EFFECTS OF SUPEROXIDE RADICALS ON MYOBLAST GROWTH AND DIFFERENTIATION

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SUMMARY. - The effects of superoxide radicals on myoblast growth and differentiation in presence and in absence of carcinogenic hydrocarbons are described. Superoxide dismutase affords strong protection in both cases and also protects the cells against the effects of γ irradiation.

We have previously described the toxic effects of superoxide radicals on various biochemical systems including nucleoproteins, proteins and lipoproteins as well as bacteria (1). This toxicity results not only from the extremely active oxidising capacity of $O_2^{\cdot -}$ but could also be due in part to the reducing properties of such radicals. It was also shown that superoxide dismutase (SOD) afforded a high degree of protection. We now describe the effects of superoxide radicals on mammalian cells in culture and the protection given by exogenous SOD. In such experiments the relatively long life time of superoxide radicals compared with diffusion rates renders possible the active participation of an exogenous enzyme which destroys $O_2^{\cdot -}$. Thus external protection becomes feasible and indeed plays a major role when the volume of the medium is compared with the total volume of the cells. Mammalian cells possess a further advantage in that not only can toxic effects be followed, but effects on cell growth, morphology and differentiation are also readily visible. This is particularly true for myoblast cells.

The present studies were initiated to examine the possible protection afforded by SOD against the effects caused by carcinogenic hydrocarbons. An earlier report had shown that certain antioxidants such as butylated hydroxytoluene (BHT) were quite efficient in diminishing chromosome breakage caused by carcinogenic hydrocarbons (2). However, in the course of this work it became evident that the effects of superoxide radicals led to much wider implications than simple activation of hydrocarbons. We therefore examined the effects on mammalian cells in culture of hydrocarbons

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alone, hydrocarbons activated by systems producing superoxide radicals and $O_2^{\cdot -}$ alone, as well as the protection afforded by SOD. In general we have used a simple photochemical system for production of $O_2^{\cdot -}$ but extension to the use of γ rays was also studied, since it has been shown that the major chemical lesion produced by high energy irradiation under aerobic conditions is indeed production of superoxide radicals (3). It was also of interest to see whether genetic effects could be observed in mammalian cells treated with $O_2^{\cdot -}$ since we have shown (4) the strong mutational effects on T 4 bacteriophage by systems producing $O_2^{\cdot -}$. Indeed, a probable cause of some spontaneous mutations is formation of superoxide radicals (5, 6) either within the cell or in the medium itself (c/f accumulation of mutants in T 4 on stockage (7) for several years).

MATERIALS AND METHODS

Cell culture : Primary myoblast cultures were derived from skeletal muscle of 3 months old foetal calves (8). All hydrocarbons (10^{-2} M) and butylated hydroxytoluene (2×10^{-4} M) were stored in acetone at - 20°, and added to give final concentrations of 10^{-5} M for the hydrocarbons and 2×10^{-7} M for BHT except in one case when 10^{-5} M BHT (final concentration) was used. Flavin mononucleotide at 10^{-5} M in 10^{-5} M EDTA (final concentration) and solutions of superoxide dismutase were sterilised by filtration through 45 micron millipore filters. Energy input was at 365 nm with a Black Ray B 100 lamp at appropriate distances. The process of production of superoxide radicals via photoreduction of FMN has been previously described (5).

Superoxide dismutase was either 100 % pure bovine erythrocuprein (9) or the bacterial ferroenzyme from Photobacterium leiognathi (10). Units were as defined previously (1). Denatured enzyme was prepared by heating dilute solutions at pH 7 to 100° for 10 mins.

γ irradiation. A caesium source giving approximately 100 rads/min under standard conditions was used. In these experiments, 5×10^{-4} M sodium formate (final concentration) was added to the culture medium to convert other oxygen radicals to $O_2^{\cdot -}$.

RESULTS

Calf primary myoblast cultures undergo cell division (0 - 42 h), followed

by a stationary phase during which the cells become aligned. This precedes the onset of cell fusion (52 h). Initially two to three nuclei fusions are observed, followed by the development of long multinucleate fusions and the appearance of the muscle contractile apparatus. The cultures underwent treatment at 24 h, during exponential cell growth ; observations were made at 12 h intervals thereafter. Striking stimulation or amplification by $O_2^{\cdot-}$ (via photoreduced FMN) of the effects of the carcinogen, methylcholanthrene was observed (Table Ia). A proportion of the cells rapidly assume an abnormal hyperplasmic morphology with a very large cell nucleus and extended cytoplasm (Fig. 1a, 1b). There is some cell death, cell division is inhibited, and very few fusions occur. This type of effect is seen to a lesser degree on treatment with flavin mononucleotide and irradiation alone, and is due essentially to the unique action of superoxide radicals. The addition of superoxide dismutase increases cell viability and the extent of cell division and fusion (Fig. 1c). Toxicity is a function of the quantity of $O_2^{\cdot-}$ generated. Increasing times of irradiation at 1700 $\mu W/cm^2$ in presence of FMN increases toxicity and after 30 min few cells survive, but again SOD alleviates the resultant abnormalities. In absence of exogenous FMN, much longer times of irradiation are necessary to achieve the same effects. It is the accumulative dose of irradiation (i. e. of $O_2^{\cdot-}$) which is important over various time periods and intensities. Irradiation for 10 mn at 850 $\mu W/cm^2$, for 5 min at 1700 $\mu W/cm^2$ or for 2. 5 min at 3400 $\mu W/cm^2$ all gave results similar to those shown in the table.

Table 1b shows the results of experiments similar to those with methylcholanthrene but with anthracene or benzpyrene. Both were highly toxic in the presence of $O_2^{\cdot-}$. Irradiation with anthracene alone added to the cultures (no FMN) had little effect whereas benzpyrene continued to be toxic in the absence of flavin. It may be noted that under the conditions used, the various hydrocarbons were completely non-toxic (or produced very minor effects) in the absence of irradiation (plus or minus FMN).

The toxic effects of irradiation on cultures to which hydrocarbon and flavin had been added at different times prior to and during myoblast differentiation were examined (Table 1c). During the period of cell division, messenger RNA species, specifying differentiated proteins, notably myosin, are transcribed, but are unstable. In the stationary period preceding fusion these

TABLE I

The effects of superoxide radicals on cell morphology and fusion in the presence and absence of hydrocarbons. Protection by superoxide dismutase.

	Without SOD	Plus bovine erythrocuprein (50 units/ml)
Control	cell division and subsequent fusion	similar
(a) FMN	normal	normal
MC	normal	normal
FMN + MC	slight abnormality in morphology and growth fusion normal	slightly abnormal, but fusion normal
hν	some dead cells, fusion normal	normal
FMN + hν	cells abnormal, some fusions	more cells, some fusions
MC + hν	normal	normal
MC + FMN + hν	very abnormal growth and morphology, few fusions	cells more numerous, more fusions
(b) A + FMN + hν	very few live cells, abnormal	more cells, but still no fusions
A + hν	cells more normal, fusions	normal
BP + FMN + hν	very few live cells	more cells
BP + hν	more cells, abnormal, no fusions	similar
(c) MC + FMN + hν		
at 24 h	cells abnormal, some fusions	more cells and fusions
47 h	cells approximately normal, fusions short and not well aligned	fusions reduced
52 h	very minor effect on number and length of fusions	normal
70 h	normal	normal
(d) γ - irradiation		
at 24 h 4000 rads	cell death, abnormal cells, fusions reduced	more cells, fusions
8000 rads	mostly abnormal cells, no fusion, cell death	more cells, some fusions
48 h 4000 rads	fusions, but few single cells	fusions normal, mononucleate cells
8000 rads	morphology of fusions abnormal, no single cells	fusions normal, mononucleate cells

TABLE II

The effects of different concentrations and preparations of superoxide dismutase on cells exposed to superoxide radicals. Comparison with a chemical antioxidant, BHT.

Control	cell division and subsequent fusion
(a) MC + FMN + hν	cells abnormal, some fusions
MC + FMN + hν + bacterial dismutase 100 units/ml	more cells, fusions longer and more normal
MC + FMN + hν + bacterial dismutase 20 units/ml	more cells, but morphology abnormal, some fusions
bacterial dismutase 100 units/ml	normal
MC + FMN + hν + denatured bacterial dismutase 100 units/ml	cells abnormal, some fusions
MC + FMN + hν + bovine dismutase 50 units/ml	more cells, fusions longer and more normal
MC + FMN + hν + bovine dismutase 100 units/ml	more cells, fusions longer and more normal
bovine dismutase 100 units/ml	normal
(b) BHT 10^{-5} M (fresh solution)	cells and fusions fairly normal, some dead cells
BHT 2 x 10^{-7} M (fresh solution)	normal
BHT 2 x 10^{-7} M (old solution)	all cells dead
MC + FMN + hν + BHT 2 x 10^{-7} M (fresh solution)	some protection
(c) Fremy salt at 10^{-5} M	cell morphology slightly abnormal, fusions less well aligned

The following abbreviations are used : FMN, flavin mononucleotide ; MC, methylcholanthrene ; A, anthracene ; BP, benzpyrene ; hν , irradiation at 365 nm. Irradiation was at an intensity of 1700 μW/cm^2 for 7.5 min. All substances were added during logarithmic growth at 24 h, except in the case of I (c).

messenger RNA molecules are stabilised, and used in the synthesis of contractile proteins during the subsequent formation of long fusions. Transcription is therefore less necessary for differentiation, once the stable messenger

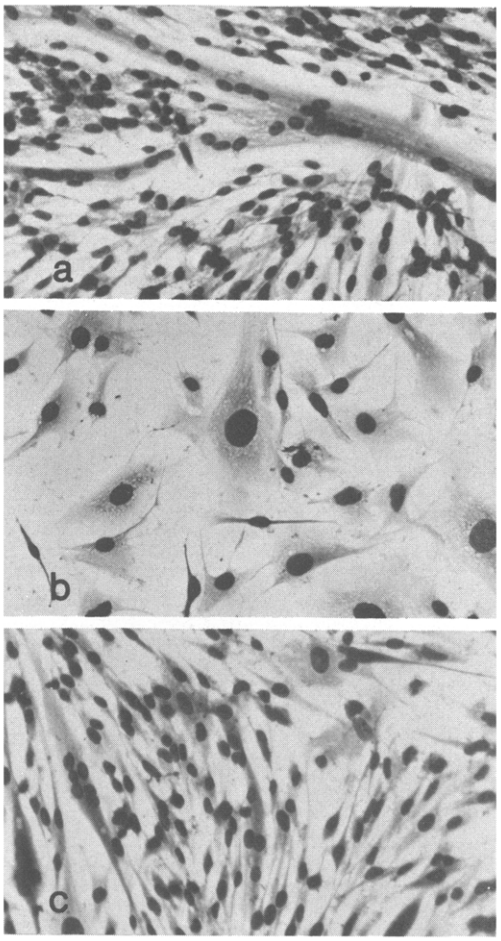

Fig. I - Morphological effects on myoblasts. (a) untreated cells, (b) cells irradiated in the presence of MC and FMN (Table I), (c) as (b), but in presence of SOD.

RNA species are present (8). In fact, myoblast differentiation was severely affected on treatment during the period of cell division. If treatment occured during the stationary period, fusion although initiated, proceeded abnormally, whereas if cells were treated once fusion had begun no effects could be detected. This would suggest that the primary toxic effect of superoxide radicals alone or via hydrocarbons is at the nuclear level affecting DNA synthesis and RNA transcription. The molecular basis of this is presently under investigation. Preliminary results indicate possible formation of purine 8-hydroperoxides and 8-hydrocarbon derivatives respectively. Such modified nucle-

osides would be forced out of the anti-conformation towards a syn structure, leading to the impossibility of complementary hydrogen bond base pairing, thus providing an explanation for a deletion type mutation or phenotypic RNA changes. In this connection it is perhaps noteworthy that we have recently isolated a specific superoxide dismutase from rate liver nuclei (11).

The effects of γ - irradiation and the protection afforded by SOD were then examined (Table 1d). With increasing doses of irradiation, changes in cell viability and morphology were essentially similar to those observed with the previous $O_2^{\cdot -}$ generating system. Superoxide dismutase greatly diminished the toxic effects. Irradiation in the stationary phase, just prior to fusion had no effect on fusion at low doses ; morphological abnormalities were induced at higher doses. Contaminating mononucleate cells were much more sensitive and were eliminated. It may thus be concluded that the primary chemical lesion leading to biological destruction by γ irradiation under aerobic conditions is in fact production of superoxide radicals, which act at the nuclear level, and that SOD provides at least a partial protection of living cells against γ rays (and presumably other high energy irradiation). Even at very high doses of γ rays (10,000 rads) SOD reduced cell death significantly.

Table II (a) provides more information on the effects of superoxide dismutase on myoblast cultures. Both the bacterial enzymes and the homologous bovine enzyme have no effect whatsoever (even at very high doses) when added alone to the cultures. Both alleviate the toxic effects resulting from exposure to irradiation in the presence of hydrocarbons and flavin mononucleotide. In contrast, the denatured enzyme has no remedial action, as was also noted in the case of γ irradiation. Table II (b) demonstrates the effects of the antioxidant, butylated hydroxytoluene. This gives some protection, although it is less effective than SOD. It should be noted that at higher concentrations BHT is toxic to some extent. If aged preparations (several weeks at -20° C in acetone) are used, this toxicity becomes extremely striking and is evident at 2×10^{-7} M, at which concentration essentially 100 % lethality is observed.

In view of theories of aging implicating free radicals (12) we examined the effect of a non-biological radical, Fremy's salt (13, 14) on mammalian cells in culture. At relatively high levels (10^{-5} M) some effects could be

seen as summarised in Table IIc, but much less than those observed with $O_2^{\cdot-}$ (approx. 10^{-8} M). Superoxide radicals, or rather a diminished cellular protection against such radicals, may well be a primary cause of aging processes.

DISCUSSION

The above results provide a convincing demonstration that the effects of carcinogenic hydrocarbons on mammalian cells are greatly stimulated and amplified in presence of a system producing $O_2^{\cdot-}$. It may even be provisionally concluded that such effects are normally mediated by endogenous production of $O_2^{\cdot-}$ within the cell. Indeed the amplification is so powerful that hydrocarbons such as anthracene (not normally regarded as carcinogenic) show most of the effects of benzpyrene or methylcholanthrene in presence of $O_2^{\cdot-}$. The degree of $O_2^{\cdot-}$ induced stimulation of hydrocarbon activity is a function of the nature of the hydrocarbon and is for example greater for methylcholanthrene than for benzpyrene activated by simple irradiation. A striking parallel exists between the results described above and the stimulation of covalent fixation of hydrocarbons on DNA in vitro by superoxide radicals (15) and the effects of SOD. It is clear that the primary target is the nucleus in dividing cells ; after fusion, the cells become much more resistant.

Of more general interest, is the effect of superoxide radicals alone in the absence of specific carcinogens. Again SOD protects against the toxic effects expressed by this radical, which as in the case of hydrocarbon mediated effects, occur principally (if not entirely at the doses used) on dividing cells. Mutational effects can be seen and indeed a significant number of cells with an abnormal morphology can be observed, the incidence of which is markedly reduced by SOD. Inhibition of differentiation is also striking and again is reduced in presence of exogenous SOD. This raises the possibility that superoxide radicals and SOD may play a role in the initiation and protection against certain malignant processes. Similar considerations may be applied to the results obtained with γ irradiation.

In view of the extremely common utilisation of chemical anti-oxidants in a wide variety of foodstuffs, the results with BHT are of some interest. It is clear that whereas fresh BHT is relatively non-toxic, aging produces products with extremely dangerous properties, at least for mammalian cells

in culture. Such observations suggest that a more restrictive legal control should be exercised with respect to food stuff additives.

It would be of great interest to repeat these experiments using another technique for production of superoxide radicals such as electron pulse radiolysis. The medical implications of this work are immediately apparent. In this respect it is noteworthy that the level of erythrocuprein in normal humans (population of 100 samples) expressed as amount of enzyme per erythrocyte, per unit of hemoglobin or unit volume of blood is remarkably constant (16). We are presently engaged in examination of a number of cases (including cancer patients) to determine a possible molecular basis of various diseases.

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