Volume 216, number 2, 253-256 FEB 04728 June 1987

Is enhanced free radical flux associated with increased intracellular proteolysis?

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Received 21 January 1987

Intracellular proteolysis was measured in cultured cells during and after free radical attack. Radicals were generated firstly, throughout the aqueous phase by gamma irradiation and secondly, selectively, either extracellularly or intracellularly by chemical and enzymic methods. With both approaches, stimulation of proteolysis was observed in certain circumstances. Phenylhydrazine stimulated proteolysis at low concentration but inhibited at higher. Depletion of the antioxidant glutathione and inhibition of catalase also increased proteolysis.

Antioxidant; Free radical; Proteolysis; (P388D1 cell)

1. INTRODUCTION

Free radicals are generated throughout biological systems [1-3]. We and others [4-8] have demonstrated that radicals fragment and otherwise modify proteins and can cause an increased susceptibility to proteinases. We have therefore proposed [9] that free radical fluxes accelerate proteolysis and have presented some evidence [10] that this may occur in isolated mitochondria. Here we test the hypothesis with reference to cultured cells.

2. MATERIALS AND METHODS

P388-D1 cells, a mouse macrophage-like cell line, were maintained in monolayer culture in Eagles MEM (Flow Laboratories, Rickmansworth, England) supplemented with 10% HIFCS and antibiotics as described [11]. 3T3 cells, a mouse fibroblast cell line, were used for some experiments and were maintained as in [12]. To measure proteolysis, confluent cell cultures were labelled for 18 h (this results in the labelling of long half-life proteins [12]) in 75 cm^2 tissue culture flasks containing 10 ml leucine-free MEM, 10% HIFCS, antibiotics and 5 μCi L-[1-^{14}C]leucine (Amersham International, England). At the end of the labelling period, cells were washed by centrifugation and resuspension three times in MEM containing 10 mM leucine in order to free the intracellular pool of radioactive leucine [12]. The resultant labelled cells were then seeded into tissue culture tubes (Sterilin, Middlesex) at a density of 3×10^5 cells per tube in 0.5 ml MEM with 10 mM leucine and 10% HIFCS together with the radical-generating systems or agents known to deplete cellular antioxidant defences described below.

For irradiation experiments, cells were seeded into 'glowed' glass tubes (cleaned in a glow oven to remove reactive material) in 0.5 ml PBS, pH 7.2, and radical species generated by using a 2000 Ci cobalt source [5,13]. In this system, the main radical species formed are OH and $O_2^{\cdot-}$. After irradiation, the PBS was removed and replaced with

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Abbreviations: PBS, phosphate-buffered saline; MEM, minimum essential medium; HIFCS, heat-inactivated foetal calf serum; HBSS, Hanks buffered salt solution

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

MEM containing 10 mM leucine and serum as above. Control cultures were treated in parallel but not irradiated. All reagents were of the best available commercial grade and radical-generating solutions freshly prepared for each experiment.

In all cases, degradation was allowed to proceed for up to 24 h. At each time point the media were harvested and the medium and cell fractions precipitated with 5% trichloroacetic acid. Measurement of degradation was essentially as in [12]. Fractions were counted using Liquiscint (National Diagnostics, Bucks) as a scintillator. Degradation (%) is expressed as (total trichloroacetic acid-soluble radioactivity/total radioactivity in the culture) × 100. Cell lysis (%) is measured by (medium trichloroacetic acid-insoluble radioactivity/total radioactivity) × 100. Results presented are means ± SD for individual experiments done in quadruplicate and are representative of several different experiments.

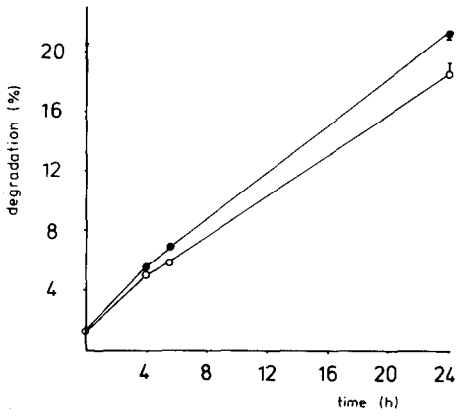

Fig.1. The effect of irradiation on protein degradation of P388 cells after 24 h. Cells irradiated as described in section 2 with 0 Gy (control) (○) or 100 Gy (●). Each value represents data from 4 replications ± SD. Differences at 6 and 24 h are significant at $p<0.01$ (Student's t-test). Several SD values are smaller than the points.

3. RESULTS AND DISCUSSION

The effect of irradiation on protein degradation in P388 cells in culture was measured by following proteolysis for 24 h after irradiation. No effects on protein degradation were found after irradiation at doses of 1, 5 and 10 Gy; after a 100 Gy irradiation a significant increase in protein degradation was observed at 24 h compared to the control (fig.1). Since P388 cells are a macrophage-like cell line and so may be adapted to a high radical flux as occurs during the respiratory burst [1–3], similar experiments were performed using 3T3 cells. However, an unacceptably high lysis occurred with these cells even in the control samples (which had been exposed to PBS alone) and thus no comparable studies could be made. In previous studies with irradiated monoamine oxidase in submitochondrial particles it was found that subsequent addition of Fe after irradiation further increased degradation [9]. This post-irradiation effect was not found in our experimental system when up to 100 μM Fe (as $FeSO_4$) was added to the incubation medium following irradiation. Possibly charged metals do not enter the cells sufficiently rapidly.

Preliminary examinations have also been made of the distribution of protein bands from whole P388 cell extracts on SDS-PAGE after an irradiation at 1000 Gy. These suggest that such irradiations in air and in the presence of N_2O/O_2 (producing $OH^{\cdot}$ radicals) cause a shift in the gel electrophoretic pattern to higher M_r values.

Subsequently (table 1), various chemical and enzymic systems were used to produce radicals inside or outside the cells. Most of the agents had no effect on protein degradation measured at four points over 24 h. At 10 μM, menadione caused a slight increase in protein degradation but at 20 μM was toxic to the cells. The high toxicity of most of these compounds was a limiting factor and it was difficult to find a concentration of most agents that could effect protein degradation without causing lysis. In addition, as shown for phenylhydrazine in fig.2 there may well be a stimulation of proteolysis at low concentration but inhibition at a higher concentration. Similar experiments were performed using 3T3 fibroblasts in place of P388 cells with similar results. The radical-generating systems may also depress levels of ATP in the cell and hence its availability for proteolysis. In the experiments summarised in table 1, both the radical-generating systems and 10% HIFCS were present throughout the time course of degradation studied. This extracellular protein may scavenge radicals and thus some experiments were performed with the radical-generating systems presented to

Table 1

The effect of various radical-generating systems on protein degradation in P388 cells in culture

Radical-generating system (concentration range)	Effect on degradation	Toxicity
Adriamycin (10–100 μM)	none	<10 μM
Alloxan (0.25–2.5 mM)	none	>2.5 mM
Ascorbic acid (0.5 mM)	none	>0.5 mM
Copper II (5–100 μM)	none	$\geqslant 100$ μM
Cu/H_2O_2	none	>50 μM
Dehydroascorbic acid (0.5–2.5 mM)	none	>2.5 mM
Hydrogen peroxide (10 μM–1 mM)	none	$\geqslant 100$ μM
Iron II (5–100 μM)	none	>50 μM
Fe/H_2O_2	none	>50 μM
Fe/ascorbate	none	>50 μM
Menadione (10 μM–1 mM)	slight increase at 10 μM	$\geqslant 20$ μM
Phenylhydrazine (50 μM–100 mM)	yes (fig.2)	$\geqslant$ 5 mM
Sodium benzoate (10 μM–1 mM)	none	$>$ 1 mM
Sodium nitrite (10 μM–100 mM)	slight decrease at 100 mM	>100 mM
Acetaminophen (10 μM–10 mM)	none	$>$ 1 mM
t-Butylhydroperoxide (10 μM–1 mM)	none	$\geqslant 100$ μM
Xanthine oxidase/ hypoxanthine (0.6 U/ml/10 μM)	none	>0.6 U/ml

Degradation was followed over 24 h as described in section 2. % lysis > 25% is defined as toxic

the cells for a period of 2 h in HBSS alone. This time was chosen to keep lysis to a minimum. After 2 h the HBSS was removed and medium containing 10% HIFCS added as before and degradation followed over 24 h. No effects on protein degradation were observed in this system without serum. However, in this case the radical-generating systems were only present for a short period of 2 h, not 24 h as previously.

To examine the balance between stimulatory and inhibitory effects of free radicals on proteolysis the response of P388 cells to phenylhydrazine was studied in detail (fig.2). 50 μM phenylhydrazine stimulated proteolysis but increasing the concentration caused an inhibition of protein degradation which was maximal at 1 mM. Higher concentrations further decreased proteolysis but also increased lysis. Lysis due to 5 mM phenylhydrazine could be reduced by the inclusion of superoxide dismutase (34 μg/ml) in the incubation medium without significantly affecting degradation. Thus low concentrations of phenylhydrazine showed both inhibition and stimulation of proteolysis dependent on concentration; these changes were only detectable after incubation for 17 h or more and were not accompanied by high toxicity.

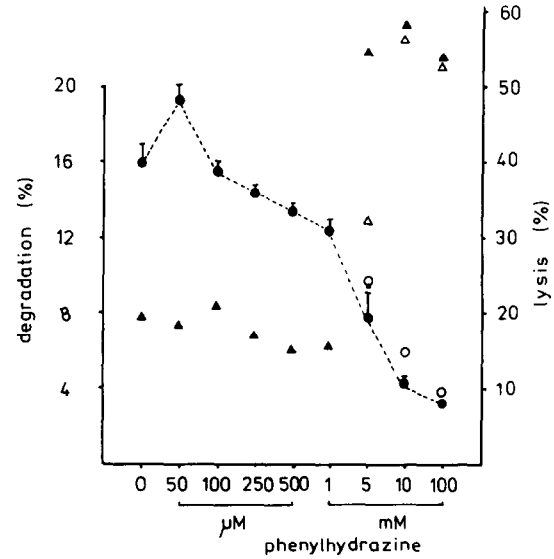

Fig.2. The effect of increasing concentrations of phenylhydrazine on proteolysis in P388 cells in culture. Degradation (●) and lysis (▲) were measured over 24 h. Superoxide dismutase (34 μg/ml) was included in some incubations (○, △). The data for degradation indicate SD for $n=4$. The degradation differences from control cell 50 μM (enhancement), and 500 μM and 1 mM (inhibition) are significant at $p<0.01$. Several SD values are smaller than the points.

We then attempted to accentuate radical flux within cells by depleting the cellular antioxidant capacity. The superoxide dismutase inhibitor diethyldithiocarbamate was toxic to P388 cells even at 10 μM (table 2). However, the other depleting agents used increased protein degradation over a wide concentration range. Subsequently aminotriazole, diamide and buthionine sulphoximine were incubated with P388 cells in conjunction with phenylhydrazine at both stimulatory and inhibitory concentrations. No consistent further enhancement of proteolysis was

Table 2

The effect of depleting agents on protein degradation in P388 cells

Depleting agent (target)	Concentration	Degradation (%)
None	-	17.17 ± 0.42
Diethyldithiocarbamate (SOD)	10 μM	toxic
Aminotriazole (catalase)	100 μM	20.17 ± 0.7
	1 mM	20.11 ± 1.1
Diamide (glutathione)	100 μM	22.79 ± 1.55
	200 μM	21.19 ± 1.9
Buthionine sulfoximine (glutathione synthetase)	100 μM	20.79 ± 0.6
	1 mM	21.09 ± 0.18

Cells incubated in MEM containing 10% HIFCS and 10 mM leucine. Degradation followed over 24 h. Results are means ± SD ($n = 4$)

observed. Further, phenylhydrazine at 500 μM together with 200 μM diamide caused considerable lysis unlike either compound alone thus supporting the idea that the depletion of the antioxidant capacity may not only accelerate proteolysis but also render cells susceptible to lysis. We have thus found several examples of the predicted [9] enhancement of proteolysis by accentuated radical stress. Cellular antioxidants seem to defend against this effect and the acceleration of protein degradation can be increased by their depletion. However, there is a narrow range of conditions in which proteolysis can be exaggerated without toxicity also ensuing. This range may possibly include that in which a stress protein response (as in the production of heat shock proteins [14]) occurs. The stress protein response is indeed associated with accelerated proteolysis [15].

ACKNOWLEDGEMENT

This work was supported by the AFRC.

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