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Increment of Photocatalytic Killing of Cancer Cells Using TiO_2 with the Aid of Superoxide Dismutase

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It was found that the efficiency of photocatalytic killing of cancer cells with TiO_2 particles under aerial condition was increased by adding superoxide dismutase (SOD). This enhancement effect of SOD was suppressed when catalase, hydrogen peroxide scavenger, was added to the system. These results can be reasonably explained by the efficient conversion of photogenerated superoxide anion into hydrogen peroxide with the aid of SOD.

Since photoexcited TiO_2 particles produce electron-hole pairs that drive various chemical reactions, they have been used in design systems for heterogeneous photosynthesis and photocatalysis.[1-5] Recently a new application with TiO_2 particles was proposed from this laboratory, which is "photokilling" of cancer cells.[6] This study has attracted considerable interest, because a large "photokilling effect" is observed not only in vitro of cultured cells, but also in vivo of experimental animals, suggesting this effect can be applied for cancer photodynamic therapy. Here we show that the efficiency of the "photokilling" of cancer cells can be increased by adding superoxide dismutase (SOD; EC 1.15.1.1) which converts superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2).

When TiO_2 is excited by a photon whose energy is greater than the TiO_2 band-gap energy, an electron and a hole are produced.

$$TiO_2 + h\nu \longrightarrow h^+ + e^- \qquad (1)$$

A single semiconductor particle can be thought as a short-circuited photoelectrochemical cell providing both the oxidizing and reducing sites for the reaction. At the oxidizing site, a water molecule is oxidized to hydroxyl radical (OH·). The generation of OH· in photoexcited TiO_2 suspension was

$$H_2O + h^+ \longrightarrow OH\cdot + H^+ \qquad (2)$$

detected by Bard et al. using spin trapping method and electron spin resonance technique.[7] The OH· can react with various chemical species and drive chemical reactions. At the reducing site,it is speculated that the photogenerated electrons mainly react with molecular oxygen presumably forming O_2^- under aerial condition.[8] Generally speaking, O_2^- is less reactive and a short lifetime, and therefore

$$O_2 + e^- \longrightarrow O_2^- \qquad (3)$$

the role of O_2^- in succeeding reactions is not understood well.

The purpose of the present study is to increase the photocatalytic activity of TiO_2 powder under aerial condition. We concentrated our attention to the reactions involving $O_2^{\cdot-}$. Our idea is to convert O_2^- into more reactive H_2O_2 efficiently with the aid of SOD catalysis.[9]

SOD is known to catalyze the conversion of O_2^- into H_2O_2 by the following reactions. In the present study, we tested the effect of SOD on the photocatalytic killing of cancer cells in TiO_2 suspension system.

$$O_2^- + \mathrm{SOD}(Cu^{2+}) \longrightarrow O_2 + \mathrm{SOD}(Cu^{+}) \qquad (4)$$

$$O_2^- + 2H^+ + \mathrm{SOD}(Cu^{+}) \longrightarrow H_2O_2 + \mathrm{SOD}(Cu^{2+}) \qquad (5)$$

HeLa cells were used in the present study, which were cultured in a minimum essential medium (MEM) with 10% fetal calf serum in a humidified incubator with an atmosphere of 5% CO_2 in air at 37 °C. A colony forming assay was used to study the effect of SOD on cell survival as described previously.[6] Firstly, 300 HeLa cells were plated in 60 mm petridishes (Becton-Dickinson Co.). They were treated with TiO_2 powders (100 μg/ml; 300 Å in diameter) containing MEM solution for 24 h, and treated with SOD [Sigma Chemical Company St. Louis, Missouri] for 3 h (during the last 3 h of TiO_2 exposure). Secondly, the TiO_2 containing MEM was removed, and a phosphate buffered saline [PBS] solution which contained the same concentration of SOD was added to the cells. TiO_2 particles are phagocitized by the cells after 24 h incubation.[6] The cells were then irradiated with a 500-W high pressure Hg lamp for 5 or 8 min. A UV pass filter was used to transmit a wavelength between 300-400 nm during irradiation. Finally, the PBS solution was replaced with fresh MEM solution and the cells were re-cultured for 10 days. Then, the formed colonies were fixed with methanol, stained with Giemsa and counted. Cultures exposed to the various drugs as described above but not exposed to light were used as control. Each experiment was done using three dishes at the same time under the same conditions.

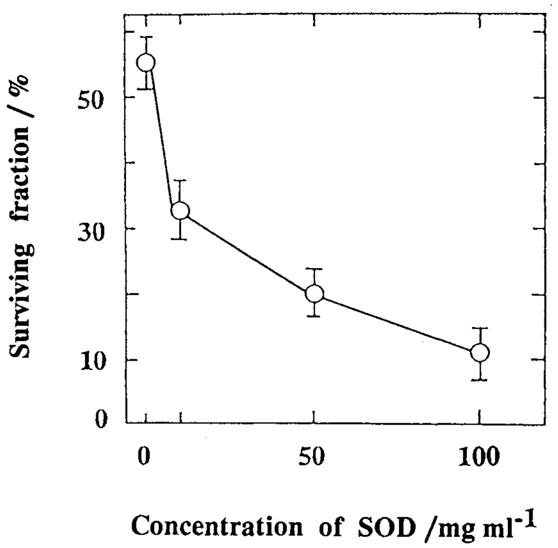

Fig. 1. The effect of SOD on the surviving fraction of HeLa cells incubated with a MEM-TiO_2.UV irradiation time: 5 min.

Relationship between the cell survival and the amount of SOD is shown in Fig. 1. In the absence of SOD, the cell survival fraction was 55% after 5-min UV irradiation. It was confirmed in the separate experiments that the cell death in the presence of photoexcited TiO_2 particles was caused by H_2O_2 and OH· formed on TiO_2 surface. When 10, 50, and 100 mg/ml of SOD were added, the cell survivals were further decreased to 32%, 20%, and 11%, respectively, during the same irradiation time, suggesting that the cells were killed effectively in the presence of SOD. Here, it should be stressed that SOD alone caused no effect on the cell survival fraction. Therefore, it can be reasonably expected that O_2^- produced by the reduction of O_2 is converted to H_2O_2 by SOD, resulting in the enhancement of photokilling activity. The formation of H_2O_2 in the TiO_2-SOD system was directly confirmed by measuring the fluorescence intensity of scopoletin (6-methoxy-7-hydroxy-1,2-benzopyrone) which is oxidized by the H_2O_2 leading the fluorescence quenching.[9)] The concentrations of H_2O_2 were determined to be 1.6×10^{-5} M and 2×10^{-6} M in the presence and absence of SOD, respectively. In these experiments, formation of H_2O_2 increased when SOD was active, but it did not when SOD was deactivated.

Further evidence for the conversion of O_2^- into H_2O_2 in the presence of SOD was obtained in the following experiment. Catalase [EC 1.11.1.6] is a scavenger of H_2O_2, which decomposes H_2O_2 into H_2O via reaction 6.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2 \qquad (6)$$

The effects of SOD were suppressed when catalase (Sigma Chemical Company St. Louis, Missouri) was added into the cells at the same time. The results are shown in Fig. 2 as closed circles. In these experiments, the irradiation time was chosen to be 8 min. In the absence of catalase, the surviving fraction of the cells was only 10% with 50 mg/ml of SOD. However, when catalase was added to the cells simultaneously, the surviving fraction recovered. The surviving fraction was larger with higher concentration of catalase. For example, it was recovered to 80% in the presence of 50 mg/l catalase. At this concentration, it was found that the absorption peak of catalase is insignificant in the wavelength region of 300-400 nm. Hence its filter effect can be neglected in the present case. Therefore, it is reasonable to conclude that the H_2O_2 produced is scavenged by catalase resulting in a lower activity.

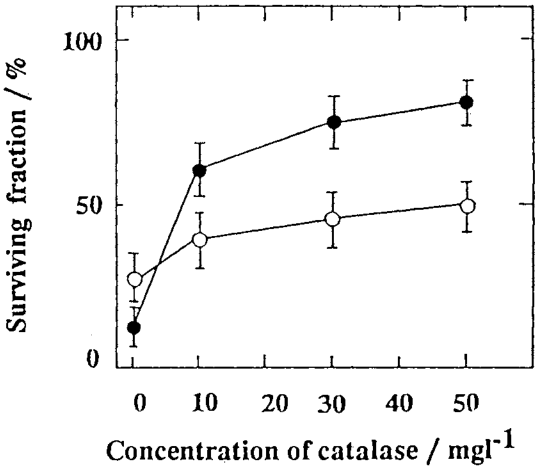

Fig. 2. The effect of catalase (H_2O_2 scavenger) on the surviving fraction of HeLa cells incubated with a MEM-TiO_2 in the presence (closed circles) and in the absence (open circles) of SOD. UV irradiation time: 8 min; SOD: 50 mg/ml.

It is speculated that H_2O_2 is slowly formed from O_2^- even in the absence of SOD by the following reaction.[10,11)]

$$O_2^- + H^+ \longrightarrow HO_2\cdot \qquad (7)$$
$$HO_2\cdot + e^- \longrightarrow HO_2^- \qquad (8)$$
$$HO_2^- + H^+ \longrightarrow H_2O_2 \qquad (9)$$

Actually, the addition of catalase increased the surviving fraction even in the absence of SOD. The effect of the H_2O_2 scavenger is, however, smaller in the absence of SOD than in the presence of SOD. The open circles in Fig. 2 represent the surviving fraction in the absence of SOD. The surviving fraction was of course larger without SOD than with SOD when catalase was not added to the cells. In the presence of catalase, however, the cells without SOD were more effectively killed. These results indicate that

O_2^- is converted not only to H_2O_2 but also some other reactive species in the absence of SOD. The details are now under investigation.

The present results can be explained as follows. Since reactivity of O_2^- is weak, it is difficult to drive a photocatalytic reaction. Therefore O_2^- itself is thought to be not so important in photocatalysis process so far. Here we succeeded in efficient conversion of such O_2^- into reactive H_2O_2 by using the enzyme (SOD), resulting in a drastic decrease of the cell survival. This finding might be applied not only to the present "photokilling" reaction, but also to many other photocatalytic reactions in which H_2O_2 is involved.

On the other hand, photoproduced H_2O_2 on TiO_2 can be converted into more reactive OH· by Fenton reagent. For instance, Fujihira et al. reported that the quantum yield of photocatalytic oxidation of aromatic compounds by TiO_2 was increased about 16 times by adding Fenton reagent.[12] Thereby it can be expected that the combination of SOD and Fenton reagent increases the efficiency of various photocatalytic reactions. Actually, preliminary experiments showed that the addition of EDTA-Fe(Fenton reagent) to the cells decreased the cell survival probability drastically.

In conclusion, we found that O_2^- produced by the reduction of O_2 can be converted into reactive H_2O_2 and OH· with the aid of a catalyst. We think that this method can be applied to various photocatalytic reactions and therefore the present results open a new way in photocatalysis in order to increase the reaction efficiency.

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