Volume 291, number 2, 173–176 FEBS 10252 October 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3 50
ADONIS 0014579391009623

Cytotoxicity of, and DNA damage by, active oxygen species produced by xanthine oxidase

Mariella Chiricolo, Pier Luigi Tazzari, Ada Abbondanza, Angelo Dinota and Maria Giulia Battelli

Departimento di Patologia Sperimentale, Universita di Bologna, Via S Giacomo 14, I-40126, Bologna, Italy

Received 19 July 1991

Toxicity to Raji cells of the xanthine oxidase/hypoxanthine system is related to the formation of single-strand DNA breaks DNA damage was proportional to the concentration of xanthine oxidase and to the time of exposure It was prevented by the absence of hypoxanthine, or by the presence of allopurinol, or both superoxide dismutase and catalase The release of ^{51}Cr from damaged cells was detectable 12 h after the inhibition of cloning efficiency and the production of DNA breakage These data suggest that DNA damage induced by the oxygen products precedes the severe lesion to the cellular membrane

Free oxygen radical, Xanthine oxidase, Single-strand DNA break, Cytotoxicity

1 INTRODUCTION

Xanthine oxidase (EC 1.1.3.22) catalyses the oxidation of hypoxanthine to xanthine and of the latter to uric acid This enzyme derives from a NAD^+-dependent dehydrogenase (EC 1.1.1 204) by proteolysis [1] or by reversible oxidation of sulphydryl groups [2,3]

During the oxidation of substrates xanthine oxidase in its oxygen-dependent form generates superoxide anions and H_2O_2, which in the presence of chelated iron are converted into highly reactive hydroxyl radicals by the Haber–Weiss and Fenton reactions [4] These oxygen products aggravate cell damage when the enzyme is converted from the dehydrogenase to the oxidase form during ischaemia–reperfusion injury (reviewed by Engerson et al [5], ethanol intoxication [6–9] and complement activation [10] The cytotoxicity of xanthine oxidase-activity products has also been investigated to obtain selective killing of target cells by conjugating the enzyme to cell-specific antibodies [11–13]

The actual mechanism by which cells become damaged while exposed to the xanthine oxidase/hypoxanthine system is still uncertain Formation of DNA strand breaks and base modification by this superoxide radical-generating system has been reported [14,15] The aim of the present study was to investigate if DNA damage is related to the cytotoxicity of the xanthine oxidase/hypoxanthine system. The effects on cells produced by this system were studied by an alkaline elution assay, inhibition of colony growth and ^{51}Cr release focusing on DNA damage, loss of replication efficiency and severe lesion to the cytoplasmic membrane, respectively

Correspondence address M G Battelli, Dipartimento di Patologia Sperimentale, Via S. Giacomo 14, 40126 Bologna, Italy Fax (39) (51) 251315

2. MATERIALS AND METHODS

2 1 *Materials*

Xanthine oxidase from buttermilk, catalase from bovine liver, superoxide dismutase from human erythrocytes, proteinase K from *Tritirachium album*, xanthine and hypoxanthine were purchased from Sigma Chemical Co , St Louis, MO, USA RPMI 1640 was from Biochrom, Berlin, Germany, fetal calf serum from Sera Lab Sussex, UK [^{3}H]thymidine (5 Ci/mmol) was from Amersham International, Amersham, Bucks, UK and $Na_2{}^{51}CrO_4$ was from Sorin, Saluggia (VC), Italy

2 2 *Xanthine oxidase assay*

Xanthine oxidase activity was determined by uric acid formation measured by A_{292} as described [16] The assay was performedat 28°C in a 3-ml final volume mixture containing 0 05 M Tris-HCl buffer, pH 8 1, 60 μM xanthine and appropriate amounts of enzyme Xanthine oxidase was omitted from references cuvettes One unit of enzyme activity is defined as the formation of 1 μmol of uric acid per min

2 3 *Cell culture and treatment*

Raji cells, derived from a human Burkitt lymphoma, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics On the day of the experiment aliquots of cell suspension corresponding to 0 5×10^6 cells were distributed into sterile tubes and incubated at 37°C for 30 min, unless otherwise specified, in a final volume of 1 ml of complete medium with appropriate amounts of xanthine oxidase in the presence of 100 μM hypoxanthine, or as specified

2 4 *Clonogenic assay*

After the incubation described in section 2 3, cells were washed, resuspended in 1 ml of culture medium supplemented with citrated human plasma (15%) and were plated on 35×10 mm Petri dishes (1000 cells/dish) The clotting of the final medium was obtained by adding 15 mM calcium chloride After a 6-day incubation, at 37°C in a

humidified atmosphere containing 5% Co_2 and 95% air, aggregates (>16 cells) were counted with an inverted microscope

2 5 *Alkaline elution assay*

[^{3}H]Thymidine (2 5 μCi/ml) was added to a Raji cell culture 30 h before the experiment commenced The cells were labelled for 24 h, then washed and incubated in fresh culture medium for the last 6 h to chase all the radioactivity into high molecular weight DNA

[^{3}H]Thymidine-labelled cells were incubated as described in section 2 3, for the indicated time At the end of the treatment the alkaline elution was performed as reported [17] The elution apparatus consisted of a filter funnel with output tubing passing through a peristaltic pump to a fraction collector The cells were loaded on Nuclepore (polycarbonate) filters (25-mm diameter, 2 0-μm pore size) and washed with cold Merchant's solution The cells were lysed for 30 min at room temperature with 4 5 ml of a 0 2% sodium lauroyl-sarcosinate, 2 M NaCl and 0 02 M EDTA solution (pH 10 2) containing 0 5 mg/ml of proteinase K Cell lysate was washed with 0 02 M EDTA (pH 10) and single-strand DNA was eluted from the filter in the dark using 0 06 M tetraethyl ammonium hydroxide containing 0 01 M EDTA (pH 12 3) at a pump speed of 0 05 ml/min by collecting 10 fractions of 2 2 ml each The radioactivity both on the filter and in each fraction was measured by a liquid-scintillation counter

2 6 *^{51}Cr-release assay*

The ^{51}Cr-release assay was performed as described [18] Briefly, 2 $\times$ 10^6 Raji cells in 0 5 ml culture medium were labelled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 h at 37°C, then washed twice with phosphate-buffered saline ^{51}Cr-labelled cells were incubated as described in section 2 3 and centrifuged The amount of ^{51}Cr incorporated by the cells and released in the culture medium after the indicated time was measured by a gamma-counter

3 RESULTS

The alkaline-elution method shows the formation of single-strand DNA breaks The kinetics of single-strand DNA breaks elution consists of two phases, the first of which is rate-limited by the size of DNA fragments, while the second unmasks the alkali-labile sites of DNA [19]. For this reason we have reported the radioactivity retained on filters after the elution of fractions 1, 3 and 10, representative of both phases and of the flex point between them.

Table I

Single-strand DNA breaks formation in Raji cells exposed to the xanthine oxidase/hypoxanthine system

Xanthine oxidase (mU/ml)	DNA retained on filter (%)		
	1[a]	3[a]	10[a]
0	82	71	53
1	81	68	51
2	54	34	21
5	44	21	10
10	25	10	6

Raji cells were treated with the indicated amounts of xanthine oxidase and processed for alkaline elution assay as described in section 2 The values represent the DNA retained on the filter after elution of the indicated fractions The results are expressed as percentage of radioactivity incorporated by the cells (8274 cpm) Data shown are representative of three experiments [a]Alkaline elution fractions

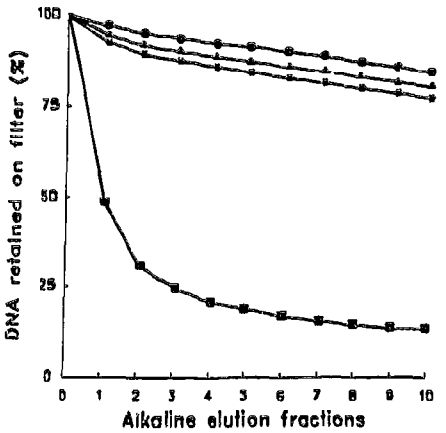

Fig 1 Effect of allopurinol and free-radical scavenger enzymes on DNA damage induced by xanthine oxidase/hypoxanthine system Raji cells were treated with 2 5 mU/ml xanthine oxidase and processed for the alkaline elution assay as described in section 2 (■), 1 U of both superoxide dismutase and catalase were added (●), no hypoxanthine was present (▲), 1 mM allopurinol was added (★) DNA retained on filter after the alkaline elution assay is expressed as percentage of the radioactivity incorporated by the cells (12 819 cpm) Data shown are representative of two experiments

The incubation of Raji cells at 37°C for 30 min in the presence of 100 μM hypoxanthine inhibited colony growth by 50% at an enzyme concentration of 2 8 mU/ml (data not shown), comparable to that reported [12]. The same experimental conditions induced the fragmentation of DNA in single-strands, as shown by the alkaline elution assay (Table I)

DNA damage was related to the concentration of xanthine oxidase (Table I) and to the time of exposure to the xanthine oxidase/hypoxanthine system (Table II). No formation of single-strand DNA breaks was observed (i) in the absence of hypoxanthine, (ii) in the presence of allopurinol, or (iii) using both superoxide dismutase and catalase (Fig 1)

A time-course study was performed to compare the results obtained with alkaline elution, clonogenic and ^{51}Cr-release assays. The percentage of DNA retained on filters started to decrease after 10-min cell treatment with the xanthine oxidase/hypoxanthine system After 30 min incubation, 50% of DNA was retained on filters and 18% was present in the last fraction Inhibition of colony growth was not detectable before 10-min and reached 95% after 30-min treatment Release of ^{51}Cr from damaged cells was not detectable at 30-min incubation and reached 50% 12 h later than DNA breakage and inhibition of cloning efficiency (Table II).

4 DISCUSSION

Toxicity to Raji cells by the xanthine oxidase/hypoxanthine system was related to DNA damage In fact, the inhibition of colony growth closely followed the

Table II

Time-course study of cytotoxicity induced by xanthine oxidase/hypoxanthine system

Time	^{51}Cr released (%)	Colony growth (%)	DNA retained on filter (%)		
			1[a]	3[a]	10[a]
0	3	100	88	76	55
1 min	2	100	88	73	55
2 "	3	100	89	78	52
5 "	3	100	88	75	50
10 "	3	92	76	57	34
20 "	4	69	65	39	26
30 "	4	5	50	28	18
12 h	51				
24 h	66				

Raji cells were treated with a single concentration of xanthine oxidase (3 mU/ml) as described in section 2 The reaction was stopped by the addition of 1 mM allopurinol at the indicated times Data shown are representative of three experiments The ^{51}Cr release was expressed as percentage of radioactivity incorporated by the cells (6728 cpm) The control cells released 226 cpm after 24 h incubation in the absence of xanthine oxidase The results of clonogenic assay are expressed as percentage of the number of colonies grown in the absence of xanthine oxidase (162) DNA retained on filter after the alkaline elution assay is expressed as percentage of the radioactivity incorporated by the cells (180 611 cpm) [a]Alkaline elution fractions

formation of single-strand DNA breaks during a time-dependent exposure of Raji cells to xanthine oxidase-activity products Thus the alkaline elution assay turned out to be a sensitive method for the detection of free radical effects

All cells contain a variable amount of DNA-strand breaks [20] as a result of either spontaneous damage or DNA-topoisomerase activities [21], particularly during cell replication. Thus the variability in alkali-elutable DNA we observed in untreated cells could be ascribed to the not-timed cycling of Raji cells

The DNA breakage depended on the activity of xanthine oxidase, since it was directly proportional to the concentration of xanthine oxidase and to the time of exposure to the xanthine oxidase/hypoxanthine system

Furthermore, the formation of single-strand DNA breaks was not observed either in the absence of the xanthine oxidase substrate hypoxanthine, or in the presence of the competitive inhibitor allopurinol

The cytotoxicity of the xanthine oxidase/hypoxanthine system has been ascribed to H_2O_2 as the major cytotoxic product of the reaction [22] and DNA-strand breaks have been described in H_2O_2-treated lymphocytes and P388D$_1$ cells [23] Under our experimental conditions, the formation of single-strand DNA breaks was unaffected by superoxide dismutase and partially decreased by catalase alone (data not shown). DNA damage was prevented by the action of both free-radical scavenger enzymes, superoxide dismutase and catalase, suggesting that the breakage was induced by both superoxide radical and H_2O_2 This finding is consistent with previous studies on cytotoxicity of the above system [11], and on DNA breakage determined by fluorimetric analysis of DNA unwinding in leukocytes exposed to superoxide radical and H_2O_2 [24,25].

A time-course study was performed in order to evaluate the role of DNA damage and of cellular membrane lesions in the loss of replication efficiency induced by the oxygen products The inhibition of colony growth was almost complete after 30 min exposure to the xanthine oxidase/hypoxanthine system At the same time (i) the alkaline elution assay showed a considerable DNA breakage, and (ii) almost no release of ^{51}Cr was observed The release of ^{51}Cr in culture medium reached 50% of the radioactivity incorporated by Raji cells only after 12 h. This was consistent with the lack of any morphological cell damage before 24-h incubation, as reported [12] The delay in ^{51}Cr release compared to the results of alkaline elution and clonogenic assays suggests that the cytotoxicity of the xanthine oxidase/hypoxanthine system involved DNA breakage as an early event which precedes the severe membrane lesion

Acknowledgements This work was supported by the Ministero per l'Universita e la Ricerca Scientifica e Tecnologica, Roma, by the Italian National Research Council, Roma, Progetto Finalizzato Biotecnologie e Biostrumentazione, by the Associazione Italiana per la Ricerca sul Cancro (AIRC), Milano, and by the Pallotti's Legacy for Cancer Research

REFERENCES

[1] Della Corte, E and Stirpe, F (1968) FEBS Lett 2, 83–84
[2] Della Corte, E and Stirpe, F (1972) Biochem J 126, 739–745
[3] Battelli, M G (1980) FEBS Lett 113, 47–51
[4] Halliwell, B (1978) FEBS Lett 92, 321–326
[5] Engerson, T D, McKelvey, T G, Rhyne, D B, Boggio, E B, Snyder S J and Jones, H P (1987) J Clin Invest 79, 1564–1570
[6] Oei, H H H, Stroo, W E, Button, K P and Schaffer, S W (1982) Res Comm Chem Pathol Pharmacol 38, 453–461
[7] Oei H H H Zoganas, H C, McCord, J M and Schaffer, S W (1986) Res Comm Chem Pathol Pharmacol 51, 195–203
[8] Abbondanza, A, Battelli, M G, Soffritti, M and Cessi, C (1989) Alcohol Clin Exp Res 13, 841–844
[9] Kato, S, Kawase, T, Alderman J, Inatomi, N and Lieber, C S (1990) Gastroenterology 98, 203–210
[10] Friedl, H P, Till, G O, Ryan, U S and Ward, P A (1989) FASEB J 3, 2512–2518
[11] Battelli, M G, Abbondanza, A, Tazzari, P L, Dinota, A, Rizzi, S, Grassi, G, Gobbi, M and Stirpe, F (1988) Clin Exp Immunol 73, 128–133
[12] Tazzari, P L, Battelli, M G, Abbondanza, A, Dinota, A, Rizzi, S, Gobbi, M and Stirpe, F (1989) Transplantation 48, 119–122
[13] Dinota, A, Tazzari, P L, Abbondanza, A, Battelli, M G, Gobbi, M and Stirpe F (1990) Bone Marrow Transplantation 6, 31–36
[14] Carson, D A, Seto, S and Wasson, D B (1986) J Exp Med 163, 746–751
[15] Aruoma, O I, Halliwell, B and Dizdaroglu M (1989) J Biol Chem 264, 13024–13028
[16] Stirpe, F and Della Corte, E (1969) J Biol. Chem 244, 3855 3863

[17] Kohn, W K , Erickson, L C , Ewig, R A G and Friedman, C A (1976) Biochemistry 15, 4629–4637
[18] Dinota, A , Gobbi, M , Tazzari, P L , Raspadori, D , Bontadini, A , Lauria, F , Foà, R and Tura, S (1988) J Immunol Methods 114, 53–59
[19] Kohn, W K (1979) Methods Cancer Res 16, 291–345
[20] Ahnstrom, G (1988) Int J Radiat Biol Relat Stud Phys Chem Med 54, 695–707
[21] Wang, J C (1985) Ann Rev Biochem 54, 665–697
[22] Link, E M and Riley, P A (1988) Biochem J 249, 391–399
[23] Schraufstatter, I U , Hinshow, D B , Hyslpo, P A , Spragg, R G and Cochrane, C G (1986) J Clin Invest 77, 1312-1320
[24] Birnboim, H C and Kanabus-Kaminska, M (1985) Proc Natl Acad Sci USA 82, 6820–6824
[25] Birnboim, H C (1988) Ann N Y Acad Sci 551, 83–94