Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 59-66

Molecular Defect in Human Acatalasia Fibroblasts

Dana R. Crawford , Marc-Edouard Mirault, Rémy Moret,
Irène Zbinden and Peter A. Cerutti

Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges/Lausanne, Switzerland

Received April 7, 1988

The human hereditary disease Acatalasia (AC) is characterized by low or no catalase activity in all body tissues. We have studied the molecular basis of AC. In order to assess their antioxidant defense status we measured the enzyme activities, protein levels and m-RNA concentrations of catalase, superoxide dismutase and glutathione peroxidase in fibroblasts from a Japanese (AC65) and a Swiss (AC64) patient and several normal individuals. Our results point to genetic heterogeneity. While strain AC64 contained normal levels of catalase mRNA and -protein, strain AC65 was completely devoid of both. A structural mutation in the catalase gene is probably responsible for the inactivation of the enzyme in AC64. Since AC65 contains at least a major portion of the catalase gene it may represent a regulatory mutation in which the gene is not transcribed.

Antioxidant defense mechanisms play a fundamental role in maintaining cellular integrity in an aerobic environment. Insufficient defence can result in a cellular prooxidant state and contribute to the etiology of several forms of human disease including cancer (1,2,3). For example a reduced level of Mn-superoxide dismutase (SOD) has been found in a majority of tumor tissues (4). Decreased catalase (CAT)-activities have been reported in skin biopsies and fibroblasts from Xeroderma pigmentosum patients and it has been speculated that a near-ultraviolet induced cellular prooxidant state might contribute to the increased susceptibility of these patients to develop cancer (5). Addition of CAT to cultured mouse cells protected them from the formation of chromosomal damage and malignant transformation by visible light (6). The genes for the major mammalian antioxidant enzymes Mn- and Cu,Zn-SOD (7), CAT (8,9) and glutathione peroxidase (GPx) (10,11) have recently been cloned allowing the analysis of the structure and function of

ABBREVIATIONS

SOD, superoxide dismutase; CAT, catalase; GP_x, glutathione peroxidase; AWT, Aniridia-Wilms tumor; AC, acatalasia; GSH, glutathione; MEM, minimal essential media; SDS, sodium dodecylsulfate.

0006-291X/88 $1.50

these genes. Hereditary abnormalities affecting these genes are expected to allow insights into their function. For example, the presence of three genes for Cu,Zn-SOD in Down's Syndrome has been suggested to play a role in the etiology of this disease (12). Aniridia-Wilms tumor (AWT) in most cases is associated with a deletion of the CAT gene which maps closely to the putative AWT-gene (13,14). However, no relationship has been established between the deletion of the CAT-gene and the development of AWT.

We have investigated the molecular basis of the hereditary human disease Acatalasia (AC). This rare disease is characterized by low or no catalase (CAT) activity in all body tissues. It is often but not always accompanied by progressive gangrene of the month cavity but otherwise there are no striking clinical symptoms (15,16,17). As indicated above the cellular antioxidant defense is composed of multiple interacting components and an abnormality in one may entail changes in others. Therefore, we have measured glutathione (GSH) concentrations and compared the activities, protein concentrations and stationary mRNA levels of the three antioxidant enzymes CAT, SOD and GP_x in fibroblasts from a Swiss (AC64) and a Japanese (AC65) patient and several normal controls.

MATERIALS AND METHODS

Cell cultures and growth medium.

The AC- and normal fibroblast strains were received from the Human Genetic Mutant Cell Repository (Camden, N.J.). The AC strains had been originally deposited by Dr. R.S. Krooth (18). The cells were cultured in monolayers on plastic dishes in Minimal Essential Medium (MEM) supplemented with 20% fetal calf serum (Gibco, Geneva) and maintained in a humidified incubator at 37°C with 5% CO_2 in air.

Survival of colony forming ability following treatment with increasing concentrations of H_2O_2 was measured as described previously. 500 or 1000 cells were plated into 6 cm petri dishes and treated after 14 hrs for 30 min with increasing amounts of H_2O_2 in MEM supplemented with 15% fetal calf serum. The culture medium was changed following treatment and colonies counted after 15 to 20 days of incubation.

Determination of antioxidant enzyme activities and glutathione concentrations.

Enzymatic activities of SOD, CAT and GP_x and total glutathione levels in normal and AC human fibroblasts were determined in extracts from subconfluent cultures. Monolayer cultures were washed twice with cold phosphate-buffered saline and the cells collected with a rubber policeman and sedimented for 4 minutes at 1600 x g. Cell pellets were resuspended in 500 ul of potassium phosphate buffer pH 7.4 containing 0.5% Triton X-100, sonicated and frozen after aliquots were removed for protein determination according to Peterson (19). For CAT, samples were spun 5 minutes at 800 x g and the supernatant assayed according to Clairborne (20). Units are expressed as cyanide inhibitable micromoles of hydrogen peroxide consumed per minute per milligram of protein. For SOD and GPx, extracts were first passed through Sephadex G-25 mini-columns. The eluate was analyzed for SOD activity accor-

ding to Paoletti et al (21) and GPx activity according to Günzler and Flohé (22). Cu,Zn-SOD and Mn-SOD activities were distinguished by pretreatment of the extracts with SDS according to Geller and Winge (23). One unit of total SOD is defined as the amount that inhibits by 50% the rate of control NADH oxidation per mg protein. Total glutathione was determined according to Griffith (24) . Units correspond to umoles NADPH oxidized per minute per mg protein using GSH equal to 1 mM and a medium selenium source of 20% Gibco fetal calf serum.

Immunoblot analysis of catalase, Mn-superoxide dismutase and Cu,Zn-superoxide dismutase in lysates from AC- and normal fibroblasts.

Lysates prepared as described above were mixed with an equal volume of 2 x SDS-tris pH 6.8-glycerol sample buffer, boiled for 3 minutes, frozen, and again boiled 3 minutes before application to a 12.5% SDS polyacrylamide gel (40 ug protein per lane) (25). The gels were electrophoresed overnight at room temperature (60 volts) and transferred to a Millipore Immobilon PVDF transfer membrane by electrotransfer. Following transfer and 2 hours incubation in triton X-100-tris pH 7.4-Denhardt saturation buffer, the filters were reacted overnight with rabbit anti-human CAT- and Mn-SOD antibody at 4°C. After washing with tris buffered saline, the filters were reacted for 2 hours at room temperature with I^{125}-labeled goat anti-rabbit antibody, washed and exposed to film. For Cu,Zn-SOD, filters were first incubated with guinea pig anti-human Cu,Zn-SOD antibody, washed, incubated with rabbit anti-guinea pig antibody, washed and reacted with I^{125}-labeled goat anti-rabbit antibody as above.

Southern-blot analysis of DNA from AC- and normal strains with a human CATcDNA probe.

The DNA was extracted, digested with Pst I and electrophoresed in 0.8% agarose gels according to standard conditions (26). The DNA was transferred to nitrocellulose essentially as described by Southern (27). The filters were probed with a ^{32}P-labeled nick-translated SP6 probe containing the 1250 bp Hind III to Pvu II fragment of the human CAT gene.

Northern-blot analysis of steady-state mRNA levels of CAT, Cu,Zn-SOD and GPx in normal and AC-fibroblasts.

mRNA levels of CAT, SOD and GPx in normal and AC fibroblasts. Total RNA was extracted and prepared according to the method of Gerstenfeld et al (28). RNA (5 or 10 ug per lane per sample) was then electrophoresed through a 1.4% agarose/formaldehyde denaturing gel and transferred to Gene Screen as described (26). The filter was simultaneouly hybridized with P^{32}-labeled transcripts from SP6 human CAT- and SP6 human Cu,Zn-SOD recombinants using 1 x 10^6 cpm per ml of hybridization fluid. The SP6 CAT recombinant contained the 1250 bp Hind III to Pvu II fragment of the human CAT gene and the SP6 Cu, Zn-SOD recombinant the 450 bp Alu to Taq fragment of the human Cu,Zn-SOD gene. A second blot was hybridized with a SP6 bovine GPx probe, also at 1 x 10^6 cpm per ml. The SP6 GPx recombinant contained the 800 bp BamI to Bgl I fragment of the bovine GPx gene which had been obtained from Dr. G. Mullenbach, Chiron Corp., California. The prehybridization, hybridization and washing procedures were according to Khandjian (29) with a final wash of 0.2x SSC at 70°C. Ribosomal markers are indicated.

RESULTS AND DISCUSSION

We first compared the activities of the antioxidant enzymes and glutathione (GSH) concentrations between normal and AC-fibroblasts which were

Table 1

Constitutive SOD, CAT and GPx enzymatic activities and total glutathione levels in normal and acatalasia human fibroblasts

Fibroblast Strain	CAT (U/mg protein)[1]	CuZn-SOD (U/mg protein)[2]	Mn-SOD (U/mg protein)[2]	GPx (U/mg protein)[3]	Total glutathione (nmole/mg protein)
NF3229	8.4 ± 0.8	9.0 ± 0.7	3.4 ± 0.5	0.090 ± .014	6.6 ± 0.5
NF1187	9.0 ± 1.4	11.7 ± 1.0	2.1 ± 0.5	0.120 ± .017	5.1 ± 0.6
NF1221	9.2 ± 1.5	6.1 ± 1.4	1.3 ± 0.3	0.082 ± .016	not done
AC64	0.3	7.3 ± 1.2	2.2 ± 0.3	0.078 ± .027	5.3 ± 0.8
AC65	0.3	1.7 ± 0.6	0.5 ± 0.3	0.066 ± .017	4.7 ± 0.6

[1] Units expressed as micromoles of hydrogen peroxide consumed per minute per milligram of protein.

[2] One unit is defined as the amount of SOD that inhibits by 50% the rate of control NADH oxidation per mg protein.

[3] One unit of GP_X is expressed as umoles NADPH oxidized per min per mg protein using $[GSH]_o$ equal to 1mM.

cultured in monolayers in MEM supplemented with 20% fetal calf serum. As presented in Table 1 the two AC strains were essentially devoid of CAT activity. Activities for Cu,Zn-SOD, Mn-SOD and GPx were present in the AC and normal strains. The very low levels of Cu,Zn- and Mn-SOD in AC65 fall below the range of inter-individual variations and may represent a characteristic of this strain.GSH-concentrations were comparable in all strains. The protein levels of the antioxidant enzymes were assessed by immuno-blots which are shown in Figure 1. The normal strains and AC64 contain CAT protein while AC65 is completely devoid of it. The band for AC64 is weaker than for the normal strains but possesses the same electrophoretic mobility on 12.5% SDS polyacrylamide gels. In contrast, Matsubara et al. (30) had observed lower mobility of CAT protein from two homozygous Swiss AC patients under different conditions of electrophoresis. The Cu,Zn- and Mn-SOD proteins were present in all strains but the bands were weaker for AC65 in agreement with the low activities of these enzymes in this strain.

Our results point towards genetic heterogeneity and a different molecular basis for the AC-phenotype in AC64 and AC65. For the first, a mutation in the structural gene of the enzyme could explain the abnormality. An inactivating mutation could be located in the catalytic domain of the protein or disturb the conformation of the enzyme. Evidence for a structural alteration of the CAT protein of Swiss AC patients (30) and CAT from the liver of

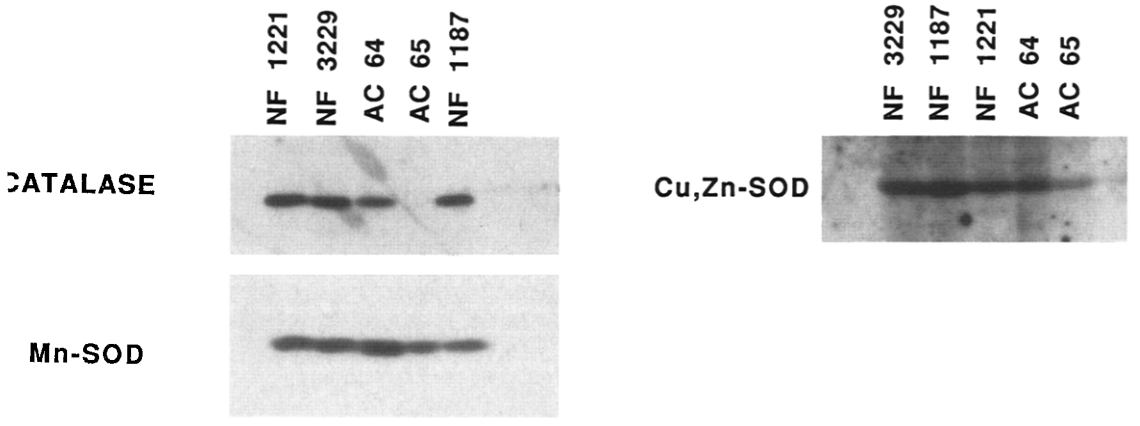
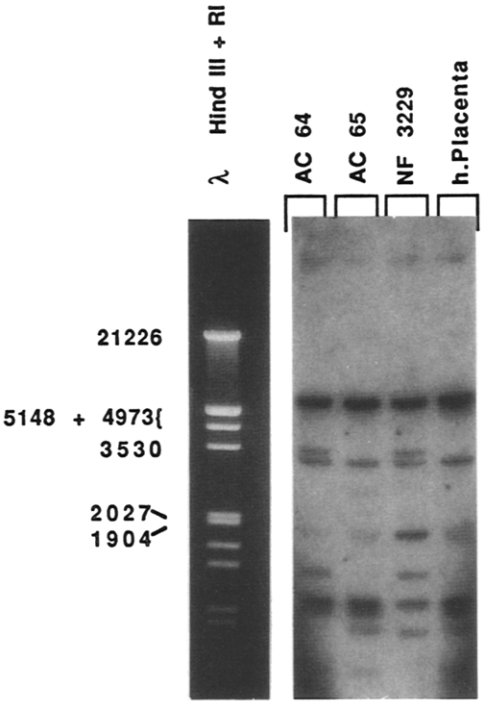


FIG. 1. Protein levels of CAT, Mn- and Cu,Zn-SOD in normal and AC fibroblasts by immunoblot analysis. Cell lysates were prepared as described in "Materials and Methods", mixed with SDS-glycerol buffer and applied to 12.5% SDS polyacrylamide gels. The electrophoresed material was electrotransferred to filters which were then reacted with rabbit anti-human CAT antibody, rabbit anti-human Mn-SOD antibody or guinea pig anti-human Cu,Zn-SOD antibody, respectively. For CAT and Mn-SOD the filters were incubated with ^{125}I-goat anti-rabbit antibody and for Cu,Zn-SOD with rabbit anti-guinea pig antibody.

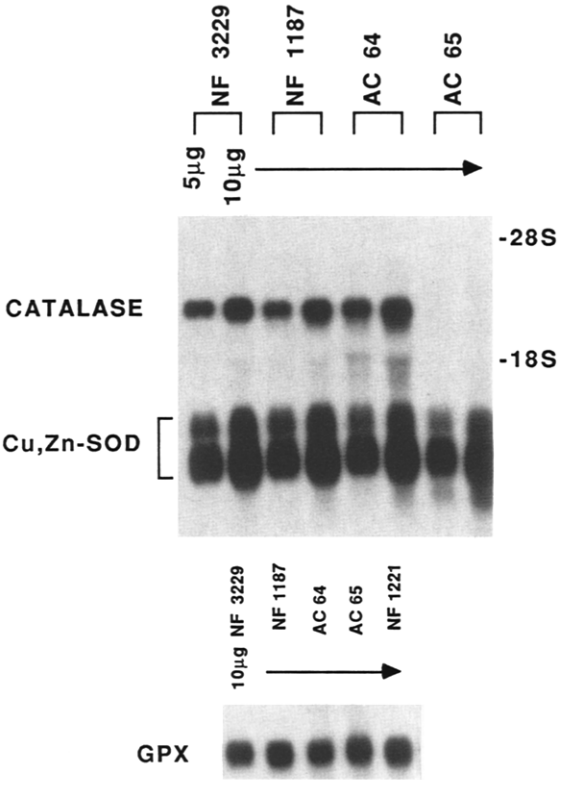
acatalasemic mice (31) has been reported. The enzymes were abnormally sensitive to denaturation. The fact that the enzyme from acatalasemic mice had lost its catalatic activity (which uses water as hydrogen donor) but not its peroxidatic activity (which uses organic hydrogen donors) pointed towards a disturbance of the tetrameric subunit structure of the enzyme (31). For AC65 several alternatives have to be considered: (1) rearrangement or deletion ofpart or all of the CAT gene (2) inactivation of the transcription of the CAT gene (3) decreased stability of the CAT mRNA or deficient m-RNA transport (4) a defect in the translation of CAT mRNA.

In order to distinguish between these alternatives we first analyzed the DNA from two normal and the two AC-strains for the presence of the CAT-gene by Southern blot hybridization with a human CAT cDNA probe.

Figure 2 shows that all strains possessed apparently normal CAT genes. No evidence was obtained for large deletions nor rearrangements of the gene in AC64 and AC65. The observed Pst I restriction fragment length polymorphism is unrelated to the Acatalasia phenotype. Similar fragmention is observed for AC65 and human placenta, on the one hand, and AC64 and NF 3229, on the other (see also ref 14). The stationary mRNA levels for the genes of the antioxidant enzymes were determined by Northern blot hybridization. From the results in Figure 3 it is evident that AC64 contained normal amounts of CAT mRNA while this message was completely absent in AC65. Northern blots with SP6 probes containing human Cu,Zn-SOD cDNA (7) and bovine GPx cDNA (11), respectively, revealed no significant differences in the mRNA levels for these genes between the normal- and AC strains. These results suggest a



(2)



(3)

FIG. 2. Southern blot analysis (27) of DNA from AC-strains and normal controls. The DNA was digested with Pst I and 5 ug aliquots were applied to each track. A nick-translated SP6 probe containing a fragment of human CAT cDNA (PvuII- Hind III, ref. 8) was used as probe.

FIG. 3. Northern-blot analysis of steady-state messenger RNA concentrations of CAT, Cu,Zn-SOD and GPx in normal and AC-fibroblasts. Total RNA was extracted, electrophoresed and transferred to filters as described in "Materials and Methods". The filters were hybridized with ^{32}P-labeled transcripts from SP6 human CAT- or SP6 human Cu,Zn-SOD recombinants, respectively. Ribosomal markers are indicated.

block in transcription in AC65 but do not exclude diminished messenger stability. The fact that AC65 possessed low activity and protein levels for CuZn-SOD despite close to normal concentrations of mRNA could be due to inefficient translation of this message.

For AC64, but not AC65, it is conceivable that the mutated CAT-protein has retained some peroxidatic activity as was the case for acatalasemic mouse liver (16,31). However, no difference in sensitivity to killing by H_2O_2 (colony forming ability) was discernible between the two AC strains and their survival curves fell into the range of the normal strains NF 1221 and 1187 (data not shown). In contrast, SV40 transformed fibroblasts from acatalasemic mice were hypersensitive to killing by H_2O_2 (32) and the growth of

human AC fibroblasts was inhibited more severely by H_2O_2 than of normal controls (33). Our data suggests that other systems of antioxidant defense, in particular GSH and GPx,determine the cellular resistance to killing by moderate doses of H_2O_2. It should be noted that GSH and GP_x which destroy H_2O_2 were comparable for all strains tested (see Table 1). In support of this notion it is remarkable that AC-patients usually exhibit only mild clinical symptoms (15,16). In strain AC65 which is completely devoid of CAT-protein the very low levels of Cu,Zn- and Mn-SOD may represent a compensatory adaptation. Although SOD is usually thought of as a detoxifying enzyme of active oxygen its activity can be harmful because it produces H_2O_2. This was clearly demonstrated in cells which contained an increased number of Cu,Zn- SOD genes introduced by transfection and may play a role in the pathogenesis of Down's Syndrome (12). Understanding the cells response to a prooxidant state in terms of a hierarchy of compensatory responses may be of importance for the elucidation of the role of active oxygen in several human pathologies.

ACKNOWLEDGEMENTS

We are grateful to Drs. R. Gravel, T. Hashimoto, Y. Groner, and P. Harrison for supplying cDNA probes for the antioxidant genes studied in this work and Dr. A. Baret for antibodies against human Cu,Zn- and Mn-superoxide dismutase and catalase. This work was supported by the Swiss National Science Foundation and the Swiss Association of Cigarette Manufacturers.

REFERENCES

1. Fridovich, I. (1978) Science 201, 875-880.
2. Clark, I., Cowden, W., and Hunt, N. (1985) Medicinal Research Reviews 5, 297-332.
3. Cerutti, P. (1985) Science 227, 375-381.
4. Oberly, L. and Buettner, G. (1979) Cancer Res. 39, 1141-1149.
5. Vuillaume, M., Calvayrac, R., Best-Belpomme, M., Tarroux, P., Hubert, M., Decroix, Y., and Sarasin, A. (1986) Cancer Res. 46, 538-544.
6. Jones, G., Sanford, K., Parshad, R., Gautt, R., Price, F., and Tarone, R. (1985) Br. J. Cancer 52, 583-590.
7. Sherman, L., Dafni, N., Lieman-Hurtwitz, J., and Groner, Y. (1983) Proc. Natl. Acad. Sci. 80, 5465-5469.
8. Korneluk, R., Quan, F., Lewis, W., Guise, K., Willard, H., Holmes, M., Gravel, R. (1984) Isolation of human fibroblast catalase cDNA clones. J. Biol. Chem. 259, 13819-13823.
9. Furuta, S., Hayashi, H., Hijikata, M., Miyazawa, S., Osumi, T., and Hashimoto, T. (1986) Proc. Natl. Acad. Sci. 83: 313-317.
10. Chambers, I., Frampton, J., Goldfarb, P., Affara, N., McBain, W. and Harrison, P. (1986) EMBO J. 5, 1221-1227.
11. Mullenbach, G. personal communication.
12. Elroy-Stein, O., Bernstein, Y., and Groner, Y., (1986) EMBO J. 5, 615-622.

13. Orkin, S. Cancer Inv. 3: 465 (1984).
14. Heyningen, V., Boyd, P., Seawright, A., Fletcher, J., Fantes, J., Buckton, K., Spowart, G., Porteons, D., Hill, R., Newton, M., and Hastie, N. (1985) Proc. Natl. Acad. Sci. 82, 8592-8596.
15. Takahara, S. (1968) in "Hereditary Disorders in Erythrocyte Metabolism" 1, (eds. Beutler), p. 21, Grune & Stratton, N.Y.
16. Aebi, H. and Suter, H. (1971) "Acatalasemia" in "Adv. in Human Genetics" 2,(eds. H. Harris and K. Hirschhorn) Plenum Press, N.Y. p. 143.
17. Feinstein, R. (1973) "Studies of Acatalasemia" in "Enzyme Therapy in Genetic Diseases" Birth Defects: Original Article Series 9, 55-61.
18. Krooth, R.S. (1967) In Vitro 2, 82-96.
19. Peterson, G.L. (1977) Analyt. Biochem. 83, 346-356.
20. Clairbone, A. (1985) "Catalase Activity" in "Handbook of Methods for Oxygen Radical Research" (ed. R. Greenwald), p 283-284 CRC Press FL.
21. Paoletti, F., Aldinucci, D., Mocali, A., and Caparrini, A., (1986) Analyt. Biochem. 154, 536-541.
22. Günzler, W. and Flohé, L. (1985) "Glutathione peroxidase" in "Handbook of Methods for Oxygen Radical Research" (ed. R. Greenwald) p 285-290 CRC Press FL.
23. Geller, B. and Winge, D. (1983) Anal. Biochem. 128, 86-92.
24. Griffith, O. (1980) Anal. Biochem. 106, 207-212.
25. Laemmli, U. (1970) Nature 227, 680-685.
26. See in "Modern Cloning" (1982) (eds. T. Maniatis, E. Fritsch and J. Sambrook), Cold Spring Harbor Laboratory.
27. Southern, E. (1975) J. Mol. Biol. 98, 503-517.
28. Gerstenfeld, L., Crawford, D., Boedtker, H., and Doty, P. (1984) Mol. Cell. Biol. 4, 1483-1492.
29. Khandjian, E. (1986) Molec. Biol. Rep. 11, 107-115.
30. Matsubara, S., Suter, H., and Aebi, H., (1967) Experientia 22, 428-429.
31. Srivastava, S. and Ansari, N. (1980) Biochim. Biophys. Acta 633, 317-322.
32. Lewis, W. (1985) Somatic Cell. & Mol. Genetics 11, 319-324.
33. Sadomoto, M. (1966) Acta Med. Okayama 20, 193-196.