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BENZOYL PEROXIDE ACTS AS A PROMOTER OF RADIATION INDUCED MALIGNANT TRANSFORMATION IN VITRO

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The experiments reported here demonstrate that benzoyl peroxide (BP) can promote radiation induced transformation in vitro. BP is shown to be capable of generating free radicals, determined by the kinetics of hydroxylation as measured by fluorescence of coumarin-3-carboxylic acid. Although the mechanisms involved in the BP enhancement of radiation transformation are unknown, we hypothesize that lipid peroxidation produced by benzoyl radicals in the vicinity of membrane associated unsaturated lipids could contribute to the promotion of transformation in vitro.

Benzoyl peroxide (BP) is known to be a promoter of skin carcinogenesis induced by the chemical carcinogen, 7, 12-dimethylbenz(a)anthracene in two-stage carcinogenesis studies in mice (1). Similar two-stage carcinogenesis studies can be performed in in vitro transformation systems (2). In both in vivo (examples are given in refs. 3-5) and in vitro (an example is given in ref. 6) two-stage carcinogenesis systems, radiation can act as the initiating agent. The studies discussed here were performed with C3H10T1/2 cells, a line of mouse embryo-derived fibroblasts that can be used for two-stage carcinogenesis studies in vitro. In this cell system, both radiation and chemical carcinogens can initiate transformation in vitro and a variety of different chemical agents can act as promoters of transformation in vitro (reviewed in reference 2). Experiments were performed to determine whether BP could promote transformation in vitro initiated by radiation.

As part of the studies reported here, we performed some experiments designed to demonstrate the ability of BP to produce free radicals. Trace metal reaction with peroxides can produce free radicals; this is referred to as Fenton Chemistry. Fenton Chemistry, discovered about 100 years ago, is very important in the understanding of hydroperoxide (H_2O_2) and peroxide toxicity. The classical Fenton reaction is:

$$Fe^{2+} + H_2O_2 + H^+ \longrightarrow Fe^{3+} + H_2O + {}^{\cdot}OH \qquad (1).$$

$^{\cdot}OH$ can be detected by 3-carboxylic acid (3-CCA) (7). This molecule is not fluorescent itself, but after $^{\cdot}OH$ radical attack, it becomes hydroxylated and a fluorescent product, identified as 7-hydroxy-coumarin-3-carboxylic acid (7-HCCA), can be detected (7).

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We have utilized 3-CCA in our studies on the production of free radicals by BP. Although the mechanisms(s) by which BP could lead to the production of 'OH is not known, we speculate that 'OH could be produced by the reactions shown below. It is assumed that BP could participate in a Fenton reaction and lead to the production of the following radicals (8, 9):

$$Fe^{2+} + (BzlCO)OO(OCBzl) + H^{+} \longrightarrow Fe^{3+} + BzlCOO^{\cdot} + BzlCOOH \quad (2).$$

$$BzlCOO^{\cdot} \longrightarrow Phenyl^{\cdot} + CO_2 \quad (3).$$

It is expected that the organic radicals, produced according to Equations 2 and 3, will react with Fe^{3+} to produce additional Fe^{2+}. The reaction of Fe^{2+} and O_2 yields superoxide and, eventually, H_2O_2. According to the Fenton reaction shown in Equation 1, H_2O_2 will then lead to the production of 'OH and resultant hydroxylation of 3-CCA.

This is a schematic representation of one of the possible ways coumarin reactive 'OH radical is produced. The actual chemistry of the hydroxylation mechanism could be considerably more complex. Additional chemical reactions can occur, but their production and lifetime are beyond the subject of this paper.

BP is insoluble in water (10); therefore, after addition to the cell culture, it is likely that it becomes associated with the plasma membrane. This association, together with transient metals present in the membrane and membrane-associated proteins, would result in BzlCOO' and the phenyl' radical generation by way of Fenton Chemistry (Eq. 2, 3). The reaction of BzlCOO' or the phenyl' radical with adjacent conjugated single or double bonds of phospholipid acyl chains will produce lipid peroxidation and other toxic intermediates.

Materials and Methods

The C3H10T1/2 assay system was originally developed by Reznikoff et al. (11, 12) and adapted for studies of two-stage transformation in vitro (2). Cultures of C3H10T1/2 cells are maintained by keeping cells in exponential growth in 60 mm Petri dishes: at 7 day intervals, the cultures are trypsinized and subcultured to low cell density. Cells in passages 9 through 14 were used in these studies. C3H10T1/2 cells are grown in Eagle's basal medium supplemented with 10% fetal bovine serum and gentamycin in a 37°C humidified--5% CO_2 atmosphere. The cells are irradiated with a 100 kV Phillips M6-100 industrial x-ray machine operating at 9.6 mA which gives a dose rate to the cells of 0.78 Gy/minute. Both 1 Gy and 4 Gy doses of radiation were used in these studies. Cells were exposed to the radiation doses approximately 24 hours after they were seeded into the dishes.

The C3H10T1/2 transformation assay system involves a 10 day period after cells have been exposed to the carcinogenic agent in which the cells are in exponential growth and then a 4 week period during which cells are in confluence. Once the cells have reached confluence, the concentration of fetal bovine serum in the medium is reduced from 10% to 5% and is then maintained at 5% during the remainder of the transformation assay period. It has previously been shown that promoting agents, such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA), enhance transformation in vitro when they are added to cultures during the 10 day-two week period during which cells are exponentially growing (13). To determine whether BP could promote transformation in vitro, it was added to cultures two times per week for the first two weeks of the transformation assay when the cells were actively growing. To determine whether agents can promote radiation transformation in vitro, the highest non-toxic dose of the agent is determined in preliminary toxicity studies. For BP, the highest non-toxic dose of the compound when assayed as a single dose was found to be 10^{-7} M.

It is planned that there will be approximately 300 cells per dish in the dishes to be utilized for determination of the yield of transformed foci. As 300 cells per dish are not a countable number of cells, plating efficiencies are determined from dishes which contain one-fifth the number of cells to be utilized for the transformation assay dishes, or approximately 60 cells per dish. The

dishes to be used for determination of the plating-efficiency are fixed and stained 10 days after the cells have been seeded into them. To determine the cell densities necessary for use in the dishes for both the transformation assay and the plating efficiency determination, it is necessary to consider both the cell killing effects of any toxic agents utilized as well as the percent of (untreated) cells which attach to the dishes and grow after the cells have been seeded into the Petri dishes.

Both types 2 and 3 foci are scored as transformed foci in this system. The comparison of the fraction of dishes containing transformed foci in different treatment groups can be used to quantitate transformation results in the C3H10T1/2 cell transformation assay system (14).

Coumarin-3-carboxylic acid (3-CCA),L-ascorbic acid, BP, and $FeSO_4$ were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and used without additional purification. The stock solution of 3-CCA (100 mM) in 20 mM PBS at pH=7.3 was stored at 5°C. The final concentration of the 3-CCA in each experiment was 0.1 mM.

The stock solution (10 mM) of the BP in N,N-Dimethylformamide (DMFA) (Aldrich, Milwaukee, WI, USA) was prepared and was stored on ice. The addition of BP to the reaction mixture did not exceed 1% of the total volume.

The fluorescence of 7-HCCA was measured with the spectrofluorometer, SPF-500C (SLM, Inc., Urbana, IL, USA), in a quartz cuvette (10 x 10 mm) under constant temperature control with a water circulator. The emission spectra were recorded at the excitation wavelength of 400 nm. The excitation spectra were recorded with emission at 450 nm; the slits in both cases were 1.5 - 2.5 nm for excitation monochromator and 2.0 - 5.0 for emission monochromator, respectively. All spectra were corrected for spectral instability of the Xe-lamp and unequal spectral sensitivity of the photomultiplier. The emission of any of the solutions and solvents used were subtracted from the final spectra.

Results

Transformation Experiments. The first experiment performed to determine whether BP could promote radiation transformation in vitro is shown in Table 1. It can be observed that, in these studies, 10^{-7}M BP was slightly toxic, leading to a lower plating efficiency than was observed in control cultures which were not treated with BP. While 10^{-7}M BP was not shown to be toxic when a single dose was used in a preliminary toxicity study, in the dishes used for the transformation assay and plating efficiency determinations, BP was added to the cultures several times and these repeated additions of BP to the cells did result in some toxicity.

While the numbers of cells per dish are somewhat different in the different treatment groups, this does not influence the results in radiation transformation experiments when the yield of transformed foci is determined from the fraction of dishes with transformed foci (2, 15, 16). When the fraction of dishes containing transformed foci for 1 Gy-treated cultures is compared to

Table 1. Results of Transformation Assay to Determine Whether Benzoyl Peroxide (BP) Can Act as a Promoter for Radiation Transformation in Vitro

Treatment Group	No. of Dishes Start	End	No. of Cells Seeded per Dish	Plating Efficiency (%)	Viable Cells per Dish	Total Cells/Total Dishes	No. of Foci Type 3	Type 2&3	Fraction of Dishes Containing Transformed Foci (Types 2&3)[2]
1. No Treatment	20	16	900	47.8	430	6880	0	0	0/16
2. 1 Gy	30	20	900	38.9	350	7000	1	2	2/20=0.10
3. 1 Gy + BP (10^{-7}M)[1]	30	14	1500	30.0	450	6300	6	8	7/14=0.50

[1]Benzoyl Peroxide (BP) was added 2 times per week for the first two weeks of the assay period (beginning 48 hours after the radiation exposure).

[2]Statistical analysis (Chi-Square): Groups 2 vs. 3, $p<0.05$.

Table 2. Results of Studies on the Ability of Benzoyl Peroxide (BP) to Transform Cells in Vitro with and without the Exposure of Cells to Radiation[1]

Treatment Group	No. of Dishes Start	End	No. of Cells Seeded	Plating Efficiency (%)	No. of Cells per Dish	No. of Cells in All Dishes	No. of Foci Type 3	Type 2&3	Fraction of Dishes Which Contain Transformed Foci (Types 2&3)[2]
1. No Treatment	10	10	900	39.4	355	3550	0	0	0/10
2. BP (10^{-7}M)	20	20	1200	13.8	166	3320	2	6	4/20=0.20
3. 1 Gy	20	18	900	30.0	270	4860	1	3	3/18=0.17
4. 4 Gy	20	20	4000	5.5	220	4400	7	14	10/20=0.50
5. 1 Gy + BP(10^{-7}M)	20	19	1200	10.0	120	2280	17	21	18/19=0.95
6. 4 Gy + BP(10^{-7}M)	20	20	10,000	3.2	320	6400	19	24	20/20=1.0

[1]BP was added to cultures two times per week for the first two weeks of the transformation assay, beginning 48 hours after the radiation exposure.

[2]Statistical analysis (Chi-Square): Groups 1 vs. 2 or 3, $p>0.05$; 1 vs. 4-6, $p<0.05$; Groups 2+3 vs. 5, $p<0.05$; Groups 2 +4 vs. 6, $p<0.05$.

the fraction observed for 1 Gy and BP treated cultures, it is clear that there was an enhanced yield of transformed foci in the cultures treated with both 1 Gy and BP. When analyzed by a chi-square test, the difference between the fraction of dishes containing foci in the two treatment groups was shown to be statistically significant at $p<0.05$.

A second experiment was performed to determine whether BP had the ability to transform cells by itself and whether it had the ability to promote transformation in vitro initiated by a higher dose of radiation than was used previously. The results of this experiment are shown in Table 2. While treatment with BP itself did result in the production of some transformed foci, the fraction of dishes containing transformed foci in the BP treated cultures was not significantly different from the fraction observed in control cultures. Similarly, the fraction of dishes containing transformed foci in the 1 Gy-irradiated cultures was not significantly different from that observed in control cultures. In cultures treated both with a 1 Gy dose of radiation and BP, the fraction of dishes containing transformed foci was significantly greater than that observed for either treatment alone or for the expected additive effect of the yields observed in cultures exposed to either BP by itself or the 1 Gy dose of radiation by itself; thus, a synergistic effect occurred for cells exposed to a 1 Gy dose of radiation followed by several doses of BP. A similar promotion of radiation transformation in vitro by BP was observed in the 4 Gy-irradiated cultures, as observed in Table 2, in that the fraction of dishes containing foci in the treatment group exposed to 4 Gy and BP was significantly different from the fraction of dishes containing foci in the treatment groups exposed to either 4 Gy or BP alone or the expected additive effect of the results in these treatment groups when added together. These results show clearly that radiation transformation in vitro induced by either a 1 Gy or a 4 Gy dose of radiation can be promoted by BP.

Experiments on the ability of BP to generate free radicals. It is our hypothesis that reaction of benzoyl peroxide with trace metals will produce reactive hydroxyl radicals. The experiments for investigating the free-radical generating ability of BP with transient metals was performed in 20 mM phosphate buffer, pH=7.3, at 37°C.

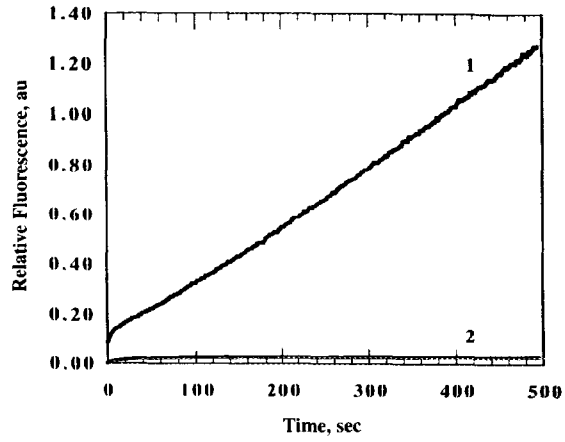

Fig. 1. The generation of ('OH)-radical by 0.01 mM Fe^{2+} and 0.2 mM ASC. The presence of hydroxyl radicals was detected by reaction with 0.1mM 3-CCA in 20mM phosphate buffer, pH=7.3, at 37°C (cf. 1). The bottom line (cf. 2) represents the lack of fluorescence for all solutions before the addition of Fe^{2+} -ASC (all concentrations are final).

Free radicals were detected using coumarin-3-carboxylic acid (3-CCA) (7). This molecule is non-fluorescent, but upon 'OH -radical attack, it becomes hydroxylated. The hydroxylated product of this reaction, 7-hydroxy-coumarin-3-carboxylic acid (7-HCCA), is known to be highly fluorescent (quantum yield $\geq$ 0.5) (7). High quantum yield of the 7-HCCA fluorescence has allowed us to detect nanomolar concentrations of 'OH -radicals with sufficient accuracy and reproducibility (Manevich, Y. and Biaglow, J.E. Unpublished data).

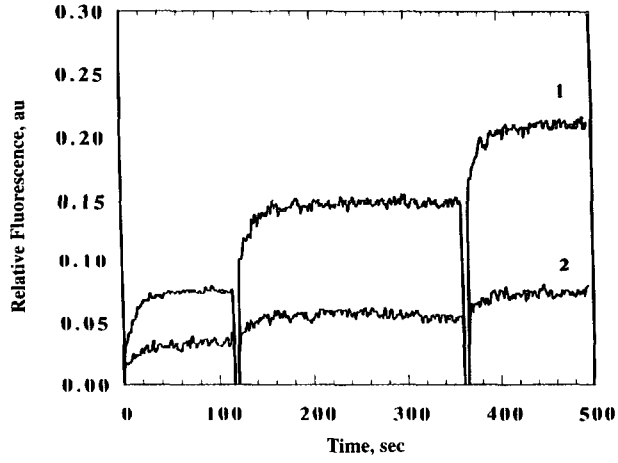

Fig. 2. The generation of 'OH-radical by addition of 0.015 mM of Fe^{2+} to 0.1 mM BP as detected by reaction with 0.1 mM 3-CCA, in 20 mM phosphate buffer, pH=7.3,at 37°C (all concentrations are final). The effects of multiple additions of .015 mM Fe^{2+} are recorded (cf. 1). The additions of 0.015 mM of Fe^{2+} were synchronized with the closing of the shutter of the spectrofluorometer (indicated on the plot as a sharp decrease of the fluorescence intensity to zero). The bottom line represents the addition of 0.015 mM of Fe^{2+} to 0.1 mM 3-CCA (cf. 2). Experiments with the same samples in organic solvents (chloroform, ethanol) did not give any fluorescent changes.

The ability to use this chemical detector is demonstrated by the use of Fe^{2+} ASC mixtures to generate the 'OH -radical. The 'OH -radical generation was initiated by the addition of 0.01 mM Fe^{2+} to 0.2 mM ASC. The rapid increase in fluorescence due to the formation of 7-HCCA can be observed in Fig. 1.

We used the increase in coumarin fluorescence to measure 'OH generation by BP plus Fe^{2+} mixtures. BP addition to 3-CCA solutions did not produce any fluorescence. However, the addition of 0.015 mM Fe^{2+} to the BP caused hydroxylation of 3-CCA; i.e., 'OH radicals were produced (Fig. 2). Some hydroxylation of 3-CCA occurs by the addition of Fe^{2+}. The Fe^{2+} induced fluorescence is slower in initial rate and magnitude than that produced by interaction with BP and Fe^{2+} alone.

Interpretation of Results

BP is insoluble in water (10). The critical concentration for self association and micelle formation for BP is unknown. Many other hydrophobic molecules form micelles in the range of 10^{-7} -10^{-8} M. In our experiments, we used DMFA to solubilize BP in buffer; however, it is conceivable that BP was in micelle form in our studies.

The mechanism for BP homolitic degradation in the presence of Fe^{2+} in water is described by Equations 2 and 3. The benzyl-radicals react with Fe^{3+} to produce Fe^{2+}. Rapid reaction of Fe^{2+} with oxygen produces peroxide and the generation of OH radicals (cf. Eq. 1).

3-CCA + 'OH ——> 7-OH-3-CCA (fluorescence at 450 nm, excitation, 400 nm) (4).

All reactions are very fast (diffusion controlled). The rapid increase of 7-HCCA emission agrees with the proposed scheme (Fig. 2). The hydroxylation of 3-CCA with Fe^{2+}/ASC mixtures shows slower kinetics because the formation of $H_2O_2$is a prerequisite for the reaction (Fig. 1).

The addition of Fe^{2+} causes a rapid increase in fluorescence. Both the first, second and third additions of Fe^{2+} result in the resumed production of hydroxyl radicals.

Discussion

The results presented here demonstrate that radiation transformation in vitro can be promoted by doses of BP. A number of other agents have been shown to promote radiation transformation (2), but this is the first demonstration that a free radical generating compound such as BP can serve as a promoter of transformation in vitro.

BP is a chemical with widespread use in our culture. It has been extensively used for the treatment of acne; thus, there has been concern about its possible effects as a promoter of skin carcinogenesis (e.g., ref. 17).

Many forms of radiation are present in our environment (18) and radiation is one of only a few carcinogens that are known to cause cancer in human populations (19). Several different types of radiation have been shown to induce malignant transformation in vitro. As examples, ionizing radiation (x-rays [reviewed in ref. 2]); ultraviolet (UV) light (20) and fluorescent light (21) are capable of transforming C3H10T1/2 cells in vitro, and could serve as initiating agents for skin carcinogenesis. Both ionizing and non-ionizing radiations can serve as initiating agents for TPA-promoted skin carcinogenesis in mice (3-5).

While the epidemiologic evidence thus far indicates that the use of BP in skin creams has not led to an increased incidence of skin cancer (e.g., ref. 17), it is far too early to conclude that its use will not have biological significance at a later date. BP has been in widespread use for only about 10 years. As it is known that most cancers induced by radiation have a very long latent period (>20 years for most radiation induced cancers) (19), not enough time has passed yet for potential skin tumors induced by radiation and promoted by BP to appear and be diagnosed clinically.

The use of BP in acne preparations might be of particular concern for individuals previously exposed to significant doses of radiation. Many individuals in this country, as well as in other countries, have been irradiated for a variety of benign conditions, including skin problems such as excema and acne, etc. (reviewed in ref. 22). For such individuals, the use of skin preparations containing BP could be particularly problematic. There is some evidence that those who have been previously exposed to radiation do have an altered sensitivity to other promoting agents for skin carcinogenesis, as has been reviewed (23); for example, individuals exposed to radiation early in life are known to be more sensitive than normal to the induction of skin cancer from a protocol utilized clinically for the treatment of psoriasis (psoralen plus several exposures to near UV [360 nm] light [PUVA] (24). Sequential doses of near UV light have been shown to act as a promoter of radiation transformation in vitro (25).

It is highly likely that the ability to generate free radicals by BP is causally associated with the observed promotion of radiation transformation in vitro. Our experiments have shown clearly that BP does indeed lead to the production of free radicals, determined by the kinetics of hydroxylation as measured by fluorescence of 3-CCA. BP is thought to be capable of producing several different types of cellular injury, including DNA damage (e.g., ref. 26). The mechanisms by which BP and other promoters enhance skin carcinogenesis in vivo or transformation in vitro are unknown, although many hypotheses have been discussed (examples, refs. 1, 2, 6, 16). Under the conditions of our transformation experiments in which biological membranes were present, it is expected that BP could penetrate inside the membrane bilayer due to its highly lipophilic nature. The subsequent production of benzoyl radicals in the vicinity of the membrane associated unsaturated lipids would result in lipid peroxidation. Our results suggest the possibility that lipid peroxidation brought about by benzoyl radicals could contribute to the promotion of radiation transformation in vitro.

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