Biochemical and Biophysical Research Communications **257,** 163–167 (1999)
Article ID bbrc.1999.0423, available online at http://www.idealibrary.com on IDEAL®

Altered Levels of Primary Antioxidant Enzymes in Progeria Skin Fibroblasts

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Received February 25, 1999

Free radicals are involved in the aging process. In this study, the profile of primary antioxidant enzymes that scavenge reactive oxygen species (ROS) was examined for the first time in human skin fibroblasts from progeria, a premature aging disease. Altered levels of antioxidant enzymes were found in progeria cells. Basal levels of MnSOD were decreased in progeria cells as well as a blunted induction in response to chronic stress. This change may contribute to the accelerated aging process in progeria cells. In contrast, the levels of CuZnSOD showed no progeria-related change. Two H_2O_2 removing enzymes demonstrated a significant reduction in progeria cells: only 50% of normal CAT activity and 30% of normal GPX activity can be detected in progeria cells. This diminished H_2O_2 removing capacity in progeria cells may lead to an imbalance of intracellular ROS and therefore may play an important role in the development of progeria.

Key Words: **progeria; aging; antioxidant enzymes; MnSOD; CuZnSOD; catalase; GPX; free radicals.**

Hutchinson-Gilford Progeria syndrome (progeria) is a premature aging disease that occurs in the first decade of life (1, 2). Progeria patients have an extremely aged appearance, growth retardation, atherosclerosis, cardiovacular disease, etc. The average lifespan of progeria patients is 13 years, and death is often due to cardiovascular complications. Progeria is considered as a good aging research model (3). Understanding the molecular mechanism underlying progeria may help to further elucidate the molecular basis of normal aging.

Aging is a highly complicated process that has been suggested as a multifactorial network (4, 5). Free radicals, primarily reactive oxygen species (ROS), may play a central role in the interplay of this network and therefore ROS are involved in many aging theories (6–8). Three primary ROS are superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the extremely reactive hydroxyl radical ($^{\cdot}OH$) that can be formed by H_2O_2 reacting with transition metals. Because of their high reactivity, ROS are capable of damaging most cellular macromolecules. One of the cellular defenses against ROS toxicity is a series of antioxidant enzymes (AE). The primary AE includes superoxide dismutase (SOD, the two intracellular forms are copper and zinc-containing SOD [CuZnSOD] and manganese-containing SOD [MnSOD]), catalase (CAT), and glutathione peroxidase (GPX). SOD as a first line of defense reduces $O_2^{\cdot -}$ to H_2O_2. CAT and GPX continue to process to convert H_2O_2 to water. The rate of the ROS generation and the level of AE defense contribute to the overall level of oxidative stress which can exert a broad regulatory influence on different levels of gene expression (9, 10).

Several lines of experimental evidence support a role of AE in aging. Overexpression of CuZnSOD and CAT increased the lifespan in transgenic *Drosophila* (11). *Drosophila* that had overexpressed CuZnSOD only in their motor neurons also showed an increased lifespan (12). Homozygous MnSOD knockout mice died within first 10 days of life (13). These results implied a strong link between the ARE levels and aging *in vivo.* The profile of ARE in progeria cells has not been reported. It would be of great interest to examine these enzymes. Here we report for the first time that progeria fibroblasts have altered AE.

MATERIALS AND METHODS

Cell lines. Two progeria and two normal skin fibroblast cell lines were purchased from the Cornell Cell Repositories (Camden, NJ). They are progeria cell line P1 (AG 03513 from a 13 years old progeria patient) and its age-matched normal control N1 (GM 02037 from a 13 years old normal donor). Progeria cell line P2 (AG 10750 from a 9 years old progeria patient) and its age-matched normal control N2 (GM 08398 from a 8 years old normal donor). All donors were Caucasian and male. Cells were grown in Eagle's minimum essential medium supplemented with 15% fetal bovine serum, 1mM pyruvate, and 0.1mM nonessential amino acids. N1 and P1 were used in passages 15–21. N2 and P2 were used in passages 10–15.

ECL Western blotting. Cells were harvested by scraping, and sonicated in 0.05 M potassium phosphate buffer (PB, pH 7.8) in ice

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0006-291X/99 $30.00

water with six bursts of 30 s each using a Vibra Cell Sonicator with a Cup Horn tip at maximum power. The protein concentration was quantitated using the Bio-Rad Protein Assay solution according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The protein were denatured with SDS loading buffer (2% SDS, 10% glycerol, 3% β-mercaptoethanol in Tris buffer, pH 6.8) at 95°C for 5 min, and separated on a SDS-12.5% polyacrylamide gel with a 5% stacking gel in SDS-Tris-glycine running buffer. The protein was then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in Tris-glycine-methanol buffer. After blocking of the membranes with 5–10% milk at room temperature for 1 h, the primary antibody (MnSOD and CuZnSOD antibodies were made in our lab, CAT antibody was purchased from Athens Research & Technology Inc., Athens, GA) reaction was carried out at 4°C overnight. The secondary antibody, anti-rabbit IgG conjugated with horseradish peroxidase (Boehringer Mannheim), was diluted by 1:10000 and reacted with the blots at room temperature for 1 h. The bands were revealed in x-ray films by enhanced chemiluminescence (ECL) staining (Amersham, Arlington, IL), and quantitated by an AlphaImager densitometer (Alpha Innotech, San Leandro, CA).

Native immunoblotting. GPX protein assay was carried out by native immunoblotting since the GPX antibody (made in our lab) seems to recognize only native GPX protein. The electrophoresis process was the same as the native activity gel described below followed by electrotransfer of the protein onto a nitrocellulose membrane. The detection procedure was the same as ECL Western blotting.

Native activity gel. Cell samples were harvested, sonicated and protein quantitated in the same way as Western blotting. A 12.5% polyacrylamide gel was made without SDS. After electrophoresis in Tris-glycine buffer, specific activity bands were visualized by the different staining methods as described below. The SOD bands were visualized by saturating the gel with 2.45 mM nitro blue tetrazolium (NBT), 28 mM riboflavin, 28 mM tetramethylethylenediamine (TEMED) in 50 mM PB buffer (pH 7.8) for 20 min in the dark, and then illuminating the gel under a fluorescent light until achromatic bands appeared on a purple background. The CAT bands were visualized by rinsing the gel with water and saturating the gel with 0.003% H_2O_2 at room temperature for 10 min, and then staining the gel with 2% ferric chloride-2% potassium ferricyanide until achromatic bands appeared on a blue background. The GPX band was visualized by saturating the gel first with 1 mM GSH for 20 min and then with 0.008% cumene hydroperoxide at room temperature for 10 min. The staining was the same as for CAT. The activity bands were quantitated by an AlphaImager densitometer.

RESULTS AND DISCUSSION

MnSOD. A comparison of MnSOD immunoreactive protein level showed that a difference exists between normal and progeria cells within each age-matched group. Progeria cells have decreased MnSOD compared with their age-matched normal counterparts: P1 lower than N1 by about 65% and P2 lower than N2 by about 45% (Fig. 1A upper panel). The MnSOD activity level was consistent with the protein level (Fig. 1A lower panel).

Because of its highly inducible trait, MnSOD in aging cells may be variable in response to cell growth status and environmental conditions. Based on this consideration, the MnSOD immunoreactive protein assay was also carried out on three different groups of cell samples. Cells were seeded at 5×10^5cells/60 mm dish in 5 ml of medium. The seeding number for progeria cells was adjusted accordingly because of their lower plating efficiency. Group I cells were harvested 24 h after seeding. Group II cells were harvested on day 6 after seeding with refeeding every other day. Group III cells were also harvested on day 6, but given only 2 ml of medium without refeeding. A significantly higher level of MnSOD protein was found in group III (Fig. 1B). Though all cell lines in this group showed an increased expression, both normal control cells showed much higher MnSOD level than their progeria counterparts. This result indicates that under group III conditions (no refeeding), MnSOD expression is highly induced in normal cells, but much less so in progeria cells.

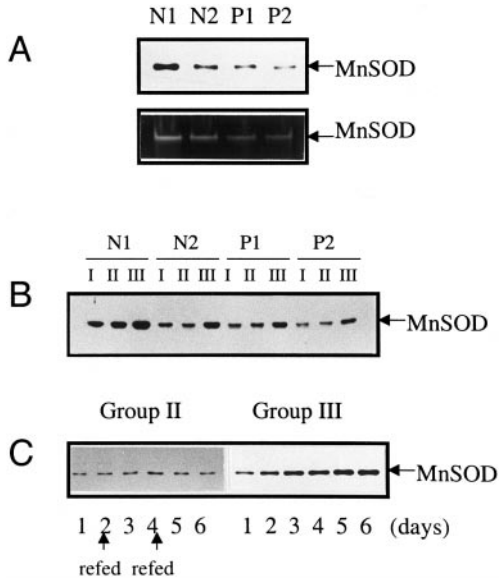

FIG. 1. The basal level of MnSOD is decreased in progeria cells as well as the induced levels in response to chronic stress within each age-matched group (P1-N1, P2-N2). (A) Western blot (upper panel, loaded with 20 μg protein/lane) and activity gel (lower panel, loaded with 150 μg protein/lane) show a decreased basal level of MnSOD protein (22 kDa) and activity in progeria cells. (B) Western blot shows a reduced induction of MnSOD expression in response to chronic stress in progeria cells. Group I cells were harvested 24 h after seeding. Group II cells were harvested on day 6 with refeeding of 5 ml of medium every other day. Group III cells were also harvested on day 6 but were grown in 2 ml of medium without refeeding (chronic stress). (C) Western blot shows that the induction of MnSOD came from stress and not from cell confluence. The same number of normal cells was grown under group II or group III conditions. Cells all reached confluence on day 3 after seeding. Data shown are representative results of three replicate experiments.

Previous work has shown that the induced MnSOD expression in normal cells is correlated with cell confluence (14). There was indeed a difference between normal and progeria cells in terms of confluence. Under our experimental conditions, normal cells reached 100% confluent on day 3, whereas progeria cells grew in clusters. But confluence is an unlikely reason for highly induced MnSOD expression in these cells, because within progeria cell clusters, cells also contacted

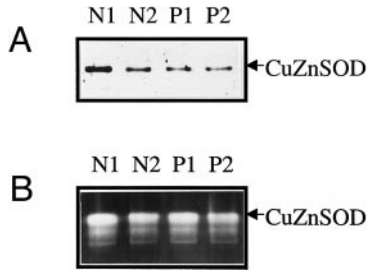

FIG. 2. CuZnSOD protein (16 kDa) is higher in N1 cells, but similar in the other cell lines (A. Western blot loaded with 20 μg protein/lane). CuZnSOD activity shows a decrease in N2 and P2, but not progeria-related (B. activity gel loaded with 150 μg protein/lane). The results are representative of three replicate experiments.

tightly with each other. A comparative experiment was conducted to examine a possible role of cell confluence on MnSOD induction. The normal cell line was seeded at 5×10^5/60 mm dish, and divided into two groups as group II and III mentioned above. Cells were then harvested daily over a 6-day period for Western analysis. Both groups reached 100% confluence on day 3. Figure 1C shows that MnSOD expression was not induced in group II which was refed with 5 ml of medium every other day. The induction of MnSOD expression occurred in group III which grown in 2 ml of medium without refeeding. The result rules out, at least in normal cells, the role of cell confluence in MnSOD induction, and suggests that chronic stress may build up gradually when cells were not fed. Normal cells respond to the chronic stress by inducing high expression of MnSOD and possibly other protective proteins. Progeria cells seem to have a certain level of deficiency in this type of stress response. This decreased induction of MnSOD expression in response to chronic stress implies that there may be an altered signal transduction pathway in progeria cells.

A question regarding protein content per cell was considered. Since our Western results were compared on the basis of equal protein loading, the comparison may not be accurate if cell lines have different protein content per cell. Progeria fibroblasts seem smaller than normal fibroblasts when attaching to dishes, but we observed that they have the similar size after trypsinization. Protein assay with the same cell number showed that the four cell lines had a very similar protein content per cell.

MnSOD is located in mitochondria, which are a major intracellular source of ROS. It is generally believed that MnSOD plays a pivotal role in detoxification of oxygen, and therefore changed MnSOD may play an important role in aging. So far, the correlative studies did not give a clear-cut picture due to the inconsistency of the reports. We believe that when evaluating MnSOD level, the inducibility should also be examined. MnSOD may contribute to aging not only through its basal activity, but also through its decreased induction in response to stresses. The lower level of MnSOD induction in progeria cells may indicate less capacity to adapt to changes in the cellular environment. Progeria patients have been reported to be insulin resistant (16), in malnutrition (17), and very thin with a ratio of 12–15 kg in weight to ~100 cm in height (2). Therefore, the cells in progeria patients could be in fact glucose starved or under other types of stress. The decreased MnSOD induction in progeria cells in response to chronic stress may be an important factor in the accelerated aging process.

CuZnSOD. CuZnSOD immunoreactive protein was found to be higher in the normal cell line N1, but the other normal cell line N2 and the two progeria cells showed a similar protein level (Fig. 2A). A decreased CuZnSOD activity level was found in N2 and P2, but not progeria-related (Fig. 2B). CuZnSOD shows several bands on the activity gel. This phenomenon happens very often under our experimental conditions. CuZnSOD did not show induced expression under our experimental stress conditions.

CuZnSOD is a cytosolic enzyme responsible for removing $O_2^{\cdot-}$ produced by numerous cytosolic oxygenases. CuZnSOD is implicated in aging because of its correlation with neurodegenerative diseases like familial amyotrophic lateral scleross (ALS) (18, 19) and Down Syndrome (DS) (20), and also because of correlative studies which were done with the CuZnSOD transgenic model (11, 12). However, our data did not show a correlation of CuZnSOD with progeria.

CAT. CAT demonstrated a constant level in both protein and activity under the different growth conditions mentioned above. Compared with normal counterparts, progeria cells had a reduced level of CAT protein by about 50% (Fig. 3A). CAT activity in progeria cells was also reduced to approximately 50% of normal level as shown on activity gels (Fig. 3B).

The aging-related decrease in CAT activity has been reported in cultured skin fibroblasts derived from aged persons (21), and in blood from patients with Parkinson's disease (PD) and stroke (22). It was also found in liver, brain, prostate, and retina from rat and mouse aging models (23–26). Since in most mammalian cell

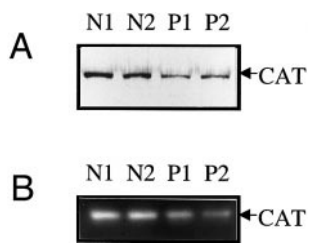

FIG. 3. CAT is reduced in progeria cells by about 50% in both protein (60 kDa) level (A. Western blot loaded with 20 μg protein/lane) and activity level (B. activity gel loaded with 100 μg protein/lane). The results are representative of three replicate experiments.

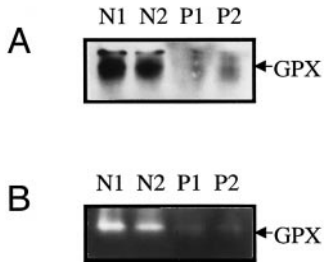

FIG. 4. GPX is significantly decreased in progeria cells in the levels of protein (21 kDa) (A. native immunoblot loaded with 300 μg protein/lane) and activity (B. activity gel loaded with 100 μg protein/lane). The results are representative of three replicate experiments.

types, CAT exists primarily within peroxisomes and removes the H_2O_2 generated by the long-chain fatty acid beta-oxidation pathway, decreased CAT may contribute to the aging process by affecting lipid metabolism and membrane function. Also the peroxisomal H_2O_2 may leak to the cytoplasm to change the overall intracellular redox status. Based on the above considerations, decreased CAT in progeria cells may play an important role in the accelerated aging process.

GPX. The GPX we detected is selenium-dependent GPX1. GPX1 immunoreactive protein assay was carried out by native immunoblotting instead of routine Western blotting. The results show that progeria cells have much less GPX protein (Fig. 4A) and activity (Fig. 4B) than normal controls. Only about 30% of normal GPX protein and activity levels were detected in progeria cells. This pattern was constant under the different experimental conditions mentioned above.

Compared with MnSOD, CuZnSOD and CAT, GPX1 shows a rather unique character. First, GPX1 is located in more than one cellular compartment including cytoplasm, mitochondria and nucleus (27). Second, GPX1 detoxifies more than one substrate including H_2O_2 and organic hydroperoxides. Third, GPX1 function needs cofactors including the GSH system directly and NADPH system indirectly. Fourth, the regulation of GPX1 gene expression is very complex occurring at different levels. It is recently identified that GPX1 gene is also a p53 target gene (28). Therefore, GPX1 is a far more complicated enzyme. This may be a reason why so many previous attempts were made to correlate GPX with aging and could only give a rather confusing picture. Our data indicates that decreased GPX in progeria cells is likely to play an important role in the progeria process.

In summary, the profile of ARE in progeria cells was found to be altered. Compared with normal controls, a basal level of MnSOD is decreased in progeria cells within each age-matched group. A decreased induction of MnSOD expression in response to chronic stress is also found in progeria cells within each age-matched group. CuZnSOD shows no progeria-related change. CAT is reduced in both protein and activity levels in progeria cells by 50%. GPX shows a significant reduction in both protein and activity in progeria cells by 70%. Taken together, in the primary ARE defense system of progeria cells, two downstream enzymes (CAT and GPX) that remove H_2O_2 show more significant change than two upstream enzymes (MnSOD and CuZnSOD) that convert $O_2^{\cdot -}$ to H_2O_2. This altered AE profile may lead to an imbalance of intracellular ROS, and further lead to a changed oxidative stress level. Therefore, diminished H_2O_2 removing capacity may play an important role in the progeria process. The decreased MnSOD basal level and the induction of MnSOD expression in response to stress may also contribute to this process.

ACKNOWLEDGMENTS

This work was supported by NIH Grants P50-DE10758 and P01-CA 66081 to L.W.O.

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