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Expression of Superoxide Dismutase in Basal Cell Carcinoma

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There have been no studies of the expression of superoxide dismutase(SOD) at the mRNA and protein level in skin cancers. Northern blot analysis and enzyme-linked immunosorbent assay (ELISA) were performed in order to analyze the expression of Cu, Zn-SOD and Mn-SOD in basal cell carcinomas (BCC) and normal skin (NS). The expression of Mn-SOD mRNA and protein was significantly higher in BCC than in NS. The expression of Cu,Zn-SOD, however, was high in BCC at the mRNA level, but not at the protein level. These results suggest that an increase in the expression of Mn-SOD relates to the development of BCCs.

Key Words: superoxide dismutase, Northern blot, enzyme-linked immunosorbent assay, basal cell carcinoma

Introduction

The number of patients with skin cancer has been increasing as the percentage of elderly people in the total population has become greater. Experimental studies of carcinogenesis have suggested that reactive oxygen species (ROS) play a role in tumor promotion[1,2)]. Also in the case of skin cancer, close attention has been paid to the roles of ROS produced by ultra violet ray and other factors. Superoxide is a representative of ROS and superoxide dismutase (SOD) is an enzyme which catalyzes the conversion of superoxide anions to hydrogen peroxide. It has been reported that SOD is also present in the skin and the major SOD isoenzymes found in the skin are Cu, Zn-SOD and Mn-SOD[3,4)]. Increased levels of Mn-SOD expression have been reported in some human cancers[5,6,7)], and serum Mn-SOD levels were found to increase in accordance with the malignant potential of disease. These studies suggested that the measurement of Mn-SOD in serum may provide a clinically useful marker.

Reduced SOD activity has been reported in skin cancers[4,8)], but no reports have demonstrated which SOD activity is reduced in the skin cancers. Furthermore, there have been no studies of SOD at the mRNA and protein level in skin cancer. We selected basal cell carcinoma (BCC) as a representative skin cancer type because it is one of the common cancers in the field of dermatology, and we examined the expression of Cu, Zn-SOD and Mn-SOD in these tumors and in normal skin (NS), using a Northern blot analysis, and an enzyme-linked immunosorbent assay (ELISA).

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Materials and methods

Nine basal cell carcinomas (BCCs) and six normal skin (NS) samples were used for Northern blot analysis and six BCCs and six NS samples were used for the enzyme-linked immunosorbent assay (ELISA). Samples were obtained during surgical operations at Nagasaki University Hospital. With regard to the sampling methods, we used the electric dermatome for NS and the resection and trimming method for BCC. We selected only mass-forming BCC and performed the trimming in such a way that the specimens used for RNA and protein extraction were not obtained from the infiltrating portion. The four corners of NS grafts were used as controls. These were obtained from the thighs of patients with various skin conditions using an electric dermatome for the purpose of skin implantation[9)]. As a result, the proportion of the epithelial component in the BCC samples was approximately the same as that

in the NS samples collected by electric dermatome. The clinical data were summarized in Tables.1 and 2. Half of the resected samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin for pathological examination. All tumors were examined histologically, and a pathological diagnosis was performed. For Northern blot analysis, the remainder of the resected samples were immediately immersed in cold phosphate buffered saline (PBS)(pH 7.4) containing 0.2% diethyl pyrocarbonate and trimmed. After trimming, one part of them was snap-frozen in liquid nitrogen and stored at -70℃ until RNA extraction. For ELISA, a portion of resected samples was immediately immersed in PBS and trimmed before being snap-frozen in liquid nitrogen and stored at -70℃ until protein extraction.

Table 1. Clinical details of patients for Northernblot analysis.

Patient number	Age/Sex	Pathology	Site
1	65 / M	BCC	Cheek
2	81 / M	BCC	Back
3	76 / M	BCC	Chin
4	73 / F	BCC	Nose
5	51 / M	BCC	Cheek
6	88 / F	BCC	Cheek
7	81 / F	BCC	Scalp
8	70 / F	BCC	Labium
9	65 / F	BCC	Abdomen
10	50 / F	NS	Thigh
11	63 / M	NS	Thigh
12	73 / M	NS	Thigh
13	89 / M	NS	Thigh
14	73 / M	NS	Thigh
15	69 / M	NS	Thigh

BCC: Basal cell carcinoma, NS: Normal skin

Table 2. Clinical details of patients for ELISA.

Patient number	Age/Sex	Pathology	Site
1	90 / F	BCC	Cheek
2	81 / F	BCC	Nose
3	83 / M	BCC	Forehead
4	74 / F	BCC	Cheek
5	73 / F	BCC	Cheek
6	88 / F	BCC	Nose
7	90 / F	NS	Thigh
8	80 / M	NS	Thigh
9	79 / M	NS	Thigh
10	63 / M	NS	Thigh
11	70 / F	NS	Thigh
12	77 / M	NS	Thigh

BCC: Basal cell carcinoma, NS: Normal skin,
ELISA: enzyme-linked immunosorbent assay

For Northern blot analysis, total RNA was extracted by the standard guanidium isothiocyanate/cesium chloride method[10], and the samples stored in ethanol at -70℃ until use. 40μg of the total RNA from each sample was electrophoresed on 1.0% agarose gel containing 8% formaldehyde. The volume and quality of RNA loaded were confirmed by the visualization of the agarose gel stained with ethidium bromide. The samples were then transferred onto a Hybond N nylon filter membrane (Amersham, Bucks, UK). The filter was baked for 2 hours at 80℃ in a vacuum. Human Cu, Zn-SOD and Mn-SOD cDNA fragments were generously provided by Dr. Taniguchi. For control hybridization on the amount of RNA, a complementary oligonucleotide probe for 28S ribosomal RNA was synthesized, and the sequence was as follows: 5'AACGATCAGAGTAGTGGTATTTCACC3'[11].

After prehybridization, hybridization was performed at 42℃ overnight using a ^{32}P-labelled human Cu, Zn-SOD or Mn-SOD probe. The hybridization solution contained 1% sodium dodecyl sulphate (SDS), 1M NaCl, 10% dextran sulfate and 100μg/ml de-natured salmon sperm DNA. The filter was washed several times with 0.1xSSC-0.1%SDS (1xSSC consists of 0.15M NaCl and 15mM sodium citrate) at 42℃. It was then exposed to a Fujix imaging plate for one day at room temperature and analyzed with a BAS 2000 bioimaging analyzer (Fujix, Tokyo, Japan). In the analysis of Mn-SOD, the summation of the values of each band was estimated as the values of Mn-SOD. After stripping, the membrane was rehybridized with an oligoprobe for 28S ribosomal (r) RNA oligonucleotide labelled with [γ-^{32}P] ATP using a Megalabel kit (Takara,Tokyo, Japan), and then the intensity of the bands at 5.0 kb was measured. The values of Cu,Zn-SOD mRNA/28S rRNA and Mn-SOD mRNA/28S rRNA, and also the ratio of these values to the mean value of NS (expression ratio) were calculated.

For ELISA, the frozen samples were placed in 9 volumes of PBS containing 0.5mM phenylmethylsulfonylfluoride and 1mM etylenediaminetetraacetic acid before being homogenized using polytron homogenizer. The homogenate was centrifuged at 900g for 10 min. The supernatants were applied to the ELISA[12] and a protein assay. The protein assay was performed using the BCA protein assay kit (PIERCE, IL, USA) and ELISA determinations were performed using the Cu,Zn-SOD ELISA system and Mn-SOD ELISA system (Amersham K.K., Tokyo, Japan).

Statistical analysis was performed by the Mann-Whitney's U test.

Results

The pathological diagnosis was confirmed in all samples.

The 0.7 kb band for Cu, Zn-SOD mRNA was recognized in all cases. The 4.0 kb and 1.0 kb bands for Mn-SOD mRNA were recognized as the two main bands in all cases, although some of 1.0 kb bands were faint in NS. The 5.0 kb band corresponding to 28S rRNA, was observed more or less equally in all 15 cases. (Fig.1)

In the comparison of quantitated values, the mean value of BCC was 1.61, while the mean value of NS was 1.00 in the expression ratio for Cu, Zn-SOD mRNA. Mann-Whitney's U test revealed a statistically significant difference in the expression ratio of Cu, Zn-SOD mRNA between BCC and NS ($P<0.01$) (Fig. 2).

On the other hand, the mean values for Mn-SOD mRNA were 1.84 for BCC and 1.00 for NS, and the statistically significant difference was confirmed between them ($P<0.05$) (Fig. 3).

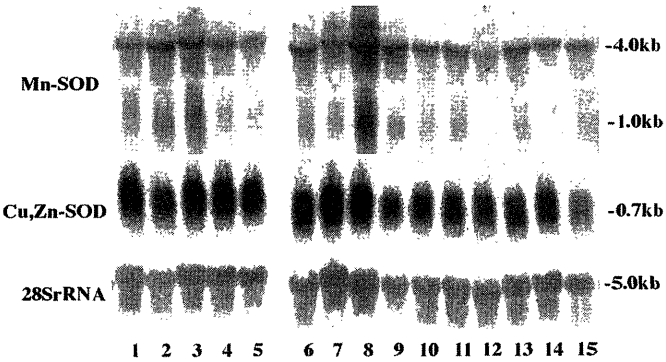

Fig. 1. Northern blot examination of total RNA (40μg in each well) for analysis of Mn-SOD mRNA at 4.0kb and 1.0kb.and Cu, Zn-SOD mRNA at 0.7kb. The lowest panel represents the 28S ribosomal RNA bands at 5.0kb. The numbers correspond to patient numbers. 1~9: BCC, 10~15: NS.

There was no statistically significant difference between the mean values of the protein level of Cu, Zn-SOD in BCC at 658.1ng/mg protein, and NS at 583.0 (Fig. 4).

Meanwhile, a statistically significant difference was recognized in the protein level of Mn-SOD in BCC at 634.1ng/mg protein and NS at 310.5 ($P<0.05$) (Fig. 5).

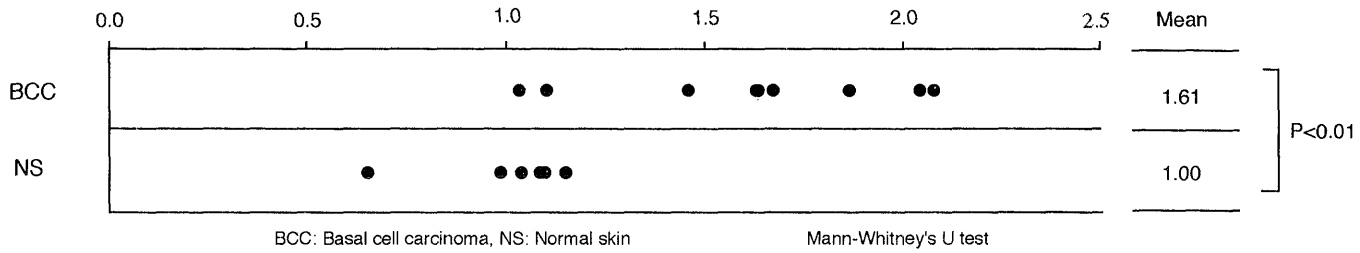

Fig. 2. Expression ratio of Cu,Zn-SOD mRNA
The ratio of each Cu, Zn-SOD mRNA level normalized by the respective 28S ribosomal RNA band to mean value of NS, along with the mean values of each group. A statistically significant difference was confirmed by the Mann Whitney's U test.

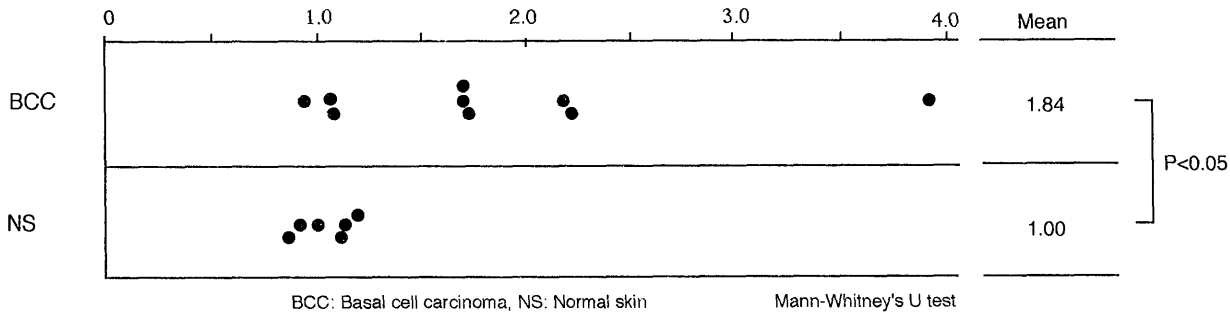

Fig. 3. Expression ratio of Mn-SOD mRNA
The ratio of each Mn-SOD mRNA level normalized by the respective 28S ribosomal RNA band to mean value of NS, along with the mean values of each group. A statistically significant difference was confirmed by the Mann Whitney's U test.

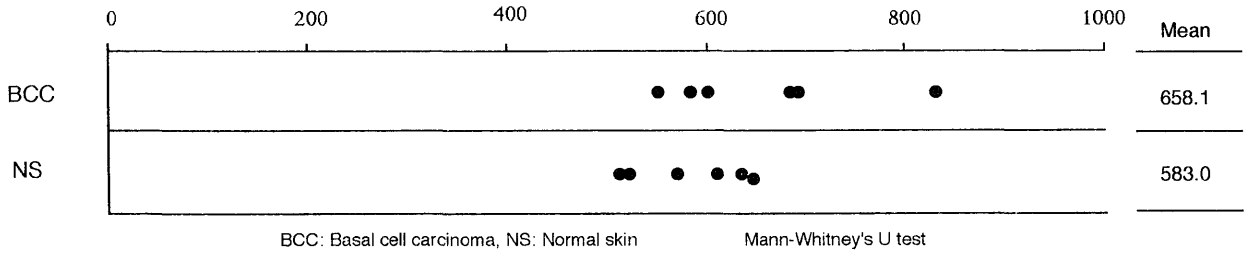

Fig. 4. The immunological level of Cu, Zn-SOD in SCC and NS. A statistically significant difference was confirmed by the Mann Whitney's U test.

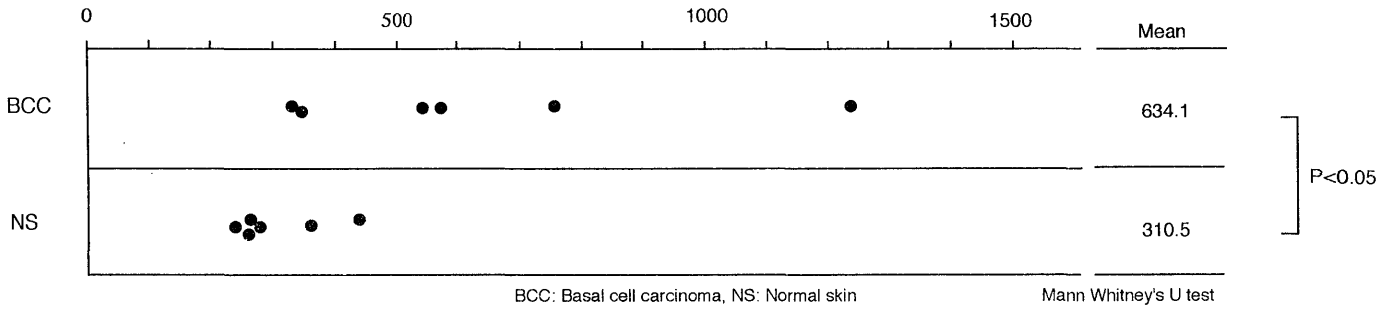

Fig. 5. The immunological level of Mn-SOD in BCC and NS. A statistically significant difference was confirmed by the Mann Whitney's U test.

Discussion

As regards the relationship between SOD and skin cancer, there has been one report concerning immunohistochemical analysis of SOD in skin cancer using monoclonal antibodies to Cu,Zn-SOD or Mn-SOD[13] and some reports for the activity of SOD in skin cancer[4,8]. However, there have been no reports concerning a quantitative analysis of SOD protein and mRNA in skin cancer. In the present study, we quantified Cu, Zn- and Mn-SOD mRNA using Northern blot analysis and performed quantitative analysis of the proteins using ELISA.

For the level of Cu, Zn-SOD and Mn-SOD mRNA, BCC showed a significantly higher expression than NS, although the levels in BCC were not more than twice the level in NS. Furthermore, we quantified the protein content in six cases of BCCs and NSs by ELISA. It has been reported that NS contained 680±78 ng Cu, Zn-SOD per mg protein and 302±08 ng Mn-SOD per mg protein[14]. Our results from NS were 583 ng/mg for Cu, Zn-SOD and 310.5 ng/mg for Mn-SOD, which were very similar to the results mentioned above. The immunological level of Cu, Zn-SOD in BCC, measured in the present study showed no significant difference from that in NS. On the other hand, the immunological level of Mn-SOD in BCC was significantly more that in NS being more than two times the normal level. Thus, the immunological levels of these enzymes determined by ELISA were approximately parallel to the mRNA levels of these enzymes, although there was some difference between them.

Cu, Zn-SOD is generally thought to be constitutive. Although there was a significant difference between BCC and NS at the Cu, Zn-SOD mRNA expression level, this enzyme was not abundantly induced in BCC in the present study. It still remains unclear why BCC showed a significant increase only at the Cu, Zn-SOD mRNA expression level. Further examination is necessary in order to answer this question.

Mn-SOD is though to be induced by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)[15,16]. It was reported[17] that IL-1 levels were high in SCC but low in BCC. We also got the high expression of Mn-SOD in SCC, which was significantly higher than that in BCC.(Data not shown) These findings on Mn-SOD in BCC and SCC are compatible with above reports[17]. Increased Mn-SOD protein in serum was reported in the patients with epithelial ovarian cancer[5], and an increase of TNF in ovarian cancer tissue has been also reported[18]. Thus it is possible that TNF levels are higher in SCC than in BCC[16]. On the other hand, Fujii et al. obtained results which showed that phorbol ester(TPA) enhanced Mn-SOD mRNA expression through protein kinase C activation, but not Cu,Zn-SOD mRNA in TNF-resistant cell lines including HeLa cell[19]. TPA is a tumor promotor which is used in carcinogenesis experiments, and so there is a possibility that some factors like TPA may work in skin cancers. An additional study is necessary to clarify this

question.

In conclusion, the evidence that the expression of Mn-SOD was augmented in BCC suggests that the ox idant/ antioxidant system is involved in skin cancer development.

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