Protective Effect of the Serum against Cellular Damage by Active Oxygen in Culture

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It has been known that there is remarkable antioxidant activity in the human sera, especially those in inflammation and pregnancy. In the present investigation, various sera were examined for the antioxidant activity with the aid of cultured cells. It was recognized that the serum added to the culture medium protected cells from harmful action of active oxygen generated by a hypoxanthine-xanthine oxidase (HX-XO) system. The inflammatory serum has the greatest protective power, followed by pregnant and normal sera in this order. The antioxidant activity of the serum was inversely related to the Fe concentrations. The addition of ceruloplasmin with SOD action could not inhibit the tissue damage, while addition of catalase or hemoglobin with catalase activity could inhibit it. The protective effect was valid against not only HX-XO, but also H₂O₂. These results show that the chief active oxygen to cause cell damage is H₂O₂ and the scavenger antioxidants in the serum are hemoglobin and catalase. ——— serum antioxidant activity; cell culture; SOD; catalase; hydrogen peroxide

The functions of active oxygens in the body as superoxide (O₂⁻), hydroxyl radical (•OH), singlet oxygen (¹O₂), hydrogen peroxide (H₂O₂) etc. have been widely studied since superoxide dismutase (SOD) was found by McCord and Fridovich (1969). The studies show that active oxygen participates in not only oxidation-reduction system in the body and bacteriocidal and phagocytic action of leukocytes, but also every pathologic state, especially inflammation, damage of membrane due to peroxidation of lipids, immune reactions, carcinogenesis, radiation injuries, arteriosclerosis, and aging, widely both inside and outside of the cells. On the other hand, protection by active oxygen scavengers and antioxidants were found. Such protective substances have been applied therapeutically (Walravens and Dequeker 1976).

It has been known that there are inherent active oxygen scavengers and
antioxidant activity in the body. SOD and catalase intracellularly act as scavengers, whereas antioxidant activities have been also demonstrated in body fluids such as serum (Imai et al. 1978) and carcinomatous ascites (Oyanagui 1981). Regarding antioxidant activity in the serum, there are a report on the measurement by ESR (Vidlakova et al. 1972) and a series of works by Dormandy et al. (1978) who employed thiobarbituric acid reaction. The studies showed that antioxidant activity was markedly increased in parallel with serum ceruloplasmin (Cp) and transferrin (Tf) in the sera obtained from pregnant women and patients with rheumatoid arthritis. Goldstein et al. (1979) demonstrated SOD-like activity in Cp by cytochrome c method. As not only Cp and Tf, but also various serum proteins including albumin are reported to possess SOD-like action, the problem is still controvertible (Oyanagui 1981). In acute reactant, which includes Cp, is transiently increased. It can readily be imaged that some of them act as an antioxidant or a scavenger. In the present study, in order to clarify these points, serum antioxidant activity was measured by a biological technique using cultured cells under conditions close to in vivo; human diploid lung fibroblasts were chosen because, compared with established cell lines, the cells are capable of exhibiting more natural reaction and of providing relatively uniform condition due to the susceptibility to contact inhibition.

**Materials and Methods**

*The cells and their culture*

Human diploid fibroblasts were obtained from 12-week-old embryo lung by the procedure described previously (Kan and Yamane. 1982). These cells were grown in Eagle's MEM (Nissui) with 10% fetal calf serum (FCS), and their doubling time was approximately 24 hours in the medium. The cells were grown in plastic Petri dishes of 60 mm in diameter (Nunc) containing 3 ml medium with a gas phase of 5% CO₂ in air at 37°C. The cells of less than 20 population doublings were used for experiments. For each experiment, cells were harvested and plated into plastic Petri dishes (24 wells multi-plate, diameter of each well 10 mm, Sumitomo Bakelite) at a cell density of $2.5 \times 10^4$/dish. After 5 days of culture, cells fully grown in the dish were examined.

*Determination of cell number*

The number of cells was determined as follows: After removal of medium, floating cells were washed out twice with Ca²⁺-Mg²⁺ free PBS (−PBS), and residual attaching cells, harvested with 0.0075% pronase-E, 0.01% EDTA in −PBS were counted in an electric hemocytometer (Micro cell counter CC-110, Toa).

*Serum*

Normal sera (*Normal*) were obtained from healthy subjects, who underwent a thorough medical examination at our hospital and were noted no abnormal findings. Inflammatory sera (*Inflammatory*) were obtained from 8 patients; 3 patients with pneumonia, 2 patients with rheumatoid arthritis, 2 patients with acute cholecystitis, 1 patient with perityphlitis. The titer of CRP of each serum was over +6. Pregnant sera (*Pregnant*) were obtained from 6 women in the last 2 weeks of normal pregnancy. Fetal calf serum (FCS) was purchased from Filtron Pty. (Altna, Australia), (Lot. No. 8124).
Active oxygen generating system

We employed a superoxide generating system originally used by McCord and Fridvich (1969) which was mediated by aerobic action of xanthine oxidase (XO) 0.1 U on hypoxanthine (HX) 10 mM/dish.

Experimental cultures

When the cells grown confluent in each dish, medium was aspirated and the cells were washed twice with -PBS. 0.5 ml of MEM with 10 mM of HX and 10 μl (2%) of the serum was added to dish. After 10 μl of XO (0.1 U/dish) was added, the culture dish was placed in a incubator promptly. Incubation period was 24 hr. As a control culture, 0.5 ml of MEM containing 2% FCS without HX-XO was added to dish. The experimental cultures were made in triplicate dishes. After 24 hr period of incubation, residual attaching cells were counted.

In order to determine the protective effect of exogenous antioxidants, SOD 150 U, catalase 400 U, Cp 15 μg, haptoglobin (Hp) 100 μg, Hp-Hb complex (2:1) 100 μg dissolved in 10 μl of -PBS, hemoglobin (Hb) 100 μg dissolved in 10 μl of FCS were added to the dish. In an experiment to determine of the protective effect of hemolytic serum, hemolytic sera with varying Hb concentration were prepared as follows: Blood obtained from a healthy subject was divided to two test tubes, one was centrifuged ordinarily, another one was hemolysed by vigorous agitation before centrifugation. The hemolytic serum was diluted variously with intact one.

SOD-like activity in the serum

Reduction of 10 mM ferricytochrome c in MEM with serum and HX-XO as the same ratio as those of culture medium was measured at 550 nm at 37°C in a Hitachi 220 A recording spectrophotometer by employing a modification of the assay system originally used by McCord and Fridvich (1969). The SOD-like activity in the serum was measured by ΔOD/sec.

Measurement of serum metals and proteins

Serum metals were measured as follows: iron (Fe), direct bathophenanthroline method (Fe direct kit, Sinotest Lab.) (ICSH 1971); copper (Cu), direct bathocuproin method (Cu direct kit, Sinotest Lab.) (Matsubara 1967); zinc (Zn), atomic absorption spectrophotometric method using a Perkin Elmer atomic absorption spectrophotometer 370 (Fuwa et al. 1968). Serum Cp, Tf, Hp, and α1 acid glycoprotein (α1 AG) were measured by single radial immunodiffusion (M-Partigen kit, Hoechst) (Mancini 1965), and serum Hb was measured by SLS hemoglobin method (Hemoglobin B Test, Wako) (Ooki et al. 1979).

Chemicals

The sources of chemicals used in this study were as follows: Cp (human) and Hp (human) from Green Cross (Osaka, Japan); Hb (type IV, human), XO (grade I), SOD (type I), cytochrome c (type V) and catalase (2X) from Sigma (St. Louis, MO, USA); hypoxanthine (HX) and hydrogen peroxide from Nakarai (Kyoto, Japan).

RESULTS

Effect of active oxygen generating system (HX-XO) on viability of cultured cells and protective effect of the serum

Individual constituents of the active oxygen generating system (HX-XO) were examined for their harmful effect on cultured cells in various media. HX alone exerted no effect on the cells in all of serum free, FBS, normal, and
inflammatory media (one case each), while XO alone slightly reduced the number of attaching cells in each group, especially the cell number decreased to about 70% of control in serum free medium. This decrease may be attributed to either direct toxic effects of XO or reaction of XO with HX released from the cells. When HX was exerted in combination with XO, the cultured cells were prominently injured in serum free, FCS, and normal media and the numbers of viable cells decreased to less than 20%, but 93% of cultured cells survived in the inflammatory serum revealing prominent protective effects against the damage (Fig. 1). The viability of both attaching and floating cells after the treatment was determined by growth ability in the medium containing 10% FCS respectively. The residual attaching cells grew with normal population doubling times, while the floating cells (20 x 10^4 cells/dish) collected and plated into 35 mm dish did not attach to the dish and almost died out (data not shown). These results indicated that the residual attaching cells were almost viable and the cells damaged lethally were floated. Therefore, it appeared to be good indicator of the cell damage to measure the number of attaching cells at 24 hr after various treatments.

**Protective effect of various sera on cell damage induced by HX-XO**

Under conditions as described in the previous experiment, FCS and sera of normal (6), pregnant (6), and inflammatory (6) origins were compared for the protective effect against HX-XO. FCS was essentially not protective, the survival rate being 13%. The rates were 64, 82, and 90% for normal, pregnant, and inflammatory groups, respectively. In the latter two groups, the protective effect.

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**Fig. 1.** Effect of active oxygen generating system (HX-XO) on viability of cultured cells and protective effect of the serum. Number of viable cells 24 hr after the addition of HX, XO or HX-XO to the medium at the confluent stage in cultures. Each medium contained FCS (△—△), normal serum (●—●), inflammatory serum (○—○) at a concentration of 2% each or without serum (▲—▲).
Table 1. Protective effect of the serum on cell damage by aerobic action of xanthine oxidase on hypoxanthine

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Viable cells ( \times 10^4 ) /dish†</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5±1.2</td>
<td>100</td>
</tr>
<tr>
<td>FCS</td>
<td>1.7±0.3</td>
<td>13</td>
</tr>
<tr>
<td>Normal</td>
<td>8.7±1.6(^{(a,b)})</td>
<td>64</td>
</tr>
<tr>
<td>Pregnant</td>
<td>11.1±1.1(^{(b)})</td>
<td>82</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>12.1±0.9(^{(b)})</td>
<td>90</td>
</tr>
</tbody>
</table>

Mean±s.d., \( n \) = n. Statistically significant at a), b) \( p < 0.01 \) (t-test).† Number of viable cells at 24 hr after treatment of HX-XO.

Table 2. Metals and proteins in the serum of each group

<table>
<thead>
<tr>
<th>Metals/Proteins</th>
<th>FCS</th>
<th>Normal</th>
<th>Pregnant</th>
<th>Inflammatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(µg/100 ml)</td>
<td>36</td>
<td>147.0±</td>
<td>9.3</td>
<td>226.0± 17.8</td>
</tr>
<tr>
<td>Fe(µg/100 ml)</td>
<td>287</td>
<td>88.7± 35.2</td>
<td>52.7± 25.5</td>
<td>44.6± 25.0</td>
</tr>
<tr>
<td>Zn(µg/100 ml)</td>
<td>364</td>
<td>67.8± 8.6</td>
<td>56.3± 8.3</td>
<td>60.3± 20.0</td>
</tr>
<tr>
<td>Ceruloplasmin(mg/100 ml)</td>
<td>—</td>
<td>29.1± 3.7</td>
<td>70.2± 7.0</td>
<td>46.6± 7.9</td>
</tr>
<tr>
<td>Transferrin(mg/100 ml)</td>
<td>—</td>
<td>392± 122</td>
<td>569± 75</td>
<td>267± 50</td>
</tr>
<tr>
<td>Haptoglobin(mg/100 ml)</td>
<td>—</td>
<td>146± 36</td>
<td>131± 29</td>
<td>358± 179</td>
</tr>
<tr>
<td>( \alpha ), AG(mg/100 ml)</td>
<td>—</td>
<td>63± 4</td>
<td>51± 5</td>
<td>149± 44</td>
</tr>
</tbody>
</table>

Mean±s.d.

was apparently significant compared with normal (Table 1).

Metals and protein composition in the serum

Three metals possibly involved in oxidations and reductions in the body, the carrier protein, and proteins called acute phase reactant which increase in inflammation were determined in sera used in the previous experiment and the results were related to the protection against HX-XO.

Cu and Cp were highest in pregnant sera and also increased in inflammatory sera. Fe was decreased in normal, pregnant, and inflammatory sera in this order. The concentrations in normal and inflammatory sera were inversely proportional to the survival rates of cultured cells \( r = -0.46, p < 0.05 \). Tf was decreased in inflammatory sera and increased in pregnant sera. The iron free Tf was increased in pregnant, normal, and inflammatory sera, in this order. No significant difference in Zn was noted among these three groups. Hp and \( \alpha \), AG were increased markedly only in inflammatory sera. The concentrations of Cu and Fe were markedly lower and higher, respectively, than those in human serum. The results indicate that the protection of cultured cells was inversely proportional to serum Fe (Table 2).
SOD-like activity in the serum

The sera obtained from various sources and used in the culture experiments were examined for the SOD-like activity. The serum was reacted with cytochrome c and a decrease in the absorption at 560 nm due to reduction of cytochrome c was taken as the SOD-like activity of the serum. In contrast with the results obtained from the culture experiment, the greatest SOD-like activity was observed in FCS; the absorption at 560 nm decreased to 66% of that of serum free experiment. Human serum reduced the absorption only to 77-85%. There was no significant difference in SOD-like activity among the groups. Accordingly, the protective effect of the serum could not be attributed to the SOD-like action (Fig. 2).
Effects of SOD and catalase on cell damage caused by HX-XO

Based on the experimental results described above, the protective effects of the serum were further studied with medium containing FCS, to which either SOD or catalase (hydroperoxidase splits H₂O₂ into H₂O and O₂) was added. The number of viable cells decreased to 29% in the medium containing FCS alone. While the addition of SOD exerted no effect at all, the addition of catalase recovered the rate to as high as 95%. Thus, the cells were virtually perfectly protected from the damage to be caused by HX-XO when catalase was added to the medium (Fig. 3).

Fig. 4. Effects of Cp, Hp, and Hb on cultured cell damage caused by HX-XO. Number of viable cells 24 hr after HX-XO treatment. Cp, Hp, and/or Hb were added to the medium with 2% FCS at the confluent stage in cultures. Column: A, 2% FCS; B, A+HX-XO; C, B+Cp (30 μg/ml); D, B+Hp (200 μg/ml); E, B+Hb (200 μg/ml); F, B+Cp+Hb; G, B+Hp-Hb complex (200 μg/ml); H, B+Cp+Hp-Hb.

Fig. 5. Effect of H₂O₂ on viability of cultured cells and protective effect of the hemolytic serum. Number of viable cells 24 hr after the addition of H₂O₂ to the medium with 2% of hemolytic serum at the confluent stage in cultures. Column: A, normal serum without H₂O₂ (control); B, serum free added H₂O₂ (0.5 mM); C, B+intact serum; D, B+hemolytic serum (Hb 16 μg/ml); E, B+hemolytic serum (Hb 20 μg/ml); F, B+hemolytic serum (Hb 24 μg/ml).
Serum components such as Cp which has SOD-like activity, Hp which is an acute phase reactant and the carrier protein for Hb, and Hb which has catalase activity were added to the medium containing FCS and their preventive action against HX-XO damages was evaluated. When cultured cells were treated with HX-XO in the medium containing FCS alone, the number of viable cells was reduced to 41% of control. The rate was slightly increased to 54% when Cp was added to the serum, but the addition of Hp revealed no effect. The rate increased remarkably to 82% when Hb was added. The rate was further improved to 87% by the addition of Cp coupled with Hb (Cp + Hb). The effect of Hp-Hb was essentially identical with that of Hb alone. When the three were added simultaneously, the rate was 86% and the effect was similar to that of Cp-Hb (Fig. 4).

**Effects of Cp, Hp, and Hb on cell damage caused by HX-XO**

Serum components such as Cp which has SOD-like activity, Hp which is an acute phase reactant and the carrier protein for Hb, and Hb which has catalase activity were added to the medium containing FCS and their preventive action against HX-XO damages was evaluated. When cultured cells were treated with HX-XO in the medium containing FCS alone, the number of viable cells was reduced to 41% of control. The rate was slightly increased to 54% when Cp was added to the serum, but the addition of Hp revealed no effect. The rate increased remarkably to 82% when Hb was added. The rate was further improved to 87% by the addition of Cp coupled with Hb (Cp + Hb). The effect of Hp-Hb was essentially identical with that of Hb alone. When the three were added simultaneously, the rate was 86% and the effect was similar to that of Cp-Hb (Fig. 4).

**Effect of H₂O₂ on viability of cultured cells and protective effects of various sera against H₂O₂**

Based on the results obtained from the experiments described above, 0.5 mM H₂O₂ (10 μl) was used in place of HX-XO and added to the media. The cells almost died out in the absence of serum, but 32, 102, and 98% of the cells survived in normal, pregnant, and inflammatory groups, respectively. The results were essentially identical with those obtained with HX-XO. Accordingly, protective effect of the serum seem to be attributed mainly to the action of catalase (Table 3).

**Effect of H₂O₂ on viability of cultured cells and protective effect of hemolytic serum**

In order to substantiate the results obtained above, homolytic serum was diluted variously with intact serum and media containing Hb at various concentrations were prepared with the dilutions to examine Hb for the protective effect against H₂O₂. Essentially no viable cells remained in the medium containing no homolytic serum. The survival rates increased to 40, 56, and 93% at 16, 20, and 24 μg/ml Hb, respectively, and the rate was directly proportional to the concentra-

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Viable cells (×10⁴/dish)†</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (2)</td>
<td>10.0 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td>Serum free (2)</td>
<td>1.5 ± 0.1</td>
<td>15</td>
</tr>
<tr>
<td>Normal (3)</td>
<td>3.2 ± 1.2⁸,₉</td>
<td>32</td>
</tr>
<tr>
<td>Pregnant (3)</td>
<td>10.2 ± 2.6⁸</td>
<td>102</td>
</tr>
<tr>
<td>Inflammatory (4)</td>
<td>9.8 ± 2.6⁹</td>
<td>98</td>
</tr>
</tbody>
</table>

Mean ± S.D., (n) = n. Statistically significant at a), b) p < 0.025 (t-test).
† Number of viable cells 24 hr after H₂O₂ treatment (0.5 mM/dish).
DISCUSSION

The present investigation using cultured cells disclosed that the serum can protect cells from active oxygen generated by HX-XO system or H$_2$O$_2$, indicating that the serum possesses antioxidant activity = scavenging activity. Although the same results had been demonstrated in vitro, it appears to be of great significance that the protective effect was proved with cultured cells which can provide experimental conditions close to in vivo. In many of reports, this protective action was described as being mainly attributed to Cp and O$_2^{-}$ scavenging activity of the serum, but increases in concentrations of Cp and Cu in the pregnant and inflammatory sera could not necessarily be related to the number of viable cells. Only few viable cells were recovered from the medium to which Cp was added. SOD revealed essentially no protective effect. The differences in SOD-like activity among media containing serum from various origins were insignificant and the activity tended even to vary to opposite direction. These results suggest that the direct action of O$_2^{-}$ is not responsible for the cell injuries in the present experiments.

Cranfield (1979) reported that the antioxidant activity was partly related to iron free Tf as well as Cp, but in the present experiment pregnant, normal, and inflammatory sera in this order of concentration contained iron free Tf independently of antioxidant activity. Serum Fe was, however, inversely related to the number of viable cells. As peroxidation of lipids requires the presence of Fe ions (Gutteridge 1979), a decrease in pooled Fe in the medium, which include free Fe ions, exerted suppression of cell injuries. Fe concentration in FCS was much higher than in the human serum and this seems to be responsible for the low antioxidant activity in FCS. In addition, as serum Zn concentration was not significantly different for each other, and Hp and $\alpha_1$ AG, each of which is one of acute phase reactants, were increased only in the inflammatory serum, these three elements appeared to be not directly involved.

SOD could not be protective against cell damage, but the cell injuries due to HX-XO were almost completely prevented when the cells were cultured in a medium to which catalase was added beforehand. When Hb to which catalase activity of the blood (Colson-Guastalla et al. 1975) is attributed was added to the medium in advance, Hb showed cell protection identical with one observed in catalase; the numbers of viable cells were the same in both experiments. The use of Hp-Hb complex in combination with Cp produced the protective effect greater than that by Hb alone. In an experiment in which H$_2$O$_2$ was directly added to the medium in place of HX-XO, the sera of various types revealed antioxidant activity essentially identical with those noticed in experiment with HX-XO. The number of viable cells was in proportion to Hb concentration when H$_2$O$_2$ acted on the cells in the medium to which hemolytic serum was added as a source
of Hb.

The results mentioned above may suggest that the active oxygen, inducing cell damage in the present culture system, mainly consists of H$_2$O$_2$, and the scavengers in the blood are mainly substances that exhibit catalase activity such as Hb. Cp, exerting SOD action in the blood, provides less protective effect.

Of active oxygens from various sources, O$_2^-$, although highly reactive, does not react directly with vital target compounds such as DNA, unsaturated fatty acids, etc. O$_2^-$ is once reduced to H$_2$O$_2$ by disproportionation reaction in which SOD is involved and H$_2$O$_2$ yields more reactive •OH by Fenton reaction mediated by Fe ions. H$_2$O$_2$ and •OH thus formed induce damage of tissues (Asada 1980). In fact, in the present experiments, both HX-XO and H$_2$O$_2$ produced the same results, SOD exerted no protective effects, and involvement of Fe ions in the cell injuries was suggested, supporting a hypothesis mentioned above.

Scavengers in the serum are currently considered to be mainly Cp with SOD activity. The transient remission of rheumatoid arthritis noted during pregnancy in female patients is reported to be due to an increase in the concentration of Cp (Denko 1979). In the present cell culture experiment, however, the addition of SOD to the serum exerted little protective effect and the effect was exhibited only when catalase was added. The same results were reported by Shingu and Nobunaga (1981) who carried out the experiment with vascular endothelial cells and by Nakamura and Kamiya (1981) who examined Hela cells for the resistivity against phototoxicity. In addition to these, there is another report describing a view contradictory to protective effect of SOD in vivo (Rosner et al. 1980). Therefore, serum Cp appeared to be not so significant.

On the other hand, it can be imagined that catalase is released from leukocytes when tissue is damaged or in inflammation or released by hemolysis from erythrocytes with Hb (Goth et al. 1983), and then topically functions to remove active oxygen, especially excess H$_2$O$_2$. It is a long established fact that the catalase activity in the normal serum is much lower than that of erythrocyte and insignificant, but the activity increases in various inflammatory diseases including acute pancreatitis (Meszaros et al. 1973; Goth et al. 1982; ). The serum antioxidant may be consisting mainly of the elevated catalase activity. Although the significance of antioxidant activity noted in the pregnant serum remained to be elucidated yet, as free radical oxidation products increase in the blood during pregnancy (Wickens et al. 1981), the antioxidant may be involved in their removal. There is a report describing the presence of peroxidase activity in the uterus and placenta, but the relation between the increased radical oxidation products in the serum and the peroxidase activity in the uterus and placenta remained to be clarified. Although it is difficult to clarify whether the antioxidant activity of the serum and the reaction of cells, both of which were demonstrated in the present experiments, occur only in the particular condition of cell culture or whether the cell culture experiment system can serve as a model
representing in vivo system, the fact that the serum antioxidant activity was demonstrated by the aid of biological technique may provide a clue for the elucidation of scavenger system for active oxygen in the body.

Acknowledgments

We would like to thank Emeritus Professor Tetsuo Maki, and Dr. Tadashi Taima, Director of Department of Internal Medicine, Tohoku Rosai Hospital for their facilities and encouragement of this work. We are indebted to the technical assistance given by the staff of the clinical laboratory of the hospital.

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