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HYDROGEN PEROXIDE FORMATION BY CELLS TREATED WITH A TUMOR PROMOTER

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To determine whether oxidants capable of DNA modification are produced by cells treated with tumor promoters, we adapted a fluorometric method to our needs. HeLa cells were preincubated with 2',7'-dichlorofluorescin diacetate (DCFdAc), treated with various agents, sonicated, centrifuged and fluorescence of the oxidized product (DCF) was determined in supernatants. When cells were exposed to H_2O_2 in the presence of azide (catalase inhibitor) or *o*-phenanthroline (a lipophilic Fe chelator), an increase in fluorescence was observed. These results show that some Fe ions were interacting with the H_2O_2 which entered the cells, thus decreasing its levels available for oxidation of the substrate and potentially increasing formation of ·OH, known DNA-damaging species. Glutathione (GSH), which is present in cells in substantial amounts, was found to reduce DCF whereas azide counteracted GSH-mediated reduction.

Treatment of HeLa cells with 12-0-tetradecanoyl-phorbol-13-acetate (TPA) in the presence of DCFdAc and azide resulted in dose- and time-dependent formation of DCF. Even when cells were sonicated prior to incubation with TPA, DCF was formed at levels proportional to the number of cells as well as dose of TPA. Flow cytometry of TPA-treated cells confirmed these findings.

These results demonstrate that tumor promoters can cause oxidative activation of HeLa cells, which produce active oxygen species, most likely H_2O_2, that ultimately contribute to the formation of oxidized bases such as 5-hydroxymethyl uracil in cellular DNA. They also show that this fluorometric method can be utilized for determination of cellular H_2O_2 formation at nM concentrations.

KEY WORDS: H_2O_2, oxidized DNA bases, iron, fluorescence, HeLa cells, tumor promoters.

ABBREVIATIONS: OS - oxygen species; ·OH - hydroxyl radicals; $\cdot O_2^-$ - superoxide anion radicals; HMdU - 5-hydroxymethyl-2'-deoxyuridine; TPA - 12-0-tetradecanoyl-phorbol-13-acetate; PMNs - polymorphonuclear leukocytes; DCFdAc - 2',7'-dichlorofluorescin diacetate; DCFH - 2',7'-dichlorofluorescin; DCF - 2',7'-dichlorofluorescein; HRPO - horseradish peroxidase; GSH - glutathione.

INTRODUCTION

It has been known for some time that ionizing radiation causes formation of active oxygen species (OS), in particular hydroxyl radicals (·OH), which cause heterogeneous DNA damage in a variety of cells.[1-9] Recent publications show that also chemical carcinogens[9-15] as well as tumor promoters[15-21] can induce oxidative DNA modification, which includes DNA strand breaks and oxidation of DNA bases. Such findings indicate that some type(s) of OS has(have) been formed in response to the treatment. Since formation of oxidative DNA damage can de decreased by catalase, H_2O_2 is a likely mediator of such damage.

For example, our laboratory found that treatment of rat liver microsomes with a number of carcinogenic polycyclic aromatic hydrocarbons induced production of

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superoxide anion radicals ($\cdot O_2^-$) and H_2O_2.[13,22] When DNA was exposed to the benzo(a)pyrene-treated microsomes, oxidation of bases occured, as evidenced by the presence of at least two base derivatives 5-hydroxymethyl-2′-deoxyuridine (HMdU) and thymidine glycol. Catalase significantly inhibited formation of that damage. Exposure of HeLa cells to 12-O-tetradecanoyl-phorobl-13-acetate (TPA)-stimulated human polymorphonuclear leukocytes (PMNs) resulted in formation of HMdU in HeLa cell DNA.[15,21] Again, addition of catalase prevented formation of HMdU in cellular DNA, suggesting H_2O_2 generated during the oxidative burst of tumor promoter-activated PMNs as the mediator of oxidative damage. Interestingly, when HeLa cells were treated with TPA in the absence of PMNs, HMdU was also formed in their DNA, although at much lower levels than in the presence of PMNs. This finding shows that tumor promoters can induce oxidative activation of cells, which leads to the modification of DNA bases; the same type of modification which required the presence of H_2O_2 in other experimental systems.

Although the evidence is strong, we examined whether H_2O_2 is actually generated by tumor promoter-treated HeLa cells.[23] There are a number of methods available for the determination of H_2O_2 formation, however, they either require rather high concentrations of externally-present H_2O_2 or use of expensive equipment such as a flow cytometer.[20,24–28] Since the levels of H_2O_2 formed by TPA-induced processes within cells were anticipated to be low (nM), we set out to adapt a very sensitive fluorometric method for this assay. This report presents the results of our work and discusses the influence of cellular catalase, iron and glutathione on the measurements, as well as the advantages and disadvantages of this particular method.

MATERIALS AND METHODS

Preparation of Substrates

A stock solution (10^{-4} M) of 2′,7′-dichlorofluorescin diacetate (DCFdAc) (Kodak) was prepared in ethanol. When needed, DCFdAc was deacetylated just prior to use by treatment with NaOH.[25] Commercially-available, non-specific esterase (Type I from porcine liver, Sigma) also effectively deacetylates DCFdAc at room temperature during a 20 min incubation in PBS buffer (unpublished data).

DCFdAc and its deacetylated derivative [2′7′-dichlorofluorescin (DCFH)] are both non-fluorescent. DCFH is easily oxidized to a fluorescent derivative 2′,7′-dichlorofluorescein (DCF) in a peroxidase-mediated reaction.[25] This property was utilized as the basis for determination of H_2O_2. Two approaches were taken. First, pretreatment with DCFdAC leads to uptake of this lipophilic compound by cells, where it is deacetylated to DCFH by cellular esterase.[26] DCFH is trapped inside because it is more polar than diacetate and cannot permeate the membrane. If H_2O_2 is present in the cells, it should oxidize that DCFH to fluorescent DCF utilizing cellular peroxidase as a mediator. In the second approach, cells were sonicated first, and the sonicates were incubated with TPA in the presence of horseradish peroxidase (HRPO) and DCFH obtained by NaOH- or esterase-mediated deacetylation of DCFdAc. Figure 1 shows a schematic representation of the reactions involved. H_2O_2 is shown as the oxidizing species since it has been previously documented that it participates in that reaction. Although some authors point to the possibility that other OS might also react in a similar manner, they either can form H_2O_2 (i.e., $\cdot O_2^-$) or can be produced

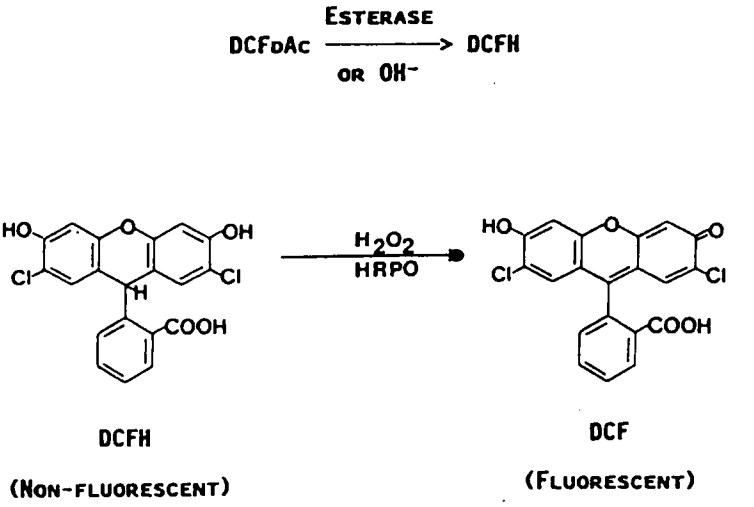

FIGURE 1. Schematic representation of reactions involved in the formation of flourescent DCF from non-fluorescent DCFdAc and DCFH.

from H_2O_2 (i.e. ·OH). For these reasons we will continue to refer to H_2O_2 as OS responsible for DCF formation.[27]

Treatment of Cells

HeLa cells were grown in suspension cultures in a Minimum Essential Medium (Joklik-modified) supplemented with 5% heat-inactivated newborn calf serum (GIBCO), 1% L-glutamine and 1% antibiotics.[3] Cells were washed twice in PBS, resuspended, then preincubated with DCFdAc and/or sodium azide, followed by treatment with TPA in the presence of human plasma or with H_2O_2. After addition of catalase, the cells were sonicated at 4° C (Heat System-Ultrasonic, Inc., Model W-385; using micro-tip: level 3, 60% duty, cycle time 5 sec, total time 50 sec, repeated twice) and centrifugated, and the fluorescence of the supernatants was determined at λ_{exc} = 475 nm and λ_{emiss} = 525 nm,[25] using a Farrand Model MK-2 spectrofluorometer. Cell viability was determined by trypan blue exclusion before and after the appropriate treatments.

In the experiments in which cells were analyzed by FACSCAN (Becton-Dickenson), HeLa cells were preincubated with DCFdAc and azide, in the presence of 5 mM EDTA and 100 μg BSA/ml PBS, followed by TPA treatment. Ten thousand cells per data point were evaluated using 488 nm emission of the argon laser operating at 200 to 500 milliwatts.

In some experiments, HeLa cells were sonicated first and then treated with TPA in the presence of azide. H_2O_2 formation was determined using DCFH[25] (obtained either from esterase- or alkali-activated DCFdAc) as a substrate for HRPO-mediated oxidation. After centrifugation, fluorescence of supernatants was determined as above.

RESULTS

Development of the Fluorometric Assay

Effect of azide on DCF formation by cells When HeLa cells were incubated with DCFdAc and up to 200 μM of H_2O_2, there was no increase in fluorescence over controls, unless cells were preincubated with sodium azide, a catalase inhibitor (Figure 2). In the presence of azide, incubation with increasing concentrations of H_2O_2 resulted in increased formation of DCF (Figure 3), as measured by the fluorometric method described above. The enchancement of fluorescence was proportional to the concentration of azide used for preincubation of cells (not shown).

Reduction of DCF by glutathione (GSH) We found that virtually all DCF can be recovered when it is co-incubated with untreated HeLa cells at 37° C, followed by centrifugation. However, we wanted to determine whether the product of oxidation DCF could be reduced back to the non-fluorescent DCFH by some cellular reductants. To test for this possibility, DCF [at a concentration approximately equivalent to the amount (pmoles) of H_2O_2 expected to be formed by cells treated with TPA] was incubated with approx. 4 mM GSH [level commonly present in cells[29]]. Figure 4 shows

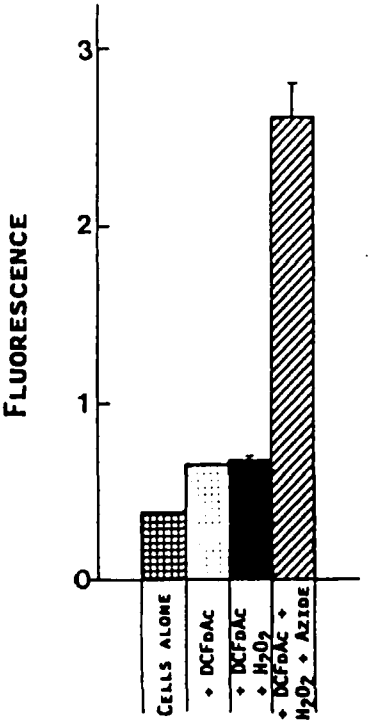

FIGURE 2. Effect of azide on DCF formation by HeLa cells. 3 × 10^5 Cells/ml PBS were preincubated either with PBS alone or with 10 μM DCFdAc in the absence or presence of 10 mM azide for 15 min at 37° C. Samples that required treatment with H_2O_2 were incubated with 10 μM H_2O_2 for the additional 15 min.

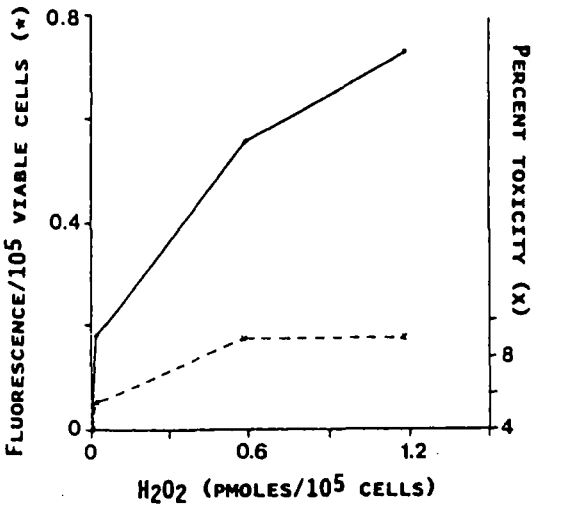

FIGURE 3. Effect of externally-applied H_2O_2 on DCF formation by HeLa cells. 4.25 × 10^6 Cells/ml PBS were preincubated with 2.1 μM DCFdAc and 5 mM azide for 15 min, and then treated with 1–50 nM H_2O_2 at 37° C for 45 min. After removal of remaining H_2O_2 with catalase and of cells by centrifugation, fluorescence of the supernatant was determined. (—) DCF formation; (- - -) toxicity as determined by trypan blue exclusion.

that under these conditions, DCF can be reduced by GSH. That reduction was enhanced by the increased concentration of GSH and incubation time (not shown). It is interesting to note that azide counteracted the GSH-mediated reduction of DCF (Figure 4). Hence, the increased fluorescence in cells pretreated with azide and incubated with H_2O_2 was not only due to the inhibition of catalase but probably also to the prevention of DCF reduction by cellular GSH.

Influence of intracellular iron on DCF formation H_2O_2 can react not only with the substrate DCFH, but with other cellular molecules as well,[20,30-33] which would lead to an apparent lowering of DCF formation. In particular, Fe(II) ions and some Fe-containing macromolecules are known to interact with H_2O_2. To establish whether cellular Fe decreases formation of DCF, Hela cells were pretreated with *o*-phenanthroline [a lipophilic Fe chelator that can migrate through the membranes[34]] in the absence of azide. Figure 5 shows that in the presence of *o*-phenanthroline (no azide), increasing levels of DCF were formed in an incubation time- and H_2O_2-dependent manner. Although the *o*-phenanthroline-mediated increase was much smaller than in the presence of azide (not shown), it indicates that some chelatable Fe(II) interacted with the H_2O_2 which entered the cells, thus decreasing its levels available for oxidation of the substrate DCFH. Moreover, such an interaction of H_2O_2 with Fe(II) may generate ·OH in a Fenton reaction,[18,31,32] the OS that are known to cause oxidation of DNA bases and formation of strand breaks.

TPA-Induced Formation of H_2O_2 by Cells

Determination by fluorometric measurements Table I shows that when HeLa cells

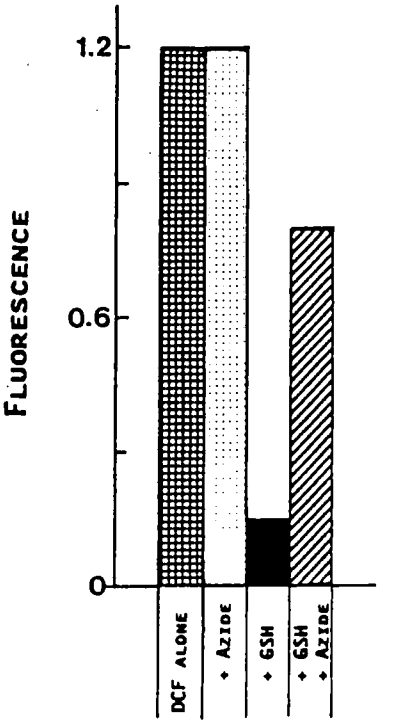

FIGURE 4. Reduction of DCF by GSH and protection by azide. DCF (8.3 nM) was incubated either alone or with GSH (4.2 mM) in the absence or presence of azide (8.3 mM) for 40 min at room temperature.

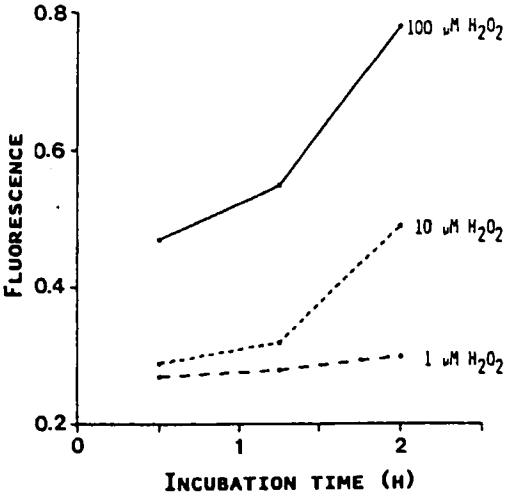

FIGURE 5. Effect of *o*-phenanthroline, an Fe chelator, on DCF formation in cells. 4×10^5 HeLa cells/ml PBS were preincubated with 10 μM DCFdAc and with 100 μM *o*-phenanthroline for 15 min at 37°C, then with H_2O_2 for an additional 15, 60 and 105 min. Control cells were incubated with DCFdAc alone and had a fluorescence of 0.23.

TABLE I
TPA-Induced formation of H_2O_2 in HeLa cells[a]

	TPA pmoles/10^5 cells	Fluorescence per 10^5 cells	% Increase over control[b]
A. DCFdAc for 15 min followed by TPA for 45 min			
C1	0	0.389	—
S1	2	0.516	33
S2	10	0.983	153
B. TPA for 30 min followed by DCFdAc for 15 min			
C2	0	0.329	—
S3	2	0.467	42
S4	10	0.788	140

[a]5 × 10^5 HeLa cells/ml were incubated with 10 or 50 nM TPA at room temperature in the presence of 0.5 μ DCFdAc, 25 mM azide and 8% human plasma. Blank (without DCFdAc) had a fluorescence of 0.22.
[b]Controls contained everything except TPA.

were incubated with TPA in the presence of DCFdAc, azide and human plasma, OS (most likely H_2O_2) were generated, the species that were capable of forming fluorescent DCF. Formation of DCF was increased by treatment with a higher concentration of TPA. This table also shows that the increase in fluorescence was comparable, regardless of whether DCFdAc or TPA was added first, provided the cells were incubated with TPA for the same period of time. Human plasma was included in these experiments because its components may affect the oxidative metabolism of cells and a consequent DNA damage.[18]

Figure 6 shows that formation of DCF depends not only on the TPA concentration but also on the number of cells exposed to that TPA. Interestingly, it also shows that (at the same amount of TPA per the same number of cells, i.e. 20 pmoles/10^5 cells), a lower concentration of TPA may be more effective when distributed among fewer

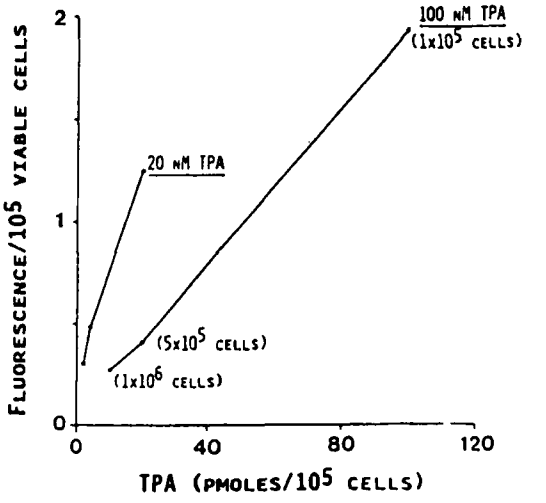

FIGURE 6. Effects of cell number and TPA concentration on DCF formation. Samples containing various numbers of HeLA cells [(1 – 10) × 10^5]/ml PBS were preincubated with 6% human plasma containing EDTA and 25 mM azide for 5 min, then with 0.5 μM DCFdAc for 15 min, followed by treatment with 20 or 100 nM TPA for 30 min at 37° C.

cells (i.e. 20 nM TPA/1 × 10^5 cells/ml) than a higher TPA concentration distributed among more cells (i.e. 100 nM TPA/5 × 10^5 cells/ml). Although viability, as measured by trypan blue exclusion, was similar (approx. 90%) at these two TPA concentrations, apparently, a higher TPA concentration exerted a more toxic effect, as measured by intracellular formation of H_2O_2 and DCF.

Determination by flow cytometry To corroborate the results obtained in the experiments in which formation of H_2O_2 was determined by measuring fluorescence after TPA-treated cells were sonicated, similar determinations were carried out on the intact cells using FACSCAN. This type of flow-cytometric method has been used recently by other investigators.[27,28] They determined the presence of H_2O_2 (or other OS) in non-phagocytic cells such as renal epithelical cells treated with external OS sources[27] or in keratinocytes treated with TPA for 6–24 h.[28] In contrast, our results show (Figure 7) that the amount of H_2O_2 formed in HeLa cells treated with increased TPA concentrations was enhanced within a 45 min incubation at room temperature.

H_2O_2 Formation by TPA-treated Sonicates of HeLa Cells

Seeing that HeLa cells respond with H_2O_2 formation to treatment with tumor promotor TPA, we decided to determine whether disrupted cells can also generate H_2O_2. In these experiments, cells were sonicated first, followed by addition of azide, HRPO and DCFH, and incubated with TPA. Table II shows that when sonicates containing equivalents of an increasing number of cells were incubated with 15 nM TPA, there was increased DCF fromation. Similar to intact cells, production of DCF by sonicates was dependent on the concentration of TPA (Table III).

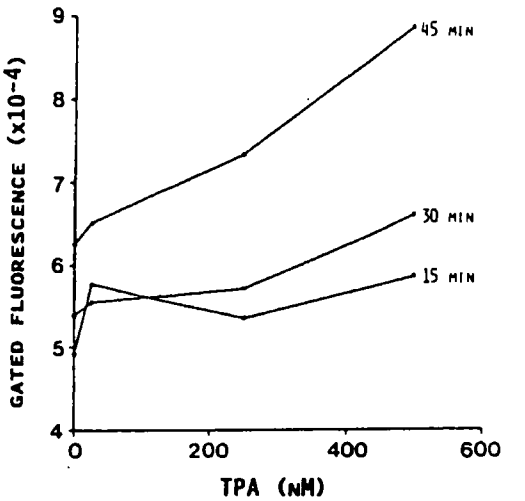

FIGURE 7. Effect of TPA on H_2O_2 formation by HeLa cells. 1.6 × 10^6 HeLa cells/ml PBS containing 5 mM EDTA and 100 μg BSA were preincubated with 10 mM azide for 30 sec, then 25 μM DCFdAc was added followed by treatment with 0–500 nM TPA for 15, 30 and 45 min at room temperature. Analyses were carried out by FACSCAN.

TABLE II
Influence of cell number on TPA-mediated H_2O_2 formation by Sonicates[a]

Number of cells/ml ($\times 10^{-5}$)	Fluorescence	% Increase over blank[b]
0.15	0.7	11
1.5	3.1	50
3.0	5.1	82
7.5	11.8	190
15.0	20.8	335

[a]Cells were sonicated, the equivalents of (0.15–15) $\times$ 10^5 cells were distributed to test tubes and, after addition of 0.4 mM azide, 75 nM DCFH (esterase-activated DCFdAc) and 30 μg/ml HRPO, sonicates were treated with 15 nM TPA at 37C for 20 min.
[b]Blank contained everything except cells.

TABLE III
Effect of TPA on H_2O_2 formation by sonicates[a]

Concentration of TPA (nM)	Fluorescence	% Increase over control[b]
12.5	1.0	40
25	2.1	84
50	3.3	132

[a]Sonicates of HeLa cells (the equivalent of 5 $\times$ 10^5 cells/ml) were treated with 12.5 to 50 nM TPA in the presence of 4 nM DCFH and 0.5 mM azide at 37°C for 20 min.
[b]Control contained everything except TPA.

DISCUSSION

The results presented in this report demonstrate that formation of even very low levels of H_2O_2 can be measured by using a simple fluorometer. Externally-applied H_2O_2 can be determined at nM concentrations using as little as a pmole of H_2O_2 per 10^5 HeLa cells (Figure 3). However, the measurements have to be carried out in the presence of azide, because otherwise the cellular catalase would destroy the H_2O_2. There is an additional benefit to using azide. We found that azide also inhibits reduction of DCF (the fluorescent producet of H_2O_2 oxidation) to the non-flourescent derivative; a reduction that is mediated by GSH, a cellular antioxidant (Figure 4).

Iron also can decrease formation of DCF by H_2O_2 because it can reduce H_2O_2 to ·OH, potent oxidizing agents that cause various types of damage,[31,32] including DNA.[15,18] We showed that this is the case by utilizing *o*-phenanthroline, an Fe(II)-chelating agent that can cross through cellular membranes[34,35] (Figure 5). The involvement of Fe in H_2O_2-mediated cellular damage to DNA was described before,[34] however, this work shows that the levels of H_2O_2 present in cells are actually decreased by chelatable Fe(II). Although copper ions can be chelated by *o*-phenanthroline as well, such copper chelates decompose H_2O_2 even more readily than Cu ions alone.[34–36] This is in contrast to the Fe(II)-*o*-phenanthroline complex which does not react with H_2O_2.

Treatment of cells with the tumor promoter TPA induces pleotropic biochemical changes. It also mediates formation of HMdU in cellular DNA.[15,21] This is the same thymidine derivative produced by ·OH that were generated by ionizing radiation[5–7] or through the interaction of Fe(II) with H_2O_2.[15,18,20,22] That H_2O_2 may be produced

by cells treated with TPA and cause DNA damage is indicated by the fact that externally-applied or -formed H_2O_2 was shown to be responsible for such damage.[15,16,21] In this work we found that H_2O_2 is formed by TPA-treated HeLa cells as determined by spectrofluorometric measurements. Only 0.1–1 $\times$ 10^6 cells were needed to see the effects of 10 to 100 nM of TPA (Table I, Figure 6), whereas 1–2 $\times$ 10^6 cells and 25 to 500 nM of TPA were required for flow cytometry (Figure 7). During the course of this work we also found that TPA could induce H_2O_2 formation even in cells disrupted by sonication. A comparison of the results presented in Tables I and III suggests that there were similar increases in H_2O_2 formation at similar TPA concentrations when the same number (5 $\times$ 10^5) of intact or sonicated HeLa cells were used. In the case of intact cells, there was a 33% and 42% increase over controls with 10 nM of TPA, and 153% and 140% with 50 nM of TPA (Table I). In the sonicated cells, 12.5 nM of TPA induced a 40% increase over the control, whereas 50 nM of TPA enhanced it by 132% (Table III).

This method appears to have several advantages for determining which cell types are capable of H_2O_2 generation in response to the action of tumor promoters as well as chemical carcinogens, such as benzo(a)pyrene, N-hydroxynaphthylamine and 2-nitropropane that were shown to cause oxidative DNA damage.[9–15] However, there are some limitations that will have to be overcome before this method can be used reproducibly and quantitatively. The first shortcoming is that the background fluorescence is often quite high, even in the flow cytometric determinations, as is evident in Figure 7. This is probably due to the facile oxidation of the substrate DCFH by cellular H_2O_2, as shown by fluorescence produced in the absence of TPA. There is likely to be increased H_2O_2 in our experiments because of the presence of azide, which prevents cellular catalase from keeping it at a normally-low, steady-state level.[37] This background fluorescence may also be a result of other cellular processes that cause oxidation of DCFH and reduction of DCF. Hence, the flourescence in the absence of TPA is likely to represent the cumulative effects of the experimental conditions that are present at the time of TPA addition.

The inconsistent effect of TPA on HeLa cells was another problem encountered, as measured by this fluorometric method. We have no explanation for this as yet. It is possible, however, that this could be due to the use of cells at different stages of growth. We are in the process of determining whether the growth rates of cells have an effect on the TPA-mediated formation of H_2O_2. These limitations notwithstanding, this fluorometric method could be successfully utilized to measure cellular production of H_2O_2 under different experimental conditions, at least qualitatively if not quantitatively. The effects of different agents can be compared and the influence of time and concentration established. Moreover, it can be used whenever formation of H_2O_2 is suspected but, until now, not established.

Acknowledgements

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