Prevention of inflammation-mediated acquisition of metastatic properties of benign mouse fibrosarcoma cells by administration of an orally available superoxide dismutase

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Evidence has been accumulated that many of the cell alterations seen in normal ageing and in various diseases including cancer are due to oxidative damage by active oxygen species (Taniguchi, 1992). Oxygen radicals are a by-product of aerobic respiration and harmful to living cells (Halliwell et al, 1992). In tumour tissues, oxygen radicals are generated by cancer cells themselves (Shaughnessy et al, 1989; Szatrowski and Nathan, 1991; Oberley and Oberley, 1997), and infiltrating inflammatory cells such as neutrophils (Babior et al, 1973; Weismann et al, 1980) are believed to exert tumoricidal effects at their relatively high concentrations. On the other hand, the normal or premalignant cells which are chronically exposed to or escape from a highly cytotoxic concentration of oxygen radicals tend to be transformed to malignant ones or acquire malignant properties as evidenced under certain conditions; namely, oxygen radicals stimulate tumour cell invasion (Shinkai et al, 1986) or enhance metastasis (Orr et al, 1988).

Most aerobic cells have an enzymatic system to eliminate active oxygen species, because some of these active species are toxic to host. Superoxide dismutases (SODs), catalase, and glutathione peroxidase comprise the major defence system against oxygen toxicity (McCord and Fridovich, 1969). Superoxide dismutases catalyse the dismutation of superoxide anion $\textit{O}_2^-$ to produce hydrogen peroxide $\textit{H}_2\textit{O}_2$ and $\textit{O}_2$. Superoxide anion is one of the reduced oxygen species generated in cellular metabolism or produced by inflammatory cells at their respiratory burst.

There are three isozymes of SODs in mammalian system (Taniguchi, 1992). Among them, manganese-SOD (manganese-superoxide dismutase (Mn-SOD)) and copper, zinc-SOD (Cu,Zn-SOD) are widely believed to play an important role in carcinogenic processes (Oberley and Buettner, 1979; Dovrat and Gerhorn, 1981). It has been found that tumour cells tend to have reduced activities of those SODs compared to normal counterpart (Sykes et al, 1978; Oberley and Buettner, 1979; St Clair and Holland, 1991; Brorrello et al, 1993), and overexpression of SOD decreases malignant phenotypes in various cancers including breast cancer (Li et al, 1995), melanoma (chruch et al, 1993) and glioma (zhong et al, 1997). Moreover, it is known that levels of SODs inversely correlate with metastatic ability of tumour cells (Kwee et al, 1991), and that SOD suppresses metastasis of tumour cells in vivo (safford et al, 1994; Yoshizaki et al, 1994) and inhibits their motility and invasiveness (muramatsu et al, 1995). Superoxide dismutase also acts as differentiation inducer for erythroleukaemia cells (beckman et al, 1989). In other words, deficiency of SOD may
disturb redox status in cells, which influences neoplastic transformation and/or the maintenance of the malignant phenotype (Oberley and Buettner, 1979; Loven et al, 1984; Oberley and Oberley, 1988). In fact, in vitro transformation of normal fibroblast cell lines was carried out by infection with simian virus 40 (SV40) for comparing SOD amounts before and after transformation (Marlhens et al, 1985; Oberley et al, 1989). The results showed that all of the SV40-transformed cell lines decreased the SOD amount as compared to the original normal fibroblast cell line. Recently, enforced expression of SOD in SV40-transformed cells partially reversed their malignant phenotypes (Yan et al, 1996). Also, constitutively expressed high levels of SOD in mouse C3H10T1/2 cells coincided with a decrease in the frequency of radiation-induced neoplastic transformation (St Clair et al, 1992). Since an inverse correlation thus exists between its expression and tumour development and progression (Safford et al, 1994), it has been hypothesised that SOD is a new tumour suppressor gene (Bravard et al, 1992).

Introduction of SODs into tumour cells and/or tumour tissues is theoretically the most efficient strategy for inhibiting both tumour growth and progression. However, for induction of appropriate levels of SOD, it is necessary to use specialised techniques, for example, DNA transfection methods or modification of a specific amino-acid sequence of SOD or modification of SOD to make it stable after injection in vivo. It would be beneficial to develop peroral active SOD for wide preclinical usage. In this scheme, Giri and Misra (1984) have reported that, similarly to most proteins, orally administered SOD will be digested in the stomach and only a small portion of SOD will be absorbed in the blood stream through gastrointestinal tract. For minimising the digestion, SOD has been coated with a wheat-based biopolymer, gliadin, (Triticum vulgare, Poaceae) which not only prevents gastric digestion (Vouldoukis et al, 2003) but also promotes the delivery of the bioactive molecules into the small intestinal mucosa by enhancing the intestinal permeability through activation of a tight-junction-regulating protein, zonulin (Clemente et al, 2003).

Our group previously showed that the QR tumour cells obtained from a clonal murine fibrosarcoma were unable to grow in normal syngeneic C57BL/6 mice when injected subcutaneously (2 × 10⁶) whereas they developed tumours after coimplantation with a foreign body, gelatine sponge (Okada et al, 1992). We reasoned that the foreign-body-induced inflammation accelerated the progression of QR tumour cells, and suggested the involvement of active oxygen species produced by inflammatory cells in this process (Okada et al, 1999; Tazawa et al, 2003). The QR-32 tumour cells used in the present study were most sensitive to inflamma-
tion-promoted progression, as compared to other QR tumour clones with resistance to progression, since they had significantly decreased Mn-SOD activity (Okada et al, 1999). For this reason, we used the tumour cell line for evaluation of the newly developed SOD derivative, oxykine, in prevention of active oxygen species-mediated tumour progression.

We aimed to determine the potential inhibitory effect of oxykine in the model in which we can observe both tumour formation (primary tumour) and progression of tumour cells (acquisition of metastatic phenotype). We herein reported that oxykine reduced primary tumour growth and prevented the acquisition of metastatic property of tumour cells through suppression of superoxide anion at the tumour-growing sites.

MATERIALS AND METHODS

Chemicals

Oxykine®, melon-derived SOD and gliadin were provided by Asama Chemical Inc., Tokyo, Japan. The SOD activity of oxykine was 1000 U g⁻¹. It was diluted with PBS just before usage and kept on ice until administration. The dose was fixed at 10 mg kg⁻¹, and administered intragastrically.

Tumour cell lines and culture conditions

The origin and characteristics of the tumour cell lines have been described previously (Ishikawa et al, 1987). Briefly, BMT-11, a transplantable fibrosarcoma, was induced in a C57BL/6 mouse with 3-methyl-cholanthrene, and a tumorigenic clone BMT-11 cl-9 was subsequently isolated by limiting dilution. BMT-11 cl-9 cells were exposed in vitro to quercetin, which gave rise to a number of random subclones (Ishikawa et al, 1987). They spontaneously regressed when injected into normal syngeneic mice. The variants were named ‘QR tumour clones’, representing ‘quercetin-induced regressive tumour’. Tumour cells of one of the variant cell clones, QR-32, were used in this study. The culture cell lines established from tumours arisen after coimplantation of QR-32 tumour cells with gelatine sponge in mice were designated as ‘QRsP’, representing ‘progressive tumour variants derived from QR-32 tumour cells co-implanted with gelatine sponge’. The QR-32 tumour cells and QRsP tumour cell lines were maintained in Eagles’s minimum essential medium (MEM, Nissui Pharm, Japan) supplemented with 8% fetal bovine serum (Filtron), sodium pyruvate, nonessential amino acids and l-glutamine, at 37°C, in a humidified 5% CO₂/95% air mixture.

Mice

Female C57BL/6 mice (5 weeks old) were obtained from Nippon SLC (Hamamatsu, Japan) and used for the experiments. All the mice were maintained in the complete barrier condition, lit from 0700 to 1900, at 23 ± 3°C and 50 ± 10% humidity, fed with mouse diet (Nihon Nosan Kogyo, Yokohama, Japan) and UV-irradiated water in the germ-free section of Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine. Diet and tap water were available ad libitum throughout the experiment.

Experimental procedures

The experimental protocol was approved by the Committee of Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine (#01139).

The mice at 6 weeks of age were used after 1 week of acclimatisation. A subcutaneous pocket reaching up to the thorax was made from a 10 mm incision on the right flank of the pelvic region in each anaesthetised mouse and one piece of sterile gelatine sponge (10 × 5 × 3 mm³ piece; Spongel, Yamanouchi Pharm., Japan) was inserted and the wound was closed with clips. Then QR-32 tumour cells (1 × 10⁶ cells/0.1 ml) were immediately injected into the inserted gelatine sponge (Okada et al, 1992).

The mice were divided randomly into three groups and treated with oral administration of oxykine or gliadin at a dose of 10 mg kg⁻¹ body weight, or saline. The treatment was carried out every day from 2 days before QR-32 tumour cell implantation to the end of the experiment. Tumour diameter and body weight were measured twice a week during the experiment. All the mice were killed under ether anaesthesia at 28 days after implantation for evaluation of the arising tumours’ malignancy and autopsy; simultaneously we removed the subcutaneously growing tumours aseptically to assess whether the arising tumours had acquired malignant phenotype, and used them for establishing individual culture cell lines after mechanical disaggregation with scissors. The detailed procedure has been described elsewhere (Okada et al, 1992). The tumour lines were allowed at least four passages in culture to eliminate host cell contamination. Each tumour cell line was injected intravenously (1 × 10⁶ cells) into normal C57BL/6
mice. On day 25, the mice were killed and metastatic nodules at the surface of the lungs or other organs were counted macroscopically.

**Determination of the total number and the types of the cells infiltrated into gelatin sponge**

The gelatin sponge pieces subcutaneously injected into the mice treated with oxykine, gliadin, melon-SOD or saline were removed and digested with 0.2% collagenase in serum-free MEM medium for a few minutes at 37°C. After collecting all the infiltrated cells by centrifugation, we counted total number of the cells per piece of gelatin sponge. We also counted differential counts of more than 200 cells in smear preparations of the collected cells stained with May-Gruenwald’s and Giemsa solution (Wako Pure Chemical Inc., Osaka, Japan). Mean percentages of differential cells were obtained from the mean values of independent counts by two pathologists.

**Nitroblue tetrazolium (NBT) staining**

The mice were killed by cervical dislocation, and the tumour masses were excised and simultaneously stained for a few minutes with NBT (1 mg ml⁻¹) in Hank’s balanced salt solution (HBSS; 24020-117, Invitrogen, Tokyo, Japan). All unreacted NBT was removed from the tumour masses by washing with HBSS. The NBT-perfused tumour mass was photographed and then fixed with 10% (wt vol⁻¹) zinc/formalin for histologic examination of formazan deposits. The procedure followed the method described in a previous paper with slight modifications (Hagen et al, 1994).

**Assay for enzymatic activities**

The methods for evaluating enzymatic activities have been described previously (Okada et al, 1999). Briefly, the tumour tissues were washed with PBS and homogenised in liquid nitrogen. The homogenates were suspended in the PBS and then sonicated on ice four times, for 10 s each, by using a sonicator at intensity of 4 (Microson, Wakenyaku Co. Ltd., Kyoto, Japan). The homogenates were centrifuged at 15 000 rpm for 15 min and the resulting supernatant was used for enzymatic assay. Serum samples were diluted with PBS and used.

Superoxide dismutase activity was measured by the NBT reduction method (Beauchamp and Fridovich, 1971), with slight modifications. Manganese-superoxide dismutase activity was examined at 25°C in 1 ml of 20 mM sodium carbonate buffer, pH 10, containing 0.1 mM EDTA, 0.2 mM xanthine, 12 μM NBT and 1.9 mM xanthine oxidase, and determined from the remaining SOD activity after addition of 2 mM potassium cyanide with a spectrophotometer at 560 nm. The amount of enzyme-reducing NBT by 50% was defined as one unit of SOD activity. Catalase activity was defined as one unit of SOD activity. Catalase activity was measured from decomposition of hydrogen peroxide, which was recorded at 230 nm on a chart recorder for 1 min at 36°C. Glutathione peroxidase (GPx) activity was determined by using a Ros probe, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA) and measured intracellular ROS levels by flow cytometry. Mean fluorescence intensities of the cells harvested from the gelatin sponge in the mice orally treated with oxykine, melon-SOD (main component of oxykine), gliadin or saline were 37.4 ± 8.6, 42.5 ± 13.6, 49.9 ± 22.2, 47.5 ± 14.2, respectively. We detected positive tendency to decrease the production of ROS in the oxykine-treated group as compared to those in other groups; however, the difference was not significant.

**Statistical analysis**

The significance of the differences in tumour and metastatic incidences was calculated by χ² test and the differences in metastatic nodules or body and organ weight were evaluated by Student’s t-test.

**RESULTS**

**Inhibition of growth of QR-32 tumour cells coimplanted with gelatin sponge in syngeneic mice treated with oxykine**

Benign fibrosarcoma cells (QR-32) did not develop tumours or form metastasis after subcutaneous (2 × 10⁶ cells) or intravenous (1 × 10⁶ cells) injection into normal syngeneic C57BL/6 mice (Ishikawa et al, 1987). Table 1a shows that QR-32 tumour cells coimplanted with a gelatin sponge grew in 15 out of 19 saline-treated mice (79%), 15 out of 18 gliadin-treated mice (83%) and 10 out of 17 oxykine-treated mice (59%). We did not find any significant difference in the tumour incidence among the three groups. However, in an attempt to establish tumour cell lines from the arising tumours, we failed in three out of the 10 tumours arisen in oxykine-treated mice due to scar or necrotic tissues, none of which were viable tumours as far as examined macroscopically and histologically (data not shown). Namely, the number of established cell lines (which is equivalent to the final incidence of tumour formation) was significantly reduced in the oxykine-treated mice compared to those in saline- or gliadin-treated mice (P > 0.05, Table 1a).

Tumour growth curves of the arising tumours are shown in Figure 1A. Oxykine administration had a slight inhibitory effect on the tumour growth, though it was not significant.

**Inhibition of oxidant production by oxykine treatment at the inflammation-promoted tumour formation**

Nitroblue tetrazolium is a dye that is reduced to an insoluble formazan derivative upon exposure to superoxide (Halliwell and Gutteridge, 1999). The blue-coloured formazan crystal deposition was extensively detectable at the surface of tumour tissues from gliadin- or saline-treated mice. In contrast, tumour tissue from the oxykine-treated mice had less deposit of the crystal (Figure 2A). We performed histological examination and revealed intense deposition of formazan crystal around the gelatin sponge filament, which coincided with the presence of infiltrated inflammatory cells. The density of formazan deposits also reflected the amount of superoxides generated locally. Namely, formazan staining was evident in gliadin-treated tumour tissues (Figure 2B), and, less in oxykine-treated tissues (Figure 2C). These results indicated that tumour tissues in oxykine-treated mice contained lower concentrations of oxygen radicals.

We then examined reactive oxygen species (ROS) statuses in both tumour cells and the inflammatory cells both of which had infiltrated into gelatin sponge. After harvesting those cells by collagenase digestion, we compared cytosolic ROS levels by using an ROS probe, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA) and measured intracellular ROS levels by flow cytometry. Mean fluorescence intensities of the cells harvested from the gelatin sponge in the mice orally treated with oxykine, melon-SOD (main component of oxykine), gliadin or saline were 37.4 ± 8.6, 42.5 ± 13.6, 49.9 ± 22.2, 47.5 ± 14.2, respectively. We detected positive tendency to decrease the production of ROS in the oxykine-treated group as compared to those in other groups; however, the difference was not significant.

We then compared the antioxidative enzyme activities such as those of SOD, glutathione peroxidase and catalase in the serum or those in other groups; however, the difference was not significant.
Oral administration is necessary for inhibiting tumour development and acquisition of metastatic phenotype of the QR-32 cells

When we administered oxykine, melon-SOD, gliadin or saline to mice via intraperitoneal route, the effect of oxykine as observed in peroral administration was lost (Table 3). This finding indicated that the inhibition of tumour progression depends on the formulation of the compound and the route of administration. The inhibitory effect of peroral administration of oxykine was also explained by the capacity to have an antioxidative enzyme, SOD, in tumour cells since the oral administration of oxykine-induced SOD in tumour tissues. However, the effect was abolished by switching the administration route to intraperitoneal route (Table 2).

Table 1  Inhibition of tumour development and acquisition of metastatic ability of QR-32 tumour cells by administration of oxykine (a) tumorigenicity of QR-32 cells co-implanted with gelatin sponge in mice and (b) characteristics of the arising tumour lines

| Treated with* | Exp. 1 | Exp. 1 | Total | No. of cell lines established/ no of tumours tested (%)b |
|---------------|--------|--------|-------|--------------------------------------------------------|
| **(a) Tumorigenicity of QR-32 cells co-implanted with gelatin sponge in mice** |       |       |       |                                                        |
| Saline        | 8/9 (89)| 7/10 (70)| 15/19 (79)|                                                        |
| Gliadin       | 8/8 (100)| 7/10 (70)| 15/18 (83)|                                                        |
| Oxykine       | 4/7 (57)| 6/10 (60)| 10/17 (59)| 7/17* (41)                                               |

| Cell lines established from the arising tumour | Lung-colonising ability | Other metastasis sites |
|-----------------------------------------------|-------------------------|-----------------------|
| **Incidence (no. of mice with lung metastasis/no. of mice tested)** | **No. of lung with metastatic nodules** | **Incidence no. of mice with other metastases/no. of mice tested)** |
| QR-32                                         | 0/10                    | 0, 0, 0, 0, 0, 0, 0, 0, 0, 0 | 0/10 | None |
| QR3P/-1                                       | 3/3                     | 1, 3, 14                |       | 0/3  | None |
| QR3P/-2                                       | 3/3                     | 8, 13, 20               |       | 0/3  | None |
| QR3P/-3                                       | 4/4                     | 3, 8, 14, >150          |       | 1/4  | [O (1/4)] |
| QR3P/-4                                       | 3/4                     | 0, 1, 3, 35             |       | 0/4  | None |
| QR3P/-5                                       | 4/5                     | 0, 2, 7, 8, 15          |       | 1/5  | [O (1/5)] |
| QR3P/-6                                       | 4/4                     | 8, 43, >150, >150       |       | 2/4  | [O (2/4)] |
| QR3P/-7                                       | 4/4                     | 8, 11, >150, >150       |       | 2/4  | [O (2/4)] |
| QR3P/-8                                       | 4/4                     | >150, >150, >150, >150  |       | 0/4  | None |
| QR3P/-9                                       | 4/4                     | 16, 48, 51, 62          |       | 0/4  | None |
| **Total**                                     | 33/35*                  | 6/35                   |       | None |
| QR3P/GD-1                                     | 4/4                     | 2, 2, 4, 17             |       | 0/4  | None |
| QR3P/GD-2                                     | 4/4                     | 5, 6, 12, 14            |       | 0/4  | None |
| QR3P/GD-3                                     | 2/4                     | 0, 3, 8                |       | 0/4  | None |
| QR3P/GD-4                                     | 3/4                     | 0, 5, 7, 22             |       | 1/4  | [O (1/4)] |
| QR3P/GD-5                                     | 3/4                     | 0, 3, 6, 12             |       | 0/4  | None |
| QR3P/GD-6                                     | 4/4                     | 2, 5, 6, 7             |       | 1/4  | [O (1/4)] |
| QR3P/GD-7                                     | 3/3                     | 16, >150, >150          |       | 1/3  | [O (1/3), A (1/3)] |
| QR3P/GD-8                                     | 4/4                     | 25, 58, 132, >150       |       | 3/4  | [O (2/4), LN (1/4)] |
| QR3P/GD-9                                     | 4/4                     | 58, 73, >150, >150      |       | 0/4  | None |
| QR3P/GD-10                                    | 4/4                     | 42, 43, 123, >150       |       | 0/4  | None |
| **Total**                                     | 35/39                   | 5/39                   |       | None |
| QR3P/OK-1                                     | 0/4                     | 0, 0, 0, 0             |       | 0/4  | None |
| QR3P/OK-2                                     | 3/4                     | 0, 2, 3, 18             |       | 0/4  | None |
| QR3P/OK-3                                     | 2/4                     | 0, 0, 5, 7             |       | 0/4  | None |
| QR3P/OK-4                                     | 0/4                     | 0, 0, 0, 0             |       | 0/4  | None |
| QR3P/OK-5                                     | 1/4                     | 0, 0, 0, 8             |       | 0/4  | None |
| QR3P/OK-6                                     | 0/4                     | 0, 0, 0, 0             |       | 0/4  | None |
| QR3P/OK-7                                     | 1/4                     | 0, 0, 0, 2             |       | 0/4  | None |
| **Total**                                     | 7/28*                   | 0/28                   |       | None |

*1 x 10^5 QR-32 tumour cells were coimplanted with gelatin sponge in normal mice to which oxykine or gliadin had been administered per os (10 mg kg^-1) once daily throughout the experiment. 10^5 cell lines were separately established from tumours arisen in each mouse. P<0.05 vs saline. In a separate experiment, 1 x 10^6 cells of each cell line were injected into mice. After 25 days, the mice were killed and metastatic nodules at the surface of lung were counted macroscopically. Incidences of lung metastasis were evaluated as follows: P<0.001 vs lung-colonising incidence of saline-treated group.

Inhibition of QRsP/OK tumour lines’ acquisition of lung metastatic ability by oxykine

It is the advantage of this model that we can determine whether the arising tumour cells acquire metastatic ability without...
inflammatory cells, because the tumour cell lines had been established by culturing the cells from tumours arisen in individual mice and were originally their metastatic potential is examined in another normal syngeneic mice (Okada et al, 1992). We established in vitro culture cell lines from the arising tumours in the mice treated with oxykine, gliadin or saline, and designated them as QRsP/OK, QRsP/GD and QRsP tumour lines, respectively. As Table 1b shows, the lung metastasis incidence was significantly low; only in seven out of 28 lungs had positive metastases developed after i.v. injection of QRsP/OK tumour cell lines, whereas 35 out of 39 lungs and 33 out of 35 lungs had positive metastases after injection of QRsP/GD and QRsP tumour lines, respectively ($P<0.001$). At the time of killing, there was no evidence of typical spontaneous metastasis.

Table 4 shows that the number of colonies per lung and the lung net weight, which indirectly represent metastatic nodules in the lungs. They are significantly less in the mice with QRsP/OK tumour lines than in those with other tumour lines ($P<0.001$). Although there was no reduction of lung metastasis with control vehicle or gliadin treatment, it was suppressed by administration of oxykine (97% inhibition).

No obvious side effect brought by oxykine treatment

Subcutaneously injected gelatin sponge pieces into the mice with administration of oxykine, gliadin, melon-SOD or saline were removed and the exact number of infiltrated cells was counted per gelatin sponge. Table 5 shows that there was no significant difference among the groups. Then we stained the infiltrated cells and determined their cell types by histological examination, we found that oxykine and other compounds did not make differences in the types of cells infiltrated into gelatin sponge.

The application of oxykine or gliadin (10 mg kg$^{-1}$ day$^{-1}$ for 27 days) did not cause either any obvious side effect such as weight loss, or alteration in the appearance or behaviour of the tumour-bearing mice during the observation period. The data of average body weight are shown in Figure 1B. The values of the oxykine group were not lower than those of the control group (gliadin- or saline treated) throughout the experimental period. There were no significant differences in the final body weight among the treatments (Table 6). Moreover, no significant differences were observed in weights of organs at autopsy either in absolute or ratio to body weight values among the groups (Table 6).

DISCUSSION

In this study, we showed that an orally available SOD, named oxykine, inhibited inflammation-promoted acquisition of metastatic phenotype of weakly tumorigenic and nonmetastatic murine fibrosarcoma cells without adverse side effect. We also observed suppression of the primary tumour growth by the oxykine treatment.

Several lines of evidence implicate a relationship between induction of SODs in tumour cells and reversion of neoplastic transformation or loss of the malignant phenotype including metastatic property. From these, it has been hypothesised that SOD can be a new tumour suppressor gene (Sykes et al, 1978; Bravard et al, 1992; Safford et al, 1994). It is reported that unbalanced overexpression of SOD protein modulates cellular signal transduction cascades such as tumour – invasion-associated matrix metalloproteases through transactivation of transcription factor(s) (Wenk et al, 1999; Nelson et al, 2003). Manganese-
superoxide dismutase is known to be particularly high in primary hepatoma (Taniguchi, 1992), gastric cancer (Taniguchi, 1992), acute myeloid and/or lymphocytic leukaemias (Nishiura et al., 1992), epithelial-type ovarian cancer (Ishikawa et al., 1990), central nervous system tumours (Cobb et al., 1996) and neuroblastoma (Kawamura et al., 1992); on the other hand, lower SOD levels have generally been demonstrated in other tumour cells and clinical tumour tissues (Oberley and Buettner, 1979; Loven et al., 1984; Oberley and Oberley, 1988; Brorrello et al., 1993).

To date, eight different techniques have been established to elevate SOD levels in tumour cells or tumour tissues. All the techniques have been reported to reverse malignant phenotypes of tumour cells. They are (i) intravenous or subcutaneous administration of recombinant human SOD which substitutes specific amino acid for stable one (Yoshizaki et al., 1994); (ii) intravenous administration of SOD conjugated with a pyran copolymer, for prolongation of its activity (Oda et al., 1989); (iii) addition of exogenous liposomal SOD (Beckman et al., 1988); (iv) intraperitoneal or subcutaneous administration of a selective SOD mimetic molecule of nonpeptidic and low molecular weight (Samlowski et al., 2003); (v) elevation of SOD level by sense cDNA transfection (Safford et al., 1994); (vi) inoculation of fibroblasts that are genetically modified to secrete SOD (Tanaka et al., 2001); (vii) elevation of SOD levels by exposure to a superoxide generator and subsequent isolation of superoxide-resistant cells (Fernandez-Pol et al., 1982); (viii) secondary induction of SOD in tumour...
tissues by administration of an immunopotentiator which stimulates immune cells to produce SOD-inducible cytokines such as interferon-gamma and tumour necrosis factor-alpha (Habelhah et al., 1998). There is no doubt that an orally available SOD would be worth developing for preclinical use of SOD. However, in an experiment using mice, only a small portion (approximately 10%) of orally administered SOD is absorbed through gastrointestinal tract (Giri and Misra, 1984), and most of it was digested, similarly to other proteins, before being absorbed into the blood stream. As a solution of this problem, SOD has been coated with a protective vegetal pelarmon (wheat gliadin) layer that not only prevents gastric digestion (Vouldoukis et al., 2003) but also promotes the delivery of the bioactive molecule in the mucosa of small intestine (Clemente et al., 2003). In this study, we used cantaloupe melon (Cucumis melo LC. Cucurbitaceae)-derived SOD. The C. melo LC. derived SOD has an equivalent activity which is more than five times that of classical melon species, charentais (Vouldoukis et al., 2004). In fact, melon, barley plant, broccoli, Brussels sprouts, cabbage, wheat grass and most green plants which we in daily meals naturally contain large amounts of SOD. Kitagawa et al. (1986, 1991) reported that X-ray crystallography of plant SOD showed a high structural homology to the mammalian SOD, indicating common characteristics beyond the species barrier such as enzymatic activity (Taniguchi, 1992).

Niitsu and his colleagues have discovered an inhibitory effect of SOD on both experimental and spontaneous pulmonary metastasis in murine models (Yoshizaki et al., 1994). They intensively investigated the mechanisms and reported that SOD dramatically suppressed motility and invasion of both human and murine tumour cells (Yoshizaki et al., 1994; Muramatsu et al., 1995). A similar suppressive effect of SOD on tumour metastasis was observed in the studies of exogenous and endogenous SOD treatments (Kwee et al., 1991) and of SOD cDNA transfection (Safford et al., 1994). Besides the direct inhibitory effect of SOD on motile phenotype of tumour cells, our present study revealed reduced acquisition of metastatic phenotype in the process of tumour development. We can conclude that the effect we observed was specific to the metastatic ability itself of tumour cells, because we used the culture cell lines established for the evaluation of metastatic ability and the culture condition excluded contamination of SOD. As indicated by in situ superoxide production levels in each treatment in Figure 2, we believe that the SOD administration reduces superoxides which are produced mainly by gelatine sponge-elicited inflammatory cells and are known as a genotoxic substance to induce gene alterations. From these, we speculate that SOD may prevent metastasis-associated gene alteration(s) caused by ROS produced by inflammatory cells.

We also observed inhibition of the primary tumour growth in the group with oxykine administration. There are three possible explanations for this. One is that the elevated levels of SOD might lower intracellular levels of O2−, which in turn downmodulates signal transduction and/or activation of transcription factors to suppress cell growth (Burdon, 1995). Irani et al. (1997) have recently shown that superoxide acts especially as signal regulator for the stimulation of cell growth through a flavoprotein and Rac1 pathways. Second is that SOD might stimulate tumoricidal immune effector cells. Samlowski et al. (2003) demonstrated that administration of SOD mimetic nonpeptidic molecule enhanced the cytotoxicity of lymphokine-activated killer (LAK) cells in vivo. Indeed, the QR-32 tumour cells are highly sensitive to LAK cells (Okada et al., 1994). Third is that oxykine might stimulate immune cells to produce SOD-inducible cytokines and growth factors. Those factors would coordinate synthesis de novo SOD at the tumour-growing sites. In our experiments, we revealed that Mn-SOD was induced in tumour cells only by oxykine formulation (Table 2). However, a single component of the oxykine by itself, that is, gliadin alone or SOD alone, does not have ability to induce SOD. At this time, we do not know the precise mechanisms responsible for this, but speculate that the oxykine formulation possibly activates the host immune system besides exerting direct SOD action. Since the main compound of melon-derived SOD induces Th1-dependent immunity (Vouldoukis et al., 2003), and the compound covered with gliadin has been identified as a major allergen for wheat-dependent exercise-induced anaphylaxis (Matsuo et al., 2003), both components seem to be immunogenic to host. Furthermore, only the oral

### Table 5 Differential leukocyte counts and numbers of cells infiltrated into gelatin sponge in mice with per oral administration of oxykine, gliadin or saline

| Treatment | No. of mice examined | Total no. of gelatin sponge-infiltrated cells (× 10⁴) | Mac/MO | PMN | LC | EOS | Others |
|-----------|----------------------|----------------------------------------------------|--------|-----|----|-----|--------|
| Saline    | 5                    | 15.6 ± 3.1                                         | 12.1 ± 6.3 | 53.7 ± 7.4 | 28.0 ± 2.8 | 1.6 ± 0.5 | 3.1 ± 2.4 |
| Gliadin   | 6                    | 19.7 ± 5.8                                         | 9.9 ± 2.0  | 49.7 ± 6.0 | 26.8 ± 2.7 | 1.6 ± 1.5 | 5.0 ± 0.8 |
| Melon SOD | 6                    | 19.0 ± 5.8                                         | 13.1 ± 4.2 | 50.6 ± 3.1 | 30.7 ± 2.9 | 1.4 ± 1.8 | 4.8 ± 3.7 |
| Oxykine   | 6                    | 18.4 ± 2.7                                         | 8.6 ± 4.2  | 53.5 ± 5.4 | 30.8 ± 2.9 | 1.6 ± 1.1 | 4.5 ± 2.3 |

Mac/MO, macrophages/monocytes; PMN, polymorphonuclear neutrophils; LC, lymphocytes; EOS, eosinophils. *A piece of gelatin sponge was implanted into the subcutaneous space of normal mice to which oxykine, gliadin or melon-SOD had been administered orally (10 mg kg⁻¹) once daily for 5 days.

### Table 6 Averages of final body weights and absolute/relative organ weights of mice treated with oxykine, gliadin or saline administered

| Treatment | No. of mice examined | Final body weight | Absolute (g) | Relative (%) | Absolute (g) | Relative (%) | Absolute (g) | Relative (%) |
|-----------|----------------------|-------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Saline    | 19                   | 20.6 ± 1.1        | 1.6 ± 0.1    | 5.1 ± 0.8    | 0.29 ± 0.08  | 1.4 ± 0.4    | 0.18 ± 0.03  | 0.9 ± 0.1    |
| Gliadin   | 18                   | 19.8 ± 1.7        | 1.00 ± 0.15  | 5.0 ± 0.8    | 0.31 ± 0.09  | 1.6 ± 0.5    | 0.19 ± 0.05  | 1.0 ± 0.3    |
| Oxykine   | 17                   | 20.7 ± 1.4        | 1.10 ± 0.19  | 5.4 ± 1.1    | 0.36 ± 0.10  | 1.7 ± 0.4    | 0.22 ± 0.05  | 1.0 ± 0.2    |

The mean values of body weight in the groups showed no significant decreases compared with that of the saline-group throughout the experimental period. *Relative organ weight = organ net weight/body weight (%). "Combined weight of the two kidneys.

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Niitsu and colleagues have discovered an inhibitory effect of SOD on both experimental and spontaneous pulmonary metastasis in murine models (Yoshizaki et al., 1994). They intensively investigated the mechanisms and reported that SOD dramatically suppressed motility and invasion of both human and murine tumour cells (Yoshizaki et al., 1994; Muramatsu et al., 1995). A similar suppressive effect of SOD on tumour metastasis was observed in the studies of exogenous and endogenous SOD treatments (Kwee et al., 1991) and of SOD cDNA transfection (Safford et al., 1994). Besides the direct inhibitory effect of SOD on motile phenotype of tumour cells, our present study revealed reduced acquisition of metastatic phenotype in the process of tumour development. We can conclude that the effect we observed was specific to the metastatic ability itself of tumour cells, because we used the culture cell lines established for the evaluation of metastatic ability and the culture condition excluded contamination of SOD. As indicated by in situ superoxide production levels in each treatment in Figure 2, we believe that the SOD administration reduces superoxides which are produced mainly by gelatine sponge-elicited inflammatory cells and are known as a genotoxic substance to induce gene alterations. From these, we speculate that SOD may prevent metastasis-associated gene alteration(s) caused by ROS produced by inflammatory cells.

We also observed inhibition of the primary tumour growth in the group with oxykine administration. There are three possible explanations for this. One is that the elevated levels of SOD might lower intracellular levels of O2−, which in turn downmodulates signal transduction and/or activation of transcription factors to suppress cell growth (Burdon, 1995). Irani et al. (1997) have recently shown that superoxide acts especially as signal regulator for the stimulation of cell growth through a flavoprotein and Rac1 pathways. Second is that SOD might stimulate tumoricidal immune effector cells. Samlowski et al. (2003) demonstrated that administration of SOD mimetic nonpeptidic molecule enhanced the cytotoxicity of lymphokine-activated killer (LAK) cells in vivo. Indeed, the QR-32 tumour cells are highly sensitive to LAK cells (Okada et al., 1994). Third is that oxykine might stimulate immune cells to produce SOD-inducible cytokines and growth factors. Those factors would coordinate synthesis de novo SOD at the tumour-growing sites. In our experiments, we revealed that Mn-SOD was induced in tumour cells only by oxykine formulation (Table 2). However, a single component of the oxykine by itself, that is, gliadin alone or SOD alone, does not have ability to induce SOD. At this time, we do not know the precise mechanisms responsible for this, but speculate that the oxykine formulation possibly activates the host immune system besides exerting direct SOD action. Since the main compound of melon-derived SOD induces Th1-dependent immunity (Vouldoukis et al., 2003), and the compound covered with gliadin has been identified as a major allergen for wheat-dependent exercise-induced anaphylaxis (Matsuo et al., 2003), both components seem to be immunogenic to host. Furthermore, only the oral
行政干预在超氧化物歧化酶（SOD）诱导和抑制肿瘤生长相关的表型（表3）。因此，免疫抑制作用可能涉及由非致病性氧化反应介导的氧化应激的形成，这种形成可能在免疫系统的消化道或全身通过吸收途径导致氧化应激。我们正在进行一项研究，以确定氧化应激是否在肠内环境中有毒作用。

氧化应激在肿瘤细胞中受到关注。研究发现，氧化应激是肿瘤生长和进展的关键因素。氧化应激的影响包括DNA损伤、蛋白质氧化和脂质氧化。氧化应激促进基因突变的积累，导致细胞死亡或转化。

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