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Acceleration of Adhesion of Cancer Cells and Neutrophils to Endothelial Cells in the Absence of *de Novo* Protein Synthesis: Possible Implication for Involvement of Hydroxyl Radicals

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The adhesion of colon cancer cells (colo201) and neutrophils to endothelial cells which had been briefly exposed to either hypoxanthine/xanthine oxidase, or hydrogen peroxide, or peroxynitrite was analyzed in the absence of *de novo* protein synthesis. Such treatments accelerated the adhesions of both colo201 cells and neutrophils to endothelial cells. These effects were blocked by SOD/catalase or EDTA. The results provided evidence that hydroxyl radicals affect the cell surface of endothelial cells and accelerate cell adhesion.

Reactive oxygen species (ROS) are produced in various pathological conditions such as inflammation, ischemia-reperfusion, and during radiation therapies and chemotherapy treatments. Endothelial cells act as a barrier between blood and tissues and thus are exposed to ROS. Endothelial cells are naturally resistant to oxidative stress because they have abundant antioxidative systems including Mn-superoxide dismutase (Mn-SOD) as reported in a previous paper (1). However, in some cases production of ROS in blood vessels is capable of affecting endothelial cells which in turn affects their adhesiveness. In this paper we reported results of a study which suggests that hydroxyl radicals accelerate the adhesion of both cancer cells and polymorphonuclear neutrophils to endothelial cells without the need for *de novo* protein synthesis. The mechanism for this differs from that due to the induction of adhesion molecules as reported by several authors. These results demonstrate for the first time that hydroxyl radicals accelerate cellular adhesion.

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Abbreviations: ROS, Reactive oxygen species; Mn-SOD, Mn-superoxide dismutase; Cu,Zn-SOD, Cu,Zn-superoxide dismutase; BCECF-AM, 3′-O-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethylester; AcD, actinomycin D; CHX, cycloheximide; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; fMLP, formyl methionyl leucyl phenylalanine; EDTA, ethylene diamine tetra acetic acid; ICAM-1, intercellular adhesion molecule-1.

MATERIALS AND METHODS

Materials. Xanthine oxidase was purchased from Boehringer Mannheim Co. Ltd. and 3′-O-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethylester (BCECF-AM) was purchased from Dojindo Co. Ltd. Peroxynitrite solution was prepared according to the quenched-flow synthesis method (2). Recombinant human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was a generous gift from Ube Industries, Co., Ltd., and catalase, actinomycin D (AcD) and cycloheximide (CHX) were Sigma products. The other reagents were of the highest grade available.

Cell culture. Human endothelial cells were isolated from umbilical veins as previously described (1). These cells were grown at 37°C in 100-mm Petri dishes or 24-well flat bottom microplates in MCDB131 medium containing fetal calf serum (10%), recombinant human basic fibroblast growth factor (10 ng/ml), hydrocortisone (1 μg/ml), penicillin G (100 u/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml), under a humid atmosphere containing 5% CO_2. Cultures of subconfluent cells were used for the ROS treatment.

Colo201 colon carcinoma cells were grown at 37°C in 100-mm Petri dishes with RPMI1640 medium containing fetal calf serum (10%), penicillin G (100 u/ml), and streptomycin sulfate (100 μg/ml) under humidified 5% CO_2.

Neutrophils were isolated from whole blood samples, of healthy volunteers according to standard methods (3).

Cell adhesion assays. Adhesion assays were performed as previously reported by Izumi, et al (4). Briefly, endothelial cells were plated in 24-well multiwell plates. Two hours prior to ROS treatment, the endothelial cell medium was changed to the one containing AcD (4 μM) or CHX (10 μM). BCECF-AM was added to the cell suspensions of the colo201 cells or neutrophils to give a concentrations of 3 μM. The cell suspensions were incubated at 37°C for 30 min and then washed to remove excess BCECF-AM. Labeled colo201 cells and neutrophils were suspended at a concentration of 2×10^5 cells/ml. The endothelial cell medium was aspirated and individual aliquots of the cells were treated for exactly 5 minutes with either hypoxanthine/xanthine oxidase, or hydrogen peroxide, or peroxynitrite. Five hundred μl of phosphate buffered saline, pH 7.4 (PBS) containing the ROS generator, hypoxanthine (2 mM)/xanthine oxidase (final concentration, 20 mU), H_2O_2 (100 μM), or peroxynitrite solution (1 μM), were added to each well.

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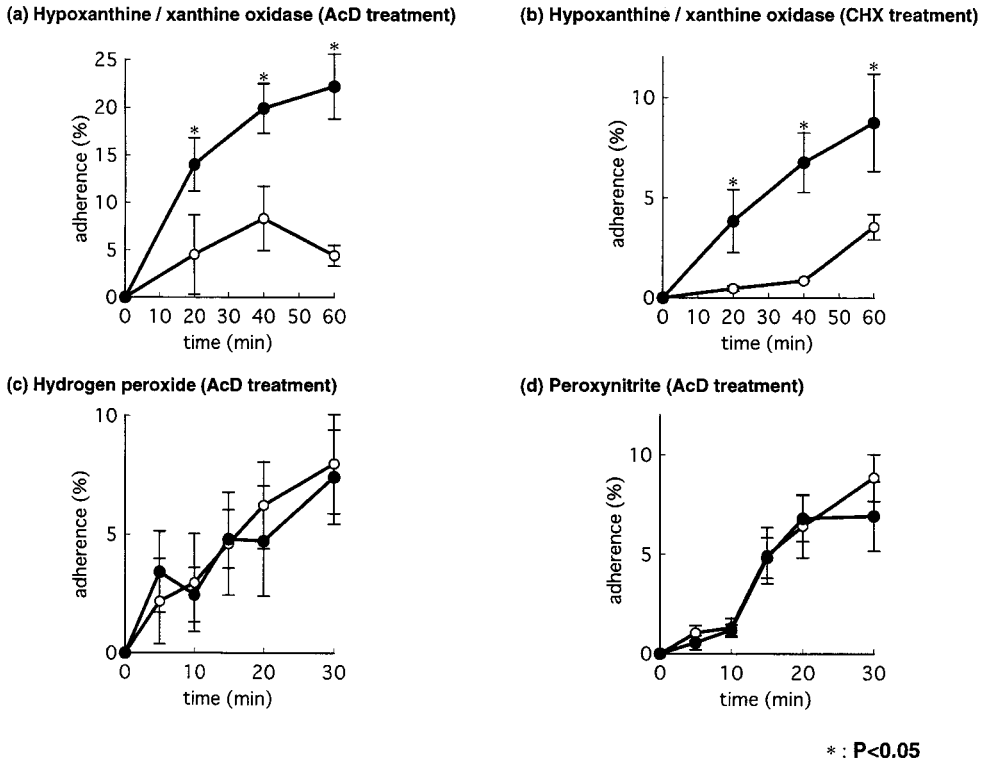

FIG. 1. ROS treatment and adhesion of colo201 to endothelial cells. Endothelial cells were treated for 5 minutes with ROS and adhesion of colo201 cells labeled with BCECF-AM was then analyzed. (○), control; (●), ROS treatment. (a), hypoxanthine (2 mM)/xanthine oxidase (20 mU) with AcD (4 μM); (b), hypoxanthine (2 mM)/xanthine oxidase (20 mU) with CHX (10 μM); (c), H_2O_2 (100 μm) with AcD (4 μM); (d) peroxynitrite solution (1 μM) with AcD (4 μM) (mean ± S.D., n = 6).

After the ROS treatments, the PBS was aspirated and 500 μl of the culture medium containing CHX or AcD were immediately added to each well, after which, a suspension of 5×10^4 cells in 250 μl of the cell suspensions both of BCECF-AM labeled colo201 and of neutrophils were added to individual wells. After incubation for various times at 37°C, each well was washed three times with the same medium and the attached cells were then lysed in 1% Triton X-100 at 37°C. The fluorescence intensity of the lysates was measured at excitation and emission wavelengths of 490 nm and 520 nm, respectively. The percent adhesion was calculated on the assumption that 250 μl of the BCECF-AM labeled cell suspension represent 100%.

RESULTS

ROS Treatment and Adhesion of colo201 to Endothelial Cells

As shown in Fig. 1-(a), (b), hypoxanthine/xanthine oxidase treatments accelerated the adhesion of colo201 cells to endothelial cells in the absence of *de novo* protein synthesis. Both CHX, an inhibitor of protein synthesis, and AcD, an inhibitor of mRNA synthesis, did not affect this acceleration. In the contrast to the effect noted for hypoxanthine/xanthine oxidase, H_2O_2, or peroxynitrite had no effect on the adhesion process (Fig. 1-(c), (d)). As judged by a cytochrome c reduction assay (5), superoxide production by the hypoxanthine/xanthine oxidase solution used in this study was comparable to the levels produced by isolated neutrophils stimulated by either phorbol myristate acetate (PMA) or formyl methionyl leucyl phenylalanine (fMLP); hypoxanthine/xanthine oxidase solution: 4.8 nM/min, neutrophils (10^7/ml) stimulated by PMA: 9.1 nM/min, neutrophils (10^7/ml) stimulated by fMLP: 3.5 nM/min. Endothelial cell viabilities with or without ROS treatment were evaluated using the methylene blue method (6) and showed no significant changes (data not shown).

ROS Treatment and Adhesion of Neutrophils to Endothelial Cells

As shown in Fig. 2-(a), hypoxanthine/xanthine oxidase treatments accelerated the adhesion of neutrophils to endothelial cells even in the presence of CHX as an inhibitor of protein synthesis. While H_2O_2 had a tendency to accelerate cell adhesion (Fig. 2-(b)), peroxynitrite solution had no significant effect (Fig. 2-(c)).

Effects of SOD/Catalase and EDTA

In order to clarify the effect of hydroxyl radicals, SOD and catalase were added to a solution of hypoxanthine/xanthine oxidase in PBS. The same amount of bovine serum albumin was also added to the ROS solution in the

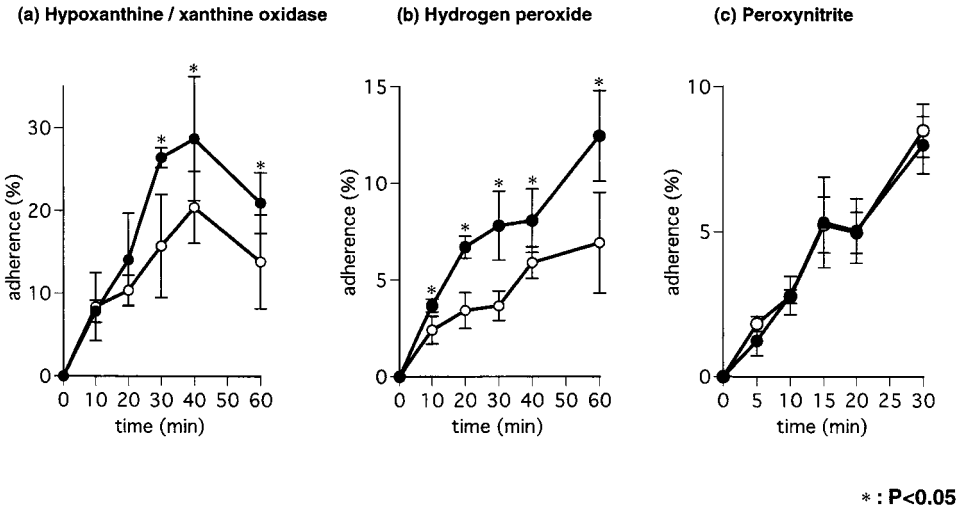

FIG. 2. ROS treatment and adhesion of neutrophils to endothelial cells. Endothelial cells were treated for 5 minutes with ROS and adhesion of neutrophils labeled with BCECF-AM was then analyzed. (○), control; (●), ROS treatment. (a), hypoxanthine (2 mM)/xanthine oxidase (20 mU) with CHX (10 μM); (b), H_2O_2 (100 μm) with CHX (10 μM); (c) peroxynitrite solution (1 μM) with CHX (10 μM) (mean ± S.D., n = 6).

hypoxanthine/xanthine oxidase group. The addition of SOD or of catalase partially prevented the acceleration of cell adhesion, but SOD plus catalase dramatically prevented this for both colo201 and neutrophils (Fig. 3-(a), (b)). Moreover, to prevent the Fenton reaction by chelating transition metals in hypoxanthine/xanthine oxidase solution, EDTA was added during ROS treatment, and as shown in Fig. 3-(c) the accelerated adhesion of colo201 cells was blocked.

DISCUSSION

In this study hypoxanthine/xanthine oxidase treatment, which is considered to be a physiologically rele-

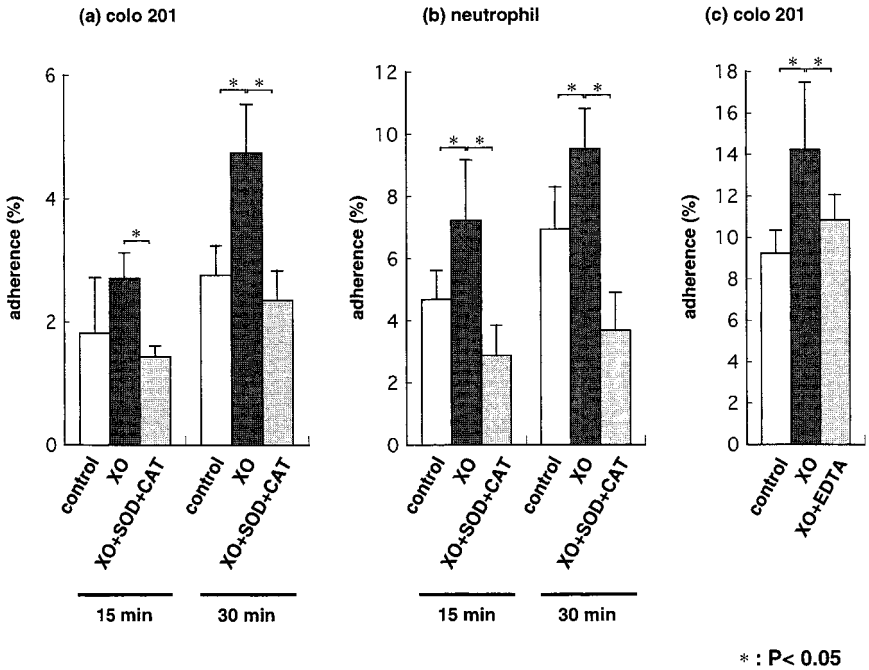

FIG. 3. Effects of SOD/catalase and EDTA. To inhibit hydroxyl radical production, SOD/catalase or EDTA were added to the hypoxanthine/xanthine oxidase solution. *De novo* protein synthesis was blocked by AcD (4μM). (a), colo201; (b), neutrophils. XO, hypoxanthine/xanthine oxidase solution + bovine serum albumin; XO+SOD+CAT, hypoxanthine/xanthine oxidase solution + Cu,Zn-SOD (100 μg/ml) + catalase (50 μg/ml). (c) colo201 and effect of EDTA. XO, hypoxanthine/xanthine oxidase solution; XO+EDTA. hypoxanthine/xanthine oxidase solution + EDTA (50 μM) (mean + S.D., n = 6).

vant condition, accelerated the cellular adhesion of colo201 cells and neutrophils to endothelial cells. The hypoxanthine/xanthine oxidase solution produces both superoxide anion and hydrogen peroxide and finally hydroxyl radicals via the Fenton reaction, provided trace amounts of transition metal ions are present. In fact, the presence of SOD/catalase completely blocked the usual acceleration of cell adhesion effected by the presence of hypoxanthine/xanthine oxidase. EDTA also blocked the acceleration of cell adhesion in this system. Neutrophil adhesion to endothelial cells was higher than that of colo201 even at control conditions and SOD/catalase treatment was more effective in blocking cell adhesion for these cells. The above data raised the possibility that neutrophils may naturally produce superoxide anions via their own NADPH oxidase systems. The ability of neutrophils to do so would account for some acceleration in cell adhesion even in the control. Moreover, in the case of neutrophils, hydrogen peroxide treatment weakly accelerated cell adhesion, while it had no effect on the adhesion of colo201 cells. These facts are consistent with neutrophils to being able to generate superoxide anions.

Several reports have appeared indicating that ROS accelerate neutrophil adhesion by the induction of adhesion molecules (7, 8, 9) or by a non-ICAM-1 dependent mechanism (10). In this study, *de novo* protein synthesis was blocked by AcD or CHX and no increase of E-selectin, P-selectin, ICAM-1 and thrombomodulin in endothelial cells was observed as judged by a FACS scan analysis using specific antibodies (data not shown). Therefore, the accelerated cell adhesion is, most likely, due to the direct effect of hydroxyl radicals. It has been recently demonstrated histochemically that the endothelial glycocalyx in rat heart was destroyed by ischemia-reperfusion treatment (11). It is known that ROS entities are capable of modifying glycosaminoglycan structures or hyaluronic acid *in vitro* (12, 13, 14, 15) in addition to various proteins (16, 17, 18, 19). A recent report indicates that cell surface carbohydrates play a role in shielding the AIDS virus from immune recognition (20). Therefore, it seemed reasonable to assume that the generation of ROS by the presence of hypoxanthine/xanthine oxidase could destroy the glycocalyx of endothelial cells and as a result the colo201 cells and neutrophils might easily approach and attached themselves to these cells. Our results are also supported by the inhibitory effect of recombinant Cu,Zn-SOD on metastasis of the tumor cells (21). These findings suggest that ROS may well accelerate cell adhesion very rapidly in the rolling of neutrophils or in the metastasis of tumor cells after radiation therapy or chemotherapy, both of which increase ROS production. ROS molecules have very short half lives and strong non-specific effects and as a result they may play an important role in the super acute phase of inflammation, ischemia-reperfusion or cancer metastasis through their effects on cell adhesion.

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