnosed cases of bladder cancer, which is estimated to affect more than 54,200 patients yearly in the United States.[1] Of all patients who present with superficial bladder tumors about 70% have 1 or more episodes of disease recurrence.[2] Intravesical chemotherapy has been given to treat recurrent disease, unresectable tumor or carcinoma in situ. Various agents, including triethylenethiophosphoramide or thiotepa, mitomycin C, bacillus Camille-Guerin and doxorubicin hydrochloride, have been administered to treat superficial bladder cancer but no single agent has emerged as clearly superior. Intravesical doxorubicin hydrochloride has been reported to achieve a 38% complete and a 35% partial response for superficial bladder tumors.[3] Mechanisms to potentiate the chemotherapeutic effect of doxorubicin or other intravesical agents would have a major impact on the clinical treatment of superficial bladder cancer.

Doxorubicin hydrochloride is one of a class of chemotherapeutic compounds known as the anthracyclines. To our knowledge its precise mechanism of action is not known but it is believed that 3 actions may contribute to its cytotoxicity. Anthracyclines intercalate between DNA base pairs, which results in unwinding of the DNA helix.[4] Anthracyclines inhibit topoisomerase II, which results in DNA strand breaks.[5] Moreover, anthracyclines metabolize to form free radicals, which interact with cell membranes, proteins and DNA.[6]

Therefore, it would appear that the cytotoxicity of doxorubicin hydrochloride is at least partially achieved through the generation of free radicals, which damage DNA. If one increased the availability of free radicals, the cytotoxicity of doxorubicin hydrochloride may increase. This phenomenon has already been demonstrated in human breast tumor cell lines.[7] The potential for using hydrogen peroxide to enhance the effect of systemic doxorubicin is limited by the presence of plasma peroxidase, which destroys hydrogen peroxide. However, administering hydrogen peroxide with intravesical chemotherapy is particularly attractive because of absent peroxidase in the urine.

C3H/He mice have been used to study the effects of chemotherapeutic agents on murine bladder cancer.[8–11] A specific mouse bladder tumor line designated MBT-2 has been raised that may be implanted into the bladder of C3H/He

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mice. On histological study the tumor has the appearance of poorly differentiated transitional cell carcinoma. This C3H/MBT model has been a reliable, economical way to study the effects of various intravesical agents on bladder cancer.

For many years investigators have been aware that oxygen free radicals may cause single strand breaks in DNA and induce oxidative modifications in DNA bases.[12, 13] Furthermore, it has been demonstrated that doxorubicin hydrochloride cleaves DNA by an oxygen free radical mediated process.[14–16] With this foundation, it seemed reasonable to examine whether doxorubicin hydrochloride and hydrogen peroxide would increase tumor killing in a murine bladder cancer cell line.

MATERIALS AND METHODS

Doxorubicin hydrochloride was obtained from Adria Laboratories, Columbus, Ohio. Hydrogen peroxide (30% solution) was a product of Fisher Laboratories, Suwanee, Georgia. α-Tocopherol vitamin E was obtained from Sigma Chemical Co., St. Louis, Missouri.

MBT-2 murine bladder cancer cells were grown in RPMI 1640 medium (Gibco, Grand Island, New York) supplemented with 10% fetal calf serum. Cells were maintained at 37C in an incubator with a 5% CO_2 atmosphere. All experiments were performed with cells in log phase growth. In experiments involving the addition of peroxide to cells peroxide was added to the medium to its final concentration before transfer of the medium to the cell cultures.

To determine the effect of doxorubicin hydrochloride on cell growth MBT-2 cells were plated at a density of approximately 15% of confluence in 6-well culture dishes. Cells were allowed to recover from trypsin treatment for 24 hours before any experimental manipulation. Doxorubicin hydrochloride was dissolved in 50 mM. Hepes buffer solution, pH 7.2. Drug concentration was determined from its extinction coefficient by measuring absorbance at 480 nm.[17] A stock solution of 2.5 mM. doxorubicin hydrochloride was used for all additions to cells in culture. Doxorubicin hydrochloride stock was made fresh for each experiment to minimize agent degradation.

At the initiation of doxorubicin hydrochloride treatment cells from 3 wells were frozen to provide material for determining baseline cellular protein content. Doxorubicin hydrochloride was added to the remaining wells in graded concentrations except for control cells for 2 hours. This time of drug exposure was chosen to simulate the maximum time that the agent would be retained in the bladder by patients treated with intravesical therapy. At the end of this time, medium containing the doxorubicin hydrochloride was removed and replaced by standard medium. Cells were then allowed to grow for 2 additional days. Medium was removed and the wells were washed with phosphate buffered saline (PBS) to eliminate debris and dead cells. Cells were then solubilized with 1 ml. 0.2% Triton X-100, 0.15 M. NaCl and 0.02 M. tris-Cl, pH 7.2 (Triton-TBS) in the cold for 30 minutes. Nuclei and insoluble material were removed from the samples by centrifugation for 5 minutes in an Eppendorf microcentrifuge. Supernatant from this step was assayed for protein using the BioRad protein assay kit (BioRad Laboratories, Hercules, California). Baseline cell samples that had been frozen at the start of the experiment were solubilized and assayed in similar fashion. Increased protein content of control cells over that of baseline cells was designated as 100% growth and growth of the other cell samples was referenced to this value.

To determine the effect of hydrogen peroxide on cell growth the stock solution of 30% hydrogen peroxide was diluted to a concentration of 8.8 mM. Various concentrations of peroxide were then added to RPMI 1640. MBT-2 cell growth medium was removed and peroxide supplemented RPMI 1640 was added to the cells for 2 hours. Subsequently, we assayed cell growth as described for doxorubicin hydrochloride experiments. The 30% hydrogen peroxide stock was used to make fresh solutions for each experiment. In the interim the 30% solution was stored at 4C in a light shielded container.

To assay cellular glutathione content we used a modification of the colorimetric assay of Beutler et al.[18] For these experiments MBT-2 cells were grown in 25 cm.^{2} culture flasks and were near confluence at the time of the assay. Briefly, after 2 hours of exposure to hydrogen peroxide cells were solubilized in Triton-TBS and insoluble material was removed by centrifugation. Supernatant from this step was assayed for glutathione.

To assay malonyl dialdehyde we measured malonyl dialdehyde as an index of lipid peroxidation in cells treated with hydrogen peroxide[19] using the thiobarbituric acid method of assay.[20] Cells in these experiments were again grown in 25 cm.^{2} culture flasks. After removing the growth medium cells were incubated for 2 hours in RPMI 1640 supplemented with various concentrations of hydrogen peroxide. The medium was removed and the plates were washed with PBS. Cells were then scraped from the plates and solubilized for 1 hour in 0.05% Triton X-100, 0.15 NaCl and 0.02 M. tris-Cl, pH 7.2. TCA-arsenite solution was added to aliquots of the supernatant for 15 minutes and samples were then centrifuged at 1,000 rpm in a Beckman RC-20 centrifuge for 5 minutes. Supernatant from this step was removed and 1 ml. thiobarbituric acid solution was added. The mixture was boiled for 15 minutes and we determined sample absorbance at 532 nm.

RESULTS

Figure 1 shows the effect on growth of 2 hours of exposure of MBT-2 cells to doxorubicin hydrochloride. A dose dependent inhibition of MBT-2 cell growth with a 50% decrease in proliferation was associated with 5 μM. doxorubicin hydrochloride. At high doses there was negative growth, representing actual cell killing rather than simply a blunting of cell growth.

We determined the effect of hydrogen peroxide on the growth of MBT-2 cells. Treating cells with low concentrations of hydrogen peroxide for 2 hours greatly decreased their proliferative capacity. Figure 2 shows that cells exposed to 80 μM. hydrogen peroxide had an approximately 70% growth

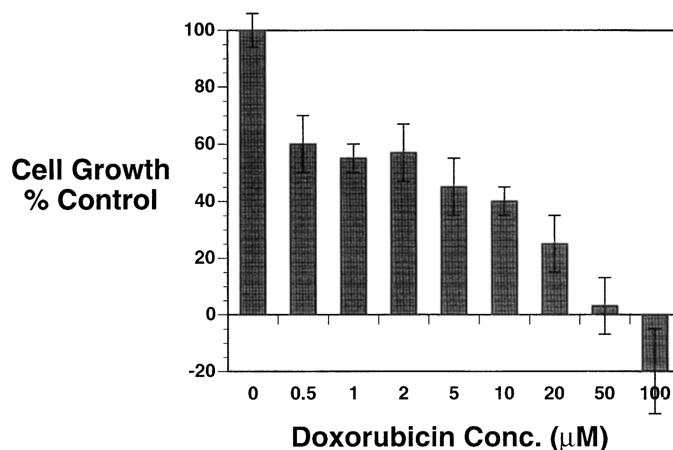

FIG. 1. Effect of doxorubicin hydrochloride on MBT-2 cell growth. Cells in log phase growth at 30% confluence were treated with various concentrations of doxorubicin hydrochloride for 2 hours in serum-free RPMI 1640 medium. Cells were washed and 1 set of 4 wells per condition was placed in standard RPMI 1640-10% fetal calf serum. After additional 48 hours of incubation cells were solubilized in detergent solution. Protein content was compared with that of parallel cells frozen at start of incubation. Increased protein content of control cells was designated 100%. Values are presented as mean plus or minus standard error of mean (SEM) for set of 4 wells.

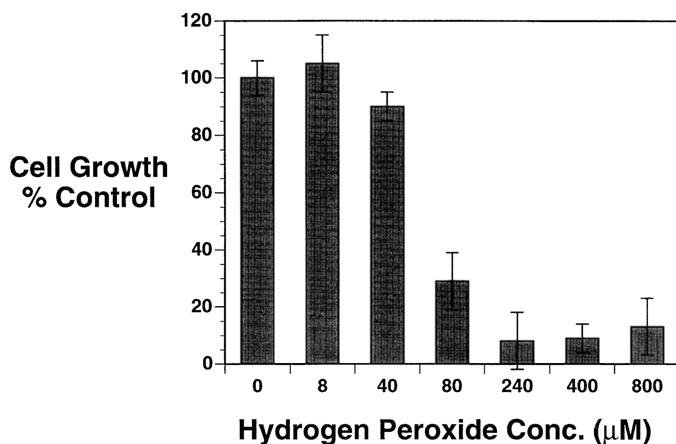

FIG. 2. Effect of hydrogen peroxide on MBT-2 cell growth. Cells were treated for 2 hours with hydrogen peroxide at various concentrations (*Conc.*) (fig. 1). Cell growth in 48 hours of incubation was normalized to that of controls.

decrease in the following 48 hours. Higher concentrations of peroxide produced even more dramatic effects. Microscopic examination of the peroxide treated cells revealed prominent vacuole formation, which was absent in controls.

Treating MBT-2 cells with combined doxorubicin hydrochloride and hydrogen peroxide resulted in cell growth inhibition that was increased over that of either agent alone. While 10 μM. doxorubicin hydrochloride decreased growth by 30%, combined 80 μM. hydrogen peroxide and 10 μM. doxorubicin hydrochloride reduced growth by 70%. Figure 3 shows the results of cell growth inhibition when exposed to doxorubicin hydrochloride as well as hydrogen peroxide.

We determined hydrogen peroxide and lipid peroxidation in MBT-2 cells. A possible mechanism by which hydrogen peroxide damages cells is by oxidant injury to vital cellular components. To determine whether significant amounts of lipid peroxidation occurred when cells were exposed to concentrations of hydrogen peroxide that substantially decreased proliferative capacity, we determined in the cells the level of malonyl dialdehyde, a product of lipid peroxidation.

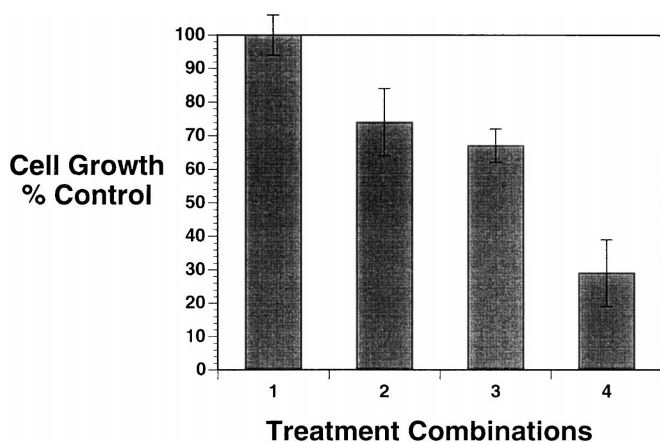

FIG. 3. Effect on MBT-2 cell growth of combined doxorubicin hydrochloride and hydrogen peroxide. Cells were grown in 24-well plates. Serum-free RPMI 1640 medium supplemented with 80 μM. hydrogen peroxide, 10 μM. doxorubicin hydrochloride or combination was added to cells for 2 hours. Medium was removed and cells were washed with fresh RPMI. Cells were incubated for 24 additional hours in standard RPMI 1640-10% fetal calf serum. Cells were washed twice in PBS and solubilized in detergent solution. Insoluble material was removed by centrifugation and supernatant protein content was determined. Control cell growth was considered 100%. Values are presented as mean plus or minus SEM for 4 sets of wells. *1*, controls. *2*, vitamin E. *3*, H_2O_2. *4*, vitamin E and H_2O_2.

Figure 4 shows the level of malonyl dialdehyde and the rate of cell growth plotted against hydrogen peroxide concentration, indicating that cell growth retardation occurred at hydrogen peroxide concentrations that produced negligible increases in the degree of lipid peroxidation in the cells. These data imply that lipid peroxidation is not the primary mechanism by which hydrogen peroxide decreases growth in these cells.

One must be aware of several caveats when interpreting these data. While the thiobarbituric acid assay is sensitive to malonyl dialdehyde, the reaction is not specific for this compound. For example, certain sugars and amino acids also reach to produce chromophores in the thiobarbituric acid assay. Therefore, the test tends to overestimate malonyl dialdehyde in the cells. The fact that the thiobarbituric acid assay produced little color change in cells exposed to levels of hydrogen peroxide that significantly inhibited cell growth makes global lipid peroxidation unlikely to be a cause of observed cell toxicity.

However, the possibility remains that damage to particular cell membranes may be responsible for the long-term effect on cell growth. For instance, peroxidation of mitochondrial membranes would significantly decrease cell proliferative capacity without changing the content of malonyl dialdehyde, as measured by whole cell extracts. This question may best be approached by fractionation of the cells into their specific components and examination of each component for peroxidative damage. These cautions when interpreting the mechanism by which hydrogen peroxide produces toxic effects in MBT-2 cells should not detract from the fact that the effect was obtained using low concentrations of the compound. Increased sensitivity of the cells to hydrogen peroxide may reflect deficiencies in the metabolism of malignant cells that may make them more susceptible to oxidant injury.

We also determined hydrogen peroxide and glutathione levels in MBT-2 cells. Glutathione peroxidase with catalase is one of the key means by which cells defend themselves against the toxic effects of hydrogen peroxide. To determine whether the levels of peroxide used in our study led to cell damage through the depletion of glutathione levels cell extracts were assayed for decreased glutathione. In cell extracts obtained from equal numbers of control cells and cells treated with 140 μM. hydrogen peroxide, which caused 70% growth inhibition in this particular experiment, the concentration of decreased glutathione was 0.09 and 0.11 μM., respectively. These data indicate that decreased glutathione is not depleted in MBT-2 cells treated with toxic levels of hydrogen peroxide.

We assessed free radical scavengers and hydrogen perox-

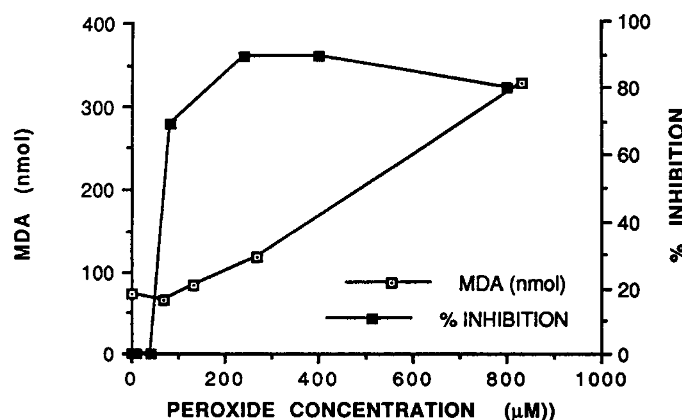

FIG. 4. Production of malonyl dialdehyde (*MDA*) in cells treated with various concentrations of hydrogen peroxide and assayed for malonyl dialdehyde using described techniques. Cell growth was determined at each concentration of hydrogen peroxide. As determined by protein assay, equal amounts of cellular material were used in evaluation of malonyl dialdehyde production.

ide toxicity. Our hypothesis was that hydrogen peroxide causes oxidant damage to MBT-2 cells, leading to growth retardation and cell death. If this hypothesis is correct, free radical scavengers should provide some protection against cell damage since free radicals, such as the hydroxyl radical, are believed to be important effectors in oxidant injury to cells.

To this end MBT-2 cells were incubated for 24 hours in medium supplemented with 100 μM. α-tocopherol vitamin E, a potent free radical scavenger. At the end of this time cells were placed in RPMI 1640 containing 160 μM. hydrogen peroxide for 2 hours. After this treatment cells were washed and placed into standard medium for 48 hours, at which time a protein assay of the samples was performed. Figure 5 shows that supplementing the cells with vitamin E completely blocked the growth inhibitory effects of hydrogen peroxide. These data are consistent with hydrogen peroxide causing cell toxicity through the generation of free radicals.

DISCUSSION

Free radicals have been evoked as a mechanism involved in a myriad of biological processes. Aging, carcinogenesis, adult respiratory distress syndrome and reperfusion injury have been partially explained by the generation of free radicals.[21–27] We investigated whether hydrogen peroxide as a source of free radical generation would enhance the cytotoxicity of doxorubicin hydrochloride. As a member of a class of chemotherapeutic compounds known as anthracyclines, doxorubicin hydrochloride has several possible mechanisms of action, including intercalation to DNA, inhibition of topoisomerase II, free radical formation and cell membrane binding.

Our data demonstrate that as a source of free oxygen radicals, hydrogen peroxide only effects significant inhibition of cell growth in the MBT-2 cell line, which reaches a maximum at a hydrogen peroxide concentration of 240 to 400 μM. (fig. 2). However, when hydrogen peroxide was added to doxorubicin hydrochloride, the cell growth inhibition achieved was greater than with either agent alone (fig. 3). When 80 μM. hydrogen peroxide was added to various concentrations of doxorubicin hydrochloride, the effect of cell growth inhibition of all concentrations of doxorubicin hydrochloride tested was potentiated.

A mechanism by which free radicals damage cells is by oxidant injury to vital cellular components. Malonyl dialdehyde, a product of lipid peroxidation, was measured as an index of the degree of lipid peroxidation in the cells. We observed that growth inhibition of the MBT-2 cells occurred at concentrations of hydrogen peroxide that caused negligible increases in the degree of lipid peroxidation. As mentioned, this finding implies that lipid peroxidation is not the primary mechanism by which hydrogen peroxide causes cell growth inhibition in the MBT-2 cell line.

α-Tocopherol, vitamin E, is a well known free radical scavenger. If the mechanism of enhanced doxorubicin cytotoxicity is mediated by free radical damage, it would be expected that free radical scavengers would confer some degree of protection against cell damage. We observed that MBT-2 cells incubated with 100 μM. α-tocopherol for 24 hours before exposure to hydrogen peroxide were completely protected from the growth inhibitory effects of hydrogen peroxide.

Mild oxidant stress, such as that produced by hydrogen peroxide, induces apoptosis in many cell lines.[28, 29] In addition, many chemotherapeutic agents, including anthracyclines, kill tumor cells by a process that involves apoptosis.[30] An intriguing possibility to explain our finding that hydrogen peroxide treatment enhances doxorubicin mediated killing of MBT-2 cells is that the agents combine their effects for inducing apoptosis in these cells. Since apoptosis is the common mechanism of cell death, a dual approach to inducing apoptosis would lessen the likelihood of cell resistance. The approach of using an oxidant, such as hydrogen peroxide, and a chemotherapy agent is uniquely applicable to the bladder. This avenue deserves further investigation.

CONCLUSIONS

Hydrogen peroxide, a known source of oxygen free radicals, appears to potentiate the growth inhibitory effects of doxorubicin hydrochloride in a murine bladder cancer cell line. The precise mechanism of cellular damage requires further investigation, although our data imply that lipid peroxidation is not involved to a significant degree. α-Tocopherol, a free radical scavenger, reverses the effect of hydrogen peroxide on cell growth inhibition, suggesting that free radicals at least partially mediate the phenomenon observed. Hydrogen peroxide may provide a relatively inexpensive, nontoxic method of augmenting the cytotoxicity of doxorubicin hydrochloride. Additional studies are planned to examine further the relationship of free radical damage and doxorubicin hydrochloride toxicity.

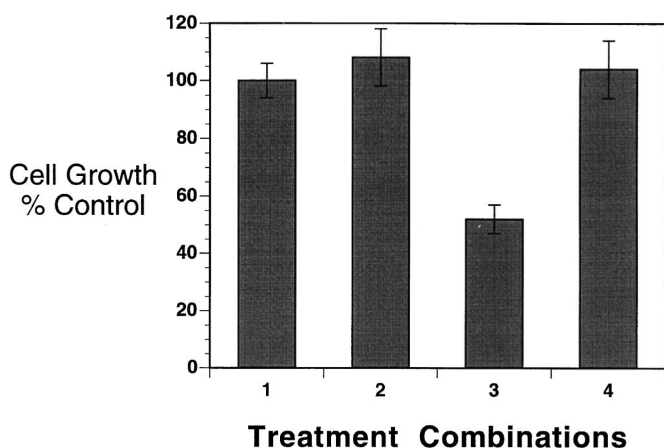

FIG. 5. Reversal of hydrogen peroxide growth inhibition by vitamin E. Cells were pretreated for 24 hours in medium supplemented with 100 μM. vitamin E. Control cells and cells treated with vitamin E were exposed to 140 μM. hydrogen peroxide for 2 hours in serum-free medium. At end of this treatment they were allowed to grow for 48 additional hours. Protein content was determined as described with change in controls from baseline considered 100%. Values are presented as mean plus or minus SEM for 4 sets of wells. *1*, controls. *2*, vitamin E. *3*, H_2O_2. *4*, vitamin E and H_2O_2.

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