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LOWERED LEVEL OF TRANSLATABLE MESSENGER RNAs FOR MANGANESE SUPEROXIDE DISMUTASE IN HUMAN FIBROBLASTS TRANSFORMED BY SV 40

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The activity of manganese superoxide dismutase (MnSOD) revealed by specific staining after gel electrophoresis of cell extracts, is decreased in human fibroblasts transformed by SV 40. The decrease in enzyme activity is attributable to decreased amount of enzyme protein as determined by radial immunodiffusion. Total fibroblast RNAs were translated in the presence of (^{35}S) methionine in a cell-free translation system and the neo synthesized proteins submitted to immunoprecipitation with an anti MnSOD antiserum. Gel electrophoresis of the immunoprecipitated material followed by fluorography shows that MnSOD is translated as a peptide which is 2000 daltons larger than the mature enzyme subunit. This precursor (pre-MnSOD) is processed in vitro to mature MnSOD by the action of an isolated mitochondrial preparation. Levels of translatable MnSOD mRNA in normal and SV 40 transformed cells were compared in terms of the radioactivities incorporated into pre MnSOD bands. The results indicate that the decreased amount of MnSOD in SV 40 transformed fibroblasts is due to a decreased level of translatable mRNA for MnSOD.

Superoxide dismutases (superoxide : superoxide oxidoreductase E.C. 1.15.11. SOD) are enzymes which are thought to play a vital role in the protection of aerobic cells against oxygen toxicity by catalysing the dismutation of the superoxide anions (O_2^-) according to the reaction :

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Eucaryotic cells contain two types of SODs ; one contains copper and zinc (CuZnSOD) and is localized in the cytosol and in the intermembrane space of mitochondria, the other contains manganese and is found in the matrix of mitochondria (MnSOD) (1-3). MnSOD is encoded by nuclear DNA, located on chromosome 6 in human (1-4). Its molecular weight is 80 000 and it is composed of four identical subunits.

A decrease in MnSOD activity has been reported in various tumors (5-9) and in human fibroblasts transformed by SV 40 (10). In these cells, gel electrophoresis

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pattern of SODs shows that the band corresponding to the mitochondrial MnSOD is diminished or absent (10). At our knowledge, the molecular basis of this observation has not been investigated.

In this paper, we show that, in human SV 40 transformed fibroblasts, the decrease in MnSOD activity corresponds to a decrease in the protein amount, and this is largely or wholly due to a decreased level of functional mRNA for MnSOD, of which the translated product is a precursor form of MnSOD.

MATERIAL AND METHODS

MnSOD was purified from human liver according to McCord et al. (11). The purified enzyme was radio-labelled by reductive alkylation with (^{14}C) formaldehyde and cyanoborohydride (12). Antisera were prepared against native and denaturated MnSOD. Denaturation of MnSOD was done by heating the enzyme during five minutes at 100°C in a phosphate buffer solution (pH 7.4, 0.1 M) containing 1 % SDS and 1 % mercaptoethanol. Rabbits were injected subcutaneously three times at two weeks intervals with 0.1 mg of the purified MnSOD mixed with Freund's complete adjuvant and boosted twice every three weeks. The antisera were used without any further purification.

Normal and SV 40 transformed fibroblasts originating from the same skin biopsy were obtained from Dr. A. Sarazin (Institut de Recherches Scientifiques sur le Cancer, Villejuif, France). They were grown to confluence in a minimum Eagle's medium supplemented with 10 % fetal calf serum and then harvested with a rubber scraper. For the preparation of cell extracts, they were suspended in 20 mM Hepes pH 8.5, 140 mM NaCl, 0,5 % NP-40 and centrifuged at 11,000 g for 10 min to remove nuclei and cell debris. Protein was determined according to Lowry et al. (13). For the isolation of total RNA, which was carried out by a guanidine HCl procedure (14), they were suspended in 0,5 ml of the guanidine containing solution used at the first step of this procedure.

Electrophoresis of cellular extracts was performed in 10 % acrylamide gels in Tris glycine buffer pH 8.8 and superoxide dismutase activity detected by the method of Beauchamp and Fridovich (15).

Mancini radial immunodiffusion was performed as previously reported (16) with the antiserum against native MnSOD.

Cell-free protein synthesis in a nuclease-treated rabbit reticulocyte lysate was carried out in the presence of (^{35}S) methionine as described in (17). Immunoprecipitation of translated products was performed by diluting the reaction mixture 3 fold with immunoprecipitation buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 9.0, 0.05 % Nonidet P-40) and adding 5 µl of antiserum. After an incubation of 16 hr at 4°C, 100 µl of a 10 % suspension of Protein A sepharose (CL4B, Pharmacia Fine Chemicals) was added and incubated for 30 min at 23°C. The mixture was centrifuged at 11,000 g for 2 min and the pellet washed four times with the incubation buffer. After the final washing the pellet was dissociated with SDS (18) and subjected to SDS-polyacrylamide (5 % to 18 % gradient) gel electrophoresis. Gels were treated with EN^3 HANCE (New England Nuclear), dried and fluorographed by exposing Kodak XAR-5 film at - 80°C.

The processing of pre-MnSOD was carried out with mitochondria isolated from mouse liver as reported in (19). To 50 µl of translation mixture was added 20 µl of mitochondria suspension (50 µg of protein), 12 µl of bidistilled water and 70 µl of the cell free translation medium without (^{35}S) methionine. The mixture was incubated at 30°C during 60 min and freezed in liquid nitrogen in order to break mitochondria. After thawing the whole mixture was subjected to immunoprecipitation and the immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography.

(^{35}S) Methionine, (800 Ci/mmol), (^{14}C) methylated protein mixture and (^{14}C) formaldehyde were obtained from Amersham International (Amersham, U.S.).

RESULTS AND DISCUSSION

Figure 1 shows that, when the same amount of normal and SV 40 fibroblasts extracts are submitted to electrophoresis and stained for SOD activity, the intensity of the mitochondrial band is lower in SV 40 fibroblasts. This result confirms the previous observation of Yamanaka and Deamer (10) showing a decrease of MnSOD activity in SV 40 fibroblasts.

The Mancini immunodiffusion assay (Fig. 2) demonstrates that this decrease in activity corresponds to a decreased amount of MnSOD protein in SV 40 fibroblasts. Standard curve with purified MnSOD allows to estimate that normal fibroblasts contain 36 µg of MnSOD per mg protein whereas SV 40 fibroblasts contain 4.7 µg of MnSOD per mg protein.

In vitro translation experiments in the presence of (^{35}S) methionine (Fig. 3) show that essentially two peptides are immunoprecipitated with the antiserum against denatured MnSOD. One has a molecular weight of 32,000, which is much larger than MnSOD whose the subunit molecular weight is 20,000. We have designated this peptide p32. Homologies between MnSOD and p32 are suggested by the following data. First, when immunoprecipitation is performed with a limited amount of anti MnSOD antibody in the presence of excess unlabeled native MnSOD, appearance of p32 is suppressed competitively by MnSOD (Fig. 3c). Secondly, comparative peptide mapping using S. aureus V-8 protease reveals strong homologies between p32 and MnSOD (data not shown).

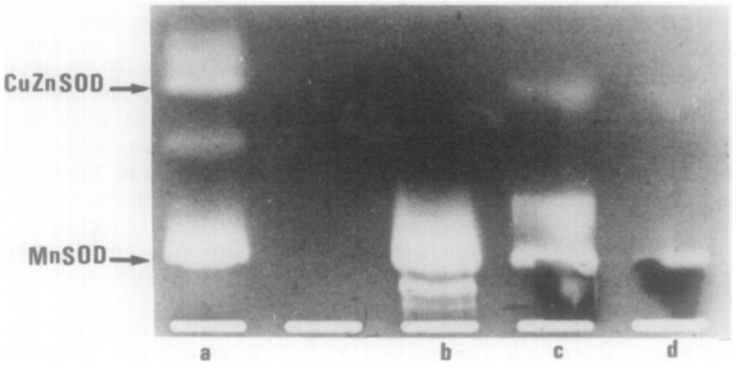

Figure 1. Polyacrylamide gel electrophoresis of tissue extracts stained for superoxide dismutase activity. Samples applied to the gel contained : a) Human liver extract : 10 µg protein ; b) Purified MnSOD : 1.75 µg ; c) Normal human fibroblast extract : 20 µg protein ; d) SV 40 transformed fibroblast extract : 20 µg protein.

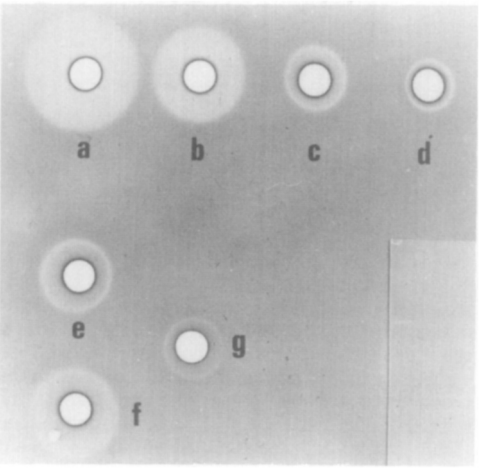

Figure 2. Mancini radial immunodiffusion assay of MnSOD. After overnight diffusion, the agar plate was stained for SOD activity. Upper wells contained purified MnSOD a : 0.700, b : 0.350, c : 0.176, d : 0.088 µg of protein. e) and f) contained respectively 10 µg and 5 µg of protein extract from normal fibroblasts, g) 23 µg protein from SV 40 transformed fibroblasts.

When the incorporation of (^{35}S) methionine by reticulocyte lysate is allowed to proceed for 60 min at 30°C and then mitochondria added to the reaction mixture for 30 min, we do not observe any change in the intensity of the p32 peptide. This indicates that there is no processing of p32 by mitochondria (Fig. 4). Thus, p32 is likely not a precursor of MnSOD. It is also observed (Fig. 4) that the levels of translatable messenger RNAs for p32

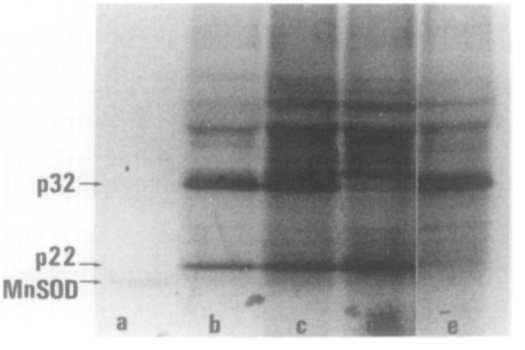

Figure 3. Electrophoresis in SDS polyacrylamide gel (5 % to 18 % gradient) of the immunoprecipitated products obtained by in vitro translation of total RNA purified from normal human fibroblasts. The translation was carried out at 30°C in 50 µl of the rabbit reticulocyte system in the presence of 350 ng of total RNA. Immunoprecipitation was carried out with an anti serum against denaturated MnSOD. a) MnSOD labeled by reductive alkylation with (^{14}C) formaldehyde ; b,e) immunoprecipitated products after a translation time of 30 min at 30°C ; c,d) immunoprecipitated products after a translation time of 90 min at 30°C ; d) immunoprecipitation in the presence of 35µg of native purified MnSOD ; e) immunoprecipitation in the presence of 35µg of purified MnSOD previously heated at 100°C during 5 min.

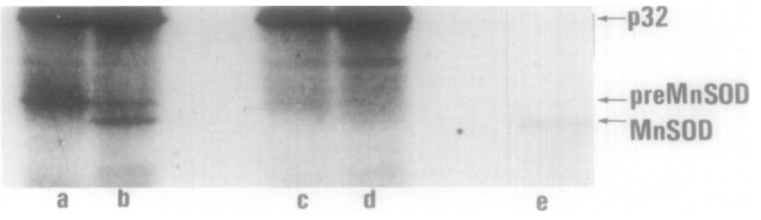

Figure 4. Comparison between the immunoprecipitated products obtained after translation of total RNA from normal fibroblasts and SV 40 transformed fibroblasts. Processing by isolated mouse liver mitochondria. Translation of 250 ng of total RNA was carried out at 30°C for 60 min in 50 µl of the rabbit reticulocyte lysate system ; then the mitochondria suspension (see Material and Methods) was added and the tubes incubated at 30°C for 60 min. Control tubes had the same treatment without addition of mitochondria. a and b : translation with RNA from fibroblasts ; a) without mitochondria ; b) with mitochondria ; c and d : translation with RNA from SV 40 transformed fibroblasts ; c) without mitochondria ; d) : with mitochondria ; e) : (^{14}C) labeled MnSOD.

are similar in both normal and SV 40 fibroblasts since, for equivalent (^{35}S) incorporation in total proteins, the intensities of p32 bands are similar. Other investigations are necessary in order to study further the homologies between MnSOD and p32 and to assign a possible biological function to p32.

An other peptide of 22,000 is synthesized in vitro from normal fibroblast mRNAs and immunoprecipitated by the antiserum SOD antibody (Fig. 3). A control experiment in which anti MnSOD antibody is replaced by non immun serum gives no radioactive band at this position (data not shown). The immunoprecipitation of this putative precursor of MnSOD (pre-MnSOD) is inhibited by adding to the immunoprecipitation medium purified MnSOD denaturated by heating 5 min at 100°C (Fig. 3e). Furthermore, when incubated with mouse liver mitochondria, there is a reduction in the amount of labeled pre-MnSOD and an appearance of a radioactive peptide that comigrates with the mature MnSOD subunit (Fig4b). All the results represent strong evidence that the 22,000 molecular weigh peptide is a precursor of MnSOD. Thus, like most of the mitochondrial enzymes (20-21), MnSOD is synthesized as a larger precursor, which is processed to the mature form of the enzyme by mitochondria. The 2000 daltons difference between the human pre-MnSOD and the mature subunit is similar to that described by A. Autor (22) between pre-MnSOD and MnSOD in Saccharomyces cerevisiae.

Fig. 4 shows that the translatable mRNAs for MnSOD in SV 40 fibroblasts is markedly decreased compared to normal fibroblasts. For the same incorporation of radioactivity into total proteins, which correspond to the translation of equivalent amounts of total RNAs (5 µg per ml of translation medium), the band corresponding to pre-MnSOD is hardly visible. When 15 µg of SV 40 fibroblast mRNA is translated, the pre-MnSOD becomes visible at the same place as for normal fibroblasts mRNA translation and is processed

to the mature mitochondrial MnSOD (data not shown). Thus, although in low amount, mRNAs for MnSOD are present in SV 40 fibroblasts and allow the synthesis of an apparently normal pre-MnSOD.

A number of studies have previously described changes in the level of various proteins in SV 40 transformed cells (23). Typically, between 5 % and 10 % of the 500 most abundant proteins are found to be quantitatively altered, and more proteins are repressed than induced. In this paper, we show that the amount of Mn SOD protein is depressed in human SV 40 transformed fibroblasts and that this decrease is due to a lowered amount of translatable mRNA for MnSOD. Interestingly, it has been observed in SV 40 transformed cells a decrease in catalase activity (24). As catalase is also involved in the metabolism of oxygen derivatives the possibility of a common regulatory mechanism leading to a decrease expression of these two enzymes in transformed cells can be hypothesized.

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