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Exposure of rat thymocytes to hydrogen peroxide increases annexin V binding to membranes: inhibitory actions of deferoxamine and quercetin

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Abstract

Effects of hydrogen peroxide (H_2O_2) on rat thymocytes were examined, using a flow cytometer and three fluorescent probes, annexin V-fluorescein isothiocyanate (annexin V-FITC) for detecting phosphatidylserine expressed on the membrane surface, ethidium bromide for estimating dead cells, and fluo-3-acetoxymethyl ester (fluo-3-AM) for monitoring changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), to characterize H_2O_2-induced cytotoxicity. Exposure to H_2O_2 (30 μM or more) increased the number of annexin V-positive live cells dose- and time-dependently while the number of dead cells increased at concentrations of 1 mM or more. H_2O_2 (30 μM or more) increased $[Ca^{2+}]_i$ in a dose-dependent manner. Threshold concentration of H_2O_2 to increase $[Ca^{2+}]_i$ was similar to that to increase annexin V binding to membranes. The H_2O_2-induced change in cell membranes was attenuated under Ca^{2+}-free conditions. Therefore, it is likely that Ca^{2+} is involved in the H_2O_2-induced cytotoxicity. Deferoxamine was effective to protect the cells suffering from H_2O_2-induced oxidative stress, suggesting a contribution of hydroxyl radicals generated by the Fenton reaction. Quercetin also exerted a potent protective action on cells suffering from H_2O_2-induced oxidative stress. The results indicate that the exposure of rat thymocytes to H_2O_2 at micromolar concentrations increases annexin V binding to cell membranes in a Ca^{2+}-dependent manner, suggesting the possibility that the oxidative stress caused by H_2O_2 (and/or hydroxyl radicals) induces apoptosis via increasing $[Ca^{2+}]_i$.

Keywords: Annexin V; H_2O_2; Ca^{2+}; Thymocyte

1. Introduction

Oxidative stress to living cells increases the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$), a process responsible for subsequent cell death or injury (Nicotera et al., 1988; Mirabelli et al., 1989; Orrenius et al., 1989). Of all biological oxidant effects, the effects of hydrogen peroxide (H_2O_2), a metabolite of superoxide anion, on $[Ca^{2+}]_i$ have been studied in several types of cells (Hyslop et al., 1986; Starke et al., 1986; Shepherd et al., 1987; Ueda and Shah, 1992). The increase in $[Ca^{2+}]_i$ is included as a common feature in H_2O_2 cytotoxicity. Since the sustained increase in $[Ca^{2+}]_i$ that activates calpain is one of the mechanisms underlying apoptosis (Jiang et al., 1994; Squier et al., 1994), the possibility is raised that H_2O_2 increases $[Ca^{2+}]_i$, resulting in apoptosis in some cells. Annexin V conjugated with fluorophore is used to detect membrane surface changes that occur early during apoptosis because annexin V binds to phosphatidylserine expressed on the membrane surface by flipping from inner membrane to outer leaflet (Koopman et al., 1994; Vermes et al., 1995; Pellicciari et al., 1997). This is important for macrophages to recognize the cells undergoing apoptosis (Fadok et al., 1992; Verhoven et al., 1995). Furthermore, if a loss of phospholipid asymmetry in plasma membranes also occurs during necrosis, the expression of phosphatidylserine would be a hallmark of dying cells. In this study, first, we examined the effects of H_2O_2 on rat thymocytes, one of the models characterized for apoptosis, to see if H_2O_2 produces such changes in membrane surface, using a flow cytometer and

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two fluorescent probes (annexin V-FITC and ethidium bromide). If so, secondly, we examined the relation of these changes to the H_2O_2-induced changes in $[Ca^{2+}]_i$ by using fluo-3-acetoxymethyl ester (fluo-3-AM) (Kao et al., 1989) and examined the effects of ionomycin as a calcium ionophore. Finally, we examined the protective effects of deferoxamine as a chelator for Fe^{2+} and quercetin as an antioxidant agent on the cells suffering from oxidative stress induced by H_2O_2.

2. Materials and methods

2.1. Preparation

Experiments were performed on thymocytes dissociated from thymus glands of 4-week-old Wistar rats (Japan SLC, Shizuoka, Japan). Thymocytes were chosen for this study because of the following reasons. First, the cells are dissociated without treatment with proteolytic enzymes which may compromise cell membranes. The cell viability of dissociated thymocytes under control conditions, examined by using ethidium bromide, was greater than 95%. Secondly, thymocytes are suitable for applying to a flow cytometer because of their spherical shape, size, and homogeneity. Thirdly, rat thymocytes are one of the models characterized for apoptosis. The technique for dissociation of thymocytes was that previously described (Chikahisa et al., 1996). In brief, thymus glands were sliced at a thickness of 400 to 500 μm. Thereafter, the slices were gently triturated in chilled control Tyrode's solution (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, glucose 5 mM, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 5 mM with an appropriate amount of NaOH to adjust to pH 7.4) to dissociate single thymocytes. Tyrode's solution containing dissociated thymocytes was passed through a mesh (a diameter of 10 μm) to remove residues. Ca^{2+}-free Tyrode's solution contained 2 mM ethylene glycol-bis(β-aminoethyl ester)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Wako, Osaka, Japan) and 50 μM 1,2-bis(2-aminophenoxylethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) (Dojin Chemical Lab., Kumamoto, Japan) after replacing 2 mM $CaCl_2$ of control Tyrode's solution by 2 mM $MgCl_2$.

2.2. Fluorescence measurements and analyses

To detect phosphatidylserine exposed on the outer surface of plasma membranes, annexin V-FITC (Trevigen, Gaithersburg, USA) was used in combination with ethidium bromide (Katayama Chemical Industries, Osaka, Japan). Early in the apoptotic process, phosphatidylserine is exposed on the outer side of plasma membranes (Koopman et al., 1994; Stuart et al., 1998). About 50 phosphatidylserine monomers are estimated to be bounded per annexin V molecule. Therefore, annexin V conjugated with FITC is used to detect the cells undergoing apoptosis. Annexin V-FITC (10 μl/ml) and ethidium bromide (5 μM) were added to the medium at 15 and 3 min, respectively, before fluorescence measurement. FITC and ethidium fluorescence from thymocytes were measured with flow cytometer (Cyto ACE-150, JASCO, Tokyo, Japan). The excitation wavelength for FITC and ethidium was 488 nm produced by an argon laser. Emissions were monitored at the wavelengths of 530 ± 20 nm for FITC and 600 ± 20 nm for ethidium. Fluorescence cytograms (FITC fluorescence vs. ethidium fluorescence, see Fig. 1A) obtained from a programmed number of thymocytes (3000 or 5000 cells) were analyzed with a software JASCO Ver.3XX (JASCO) and a personal computer (PC-9801RX, NEC, Tokyo, Japan). Area A shown on the left panel of Fig. 1A indicates the cells (intact cells) which were not stained with annexin V-FITC and ethidium. Area B shows the cells (annexin V-positive live cells) stained with annexin V-FITC but not with ethidium. Dead cells showing both FITC and ethidium fluorescence belong to area C. There-

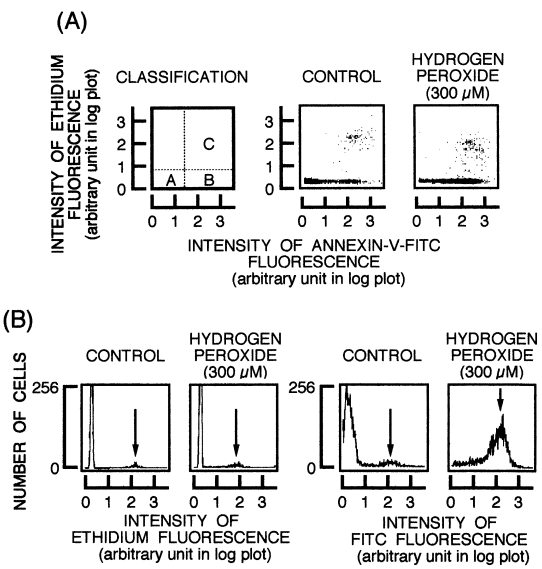

Fig. 1. Estimation of H_2O_2-induced changes in three cell populations of rat thymocytes with ethidium bromide and annexin V-FITC. (A) Flow-cytometric measurements of FITC (abscissa) and ethidium (ordinate) fluorescence from a programmed number of rat thymocytes (5000 cells) in the cell suspension. Left panel (CLASSIFICATION) shows classification of cell population. Area A indicates the cells which did not show intensive FITC and ethidium fluorescence (intact cells). Area B shows the cells only exhibiting FITC fluorescence (annexin V-positive live cells). The cells stained with ethidium (dead cells) belong to area C. Right two panels show the fluorescence cytograms before (left panel, CONTROL) and 180 min after application of 300 μM hydrogen peroxide (H_2O_2) (right panel, HYDROGEN PEROXIDE). (B) Effects of H_2O_2 on the histograms of ethidium (left two panels) and FITC (right two panels) fluorescence. Results were obtained at 180 min after application of H_2O_2. Left two panels: ethidium fluorescence histograms obtained in absence (CONTROL) and presence of H_2O_2 (HYDROGEN PEROXIDE), right two panels: FITC fluorescence histograms in absence (CONTROL) and presence of 300 μM H_2O_2. Arrows respectively indicate the cells which showed intense ethidium fluorescence (left two panels) or FITC fluorescence (right two panels).

fore, there are three populations classified by FITC and ethidium fluorescence. The cell populations under control conditions were statistically unchanged during the experiment, a period of 180 to 240 min. All experiments under oxidative stress were accompanied by a control experiment without oxidative stress.

To monitor the changes in $[Ca^{2+}]_i$ of thymocytes, fluo-3-AM (Dojindo Laboratory, Kumamoto, Japan) and ethidium bromide were used (Okazaki et al., 1996). Fluo-3-AM was added into the cell suspension to achieve a final concentration of 500 nM at least 60 min before measurement. Simultaneous measurement of fluo-3 and ethidium fluorescence from thymocytes was also done with a flow cytometer. The excitation wavelength for fluo-3 was 488 nm. Its emission was detected at a wavelength of 530 ± 20 nm. The intensity of fluo-3 fluorescence from the cells which did not show ethidium fluorescence was analyzed as described above.

3. Results

3.1. H_2O_2-induced changes in FITC and ethidium fluorescence of rat thymocytes

Under control conditions, a large population of cells (intact cells) did not show any fluorescence (Fig. 1A). However, there was a great increase in the number of cells (annexin V-positive live cells) showing only FITC fluorescence at 180 min after the start of application of 300 μM H_2O_2 (Fig. 1A). H_2O_2 at this concentration did not significantly increased the number of dead cells with ethidium fluorescence (Fig. 1B) while there was a large increase in the number of annexin V-positive live cells showing FITC fluorescence (Fig. 1B). The results suggest that continued application of 300 μM H_2O_2 significantly increased the number of cells with phosphatidylserine exposed on plasma membranes, without affecting cell viability.

Fig. 2A shows the time course of H_2O_2-induced changes in three cell populations classified by FITC and ethidium fluorescence. In the presence of 300 μM H_2O_2, the number of annexin V-positive live cells greatly increased in a time-dependent manner while the number of dead cells did not. Thus, the results suggest that the exposure to 300 μM H_2O_2 increases the population of cells undergoing apoptosis, without affecting cell viability. As shown in Fig. 2B, H_2O_2 at 30 μM or higher dose-dependently increased the number of annexin V-positive live cells while the number of dead cells increased only at millimolar concentrations. It is likely that annexin V-FITC enables us to detect one aspect of the cytotoxic actions of H_2O_2 at concentrations lower than those for ethidium. As shown in Fig. 2A, the cells were not affected at 15 min after the start of application of 300 μM H_2O_2. When the cells were treated with 300 μM H_2O_2 for 15 min then further incubated without H_2O_2 for next 165 min, the number of annexin V-positive

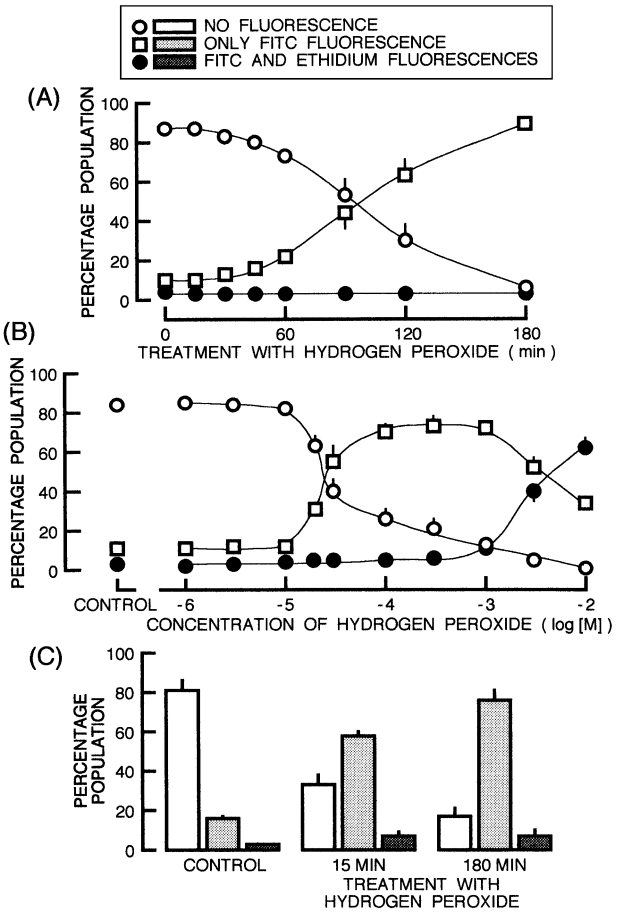

Fig. 2. Hydrogen peroxide-induced changes in three cell populations of rat thymocytes. Asterisks near symbols or columns indicate a significant difference between control group and H_20_2-treated group ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). (A) Time-dependent changes in three cell populations after the start of application of 300 μM H_2O_2. Symbol and bars indicate mean of percentage population and S.D. of four experiments, respectively. Open circles: intact cells, open squares: annexin V-positive live cells, filled cycles: dead cells. (B) Dose-dependent changes in three cell populations after the start of application of H_2O_2 (1 μM to 10 mM). Symbols are same as in (A). Symbol and bars show average and S.D. of four experiments, respectively. (C) Effects of short (15 min, middle panel, 15 MIN) and prolonged (180 min, right panel, 180 MIN) exposures to 300 μM H_2O_2 on three cell populations. Effects were examined at 180 min after start of H_2O_2 application. In the middle panel (15 MIN), H_2O_2 was removed from incubation medium at 15 min after the start of H_2O_2 application. Column and bar respectively indicate the mean of percentage population and S.D. of four experiments.

live cells also increased to a certain extent (50%–60%), but less than in the case of 180 min treatment with H_2O_2 (70%–80%) (Fig. 2C). Therefore, the exposure to H_2O_2 even for a short period seems to induce a change in cell membranes.

3.2. Involvement of Ca^{2+} in H_2O_2-induced changes in cell membrane surface

There is a possibility that the H_2O_2-induced increase in $[Ca^{2+}]_i$ initiates the H_2O_2-induced changes in membrane

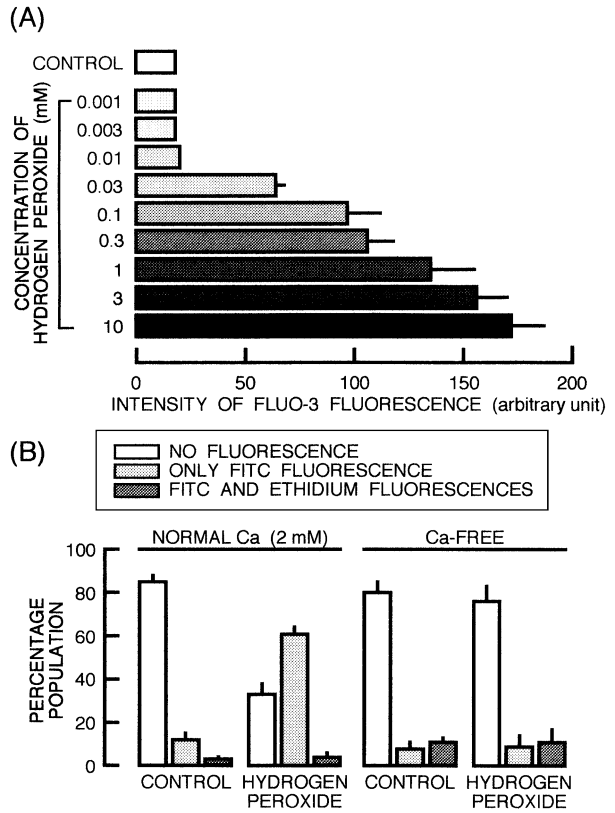

Fig. 3. Involvement of Ca^{2+} in the H_2O_2-induced changes in three cell populations. Asterisks near symbols or columns indicate a significant difference between control group and H_20_2-treated group ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). (A) Effects of H_2O_2 at concentrations ranging from 1 μM to 10 mM on the mean intensity of fluo-3 fluorescence obtained from 5000 thymocytes. Effects were examined at 180 min after the start of application of H_2O_2. Column and bar, respectively, indicate the mean and S.D. of four experiments. (B) Effects of H_2O_2 on three cell populations under Ca^{2+}-free conditions. Column and bar, respectively, indicate the mean and S.D. of four experiments.

surface. To test this possibility, we examined the effects of H_2O_2 on fluo-3 fluorescence of thymocytes incubated under normal Ca^{2+} conditions and of three cell populations under Ca^{2+}-free conditions. Fig. 3A shows the effect of H_2O_2 at concentrations ranging from 1 μM to 10 mM on fluo-3 fluorescence. H_2O_2 started to greatly increase the intensity of fluo-3 fluorescence when the concentration was 30 μM. Increasing the concentration of H_2O_2 produced further increases in fluo-3 fluorescence intensity. These concentrations (30 μM to 10 mM) of H_2O_2 corresponded to those needed to increase the number of annexin V-positive live cells (Fig. 2B). Under Ca^{2+}-free conditions the effect of 300 μM H_2O_2 on the cell populations was greatly attenuated (Fig. 3B). These results suggest the involvement of Ca^{2+} in the H_2O_2-induced changes in cell populations. To test this hypothesis, we studied the effect of ionomycin, a calcium ionophore, on fluo-3 fluorescence and cell populations of rat thymocytes. Ionomycin at 3 μM significantly increased the intensity of fluo-3 fluorescence, indicating an increase in $[Ca^{2+}]_i$. Ionomycin at this concentration significantly increased the numbers of annexin V-positive live cells and dead cells. The percentage population of live cells positive to annexin V increased from the control ($12 \pm 1\%$) to $38 \pm 3\%$ (mean ± S.D. in four experiments). For dead cells, this was from $5 \pm 1\%$ to $61 \pm 3\%$. Thus, it is likely that an increased $[Ca^{2+}]_i$ is involved in the H_2O_2-induced increase in the number of annexin V-positive cells.

3.3. Inhibition of H_2O_2-induced changes by drugs

Deferoxamine, a chelator for Fe^{2+}, at 1 mM significantly attenuated not only the increase in number of dead cells with 3 mM H_2O_2 (Oyama et al., 1998), but also the increase in number of annexin V-positive live cells with 300 μM H_2O_2 (Fig. 4A). Therefore, the hydroxyl radical is involved in both the increase in number of annexin

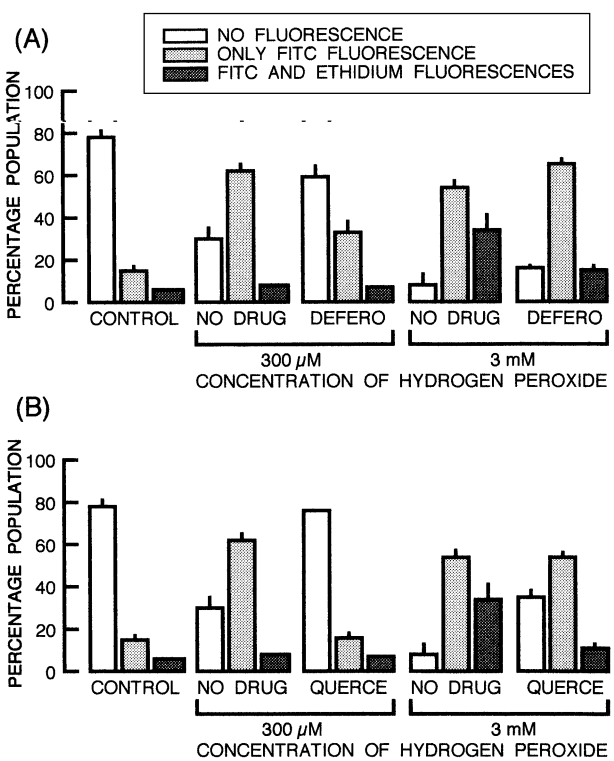

Fig. 4. Effects of deferoxamine and quercetin on H_2O_2-induced changes in the cell populations. Asterisks indicate a significant difference between no drug group and drug-treated group in presence of H_2O_2 ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). (A) Effect of 1 mM deferoxamine on H_2O_2-induced changes in the cell populations. Concentrations of H_2O_2 were 300 μM and 3 mM. Deferoxamine was applied at 1 h before the start of H_2O_2 application. Effects were examined at 180 min after the start of H_2O_2 application. Column and bar, respectively, indicate the mean of percentage population and S.D. of four experiments. (B) Effect of 10 μM quercetin on H_2O_2-induced changes in three cell populations. Concentrations of H_2O_2 were 300 μM (left panels) and 3 mM (right panels). Quercetin was applied at 1 hr before the start of H_2O_2 application. Effects were examined at 180 min after the start of H_2O_2 application. Column and bar, respectively, indicate the mean of percentage population and S.D. of four experiments.

V-positive live cells and the decrease in cell viability induced by H_2O_2.

Quercetin is known to protect cells suffering from oxidative stress induced by H_2O_2 (Nakayama et al., 1993; Yokoo and Kitamura, 1997) but was reported to show no scavenging action on hydroxyl radicals (Hanasaki et al., 1994). Therefore, as shown in Fig. 4B, the effect of quercetin was tested. Quercetin at 10 μM greatly suppressed the changes in cell populations induced by 300 μM H_2O_2. It was also effective to attenuate the changes induced by 3 mM H_2O_2 in the cell populations. Therefore, it can be concluded that quercetin exerts a potent protective effect on the cells suffering from H_2O_2-induced oxidative stress.

4. Discussion

4.1. Some implications of phosphatidylserine detection by annexin V-FITC

We have previously examined the cytotoxic action of H_2O_2 on rat thymocytes by using ethidium bromide (Chikahisa et al., 1996; Okazaki et al., 1996). The cells stained with ethidium were assumed to be dead. However, when the cells had blebs on their membranes after the application of H_2O_2, some of such cells were not stained with ethidium (Oyama et al., unpublished results). Therefore, while ethidium is suitable for detecting the dead cells, it is not suitable for live cells the membranes of which are somehow compromised. In the present study, we used annexin V conjugated with FITC for detecting phosphatidylserine exposed on the outer surface of membranes. Phosphatidylserine is normally exposed on inner membranes of intact cells and it becomes exposed on outer surface of membranes during the early stage of apoptosis (Pellicciari et al., 1997; Stuart et al., 1998). Therefore, phosphatidylserine exposed on outer membranes is one of the parameters of the cytotoxicity induced by some chemicals. The combination of annexin V-FITC and ethidium enabled us to make a classification of the intact cells, the cells with phosphatidylserine exposed on outer side of plasma membranes and the dead cells in the cell suspension, using a flow cytometer as shown in Fig. 1. Therefore, annexin V-FITC is useful to detect changes in membranes of cells affected by toxic substances, including H_2O_2, because the change in cell membranes at micromolar concentrations of H_2O_2 was detected with annexin V-FITC but not with ethidium bromide. However, it is unlikely that all cells belonging to area B of Fig. 1A in this system are apoptotic because of the following observation (Oyama et al., unpublished result). Digitonin, one of the saponins, non-specifically increases the membrane permeability of cells, resulting in necrotic cell death (Oyama et al., 1995). Digitonin increased the number of cells stained with ethidium without apparently increasing the number of cells belonging to area B. The cells belonging to area B were greatly affected by digitonin, resulting in the accumulation of cells in area C. Because there is a small population of cells in area B during necrosis, it is likely that the exposure of phosphatidylserine on membranes also occurs just before the loss of barrier functions of membranes. Therefore, it seems that some of cells belonging to area B are undergoing necrosis.

4.2. Some characteristics of H_2O_2-induced cytotoxicity

As shown in Figs. 1 and 2, the exposure to H_2O_2 at concentrations ranging from 30 to 300 μM dose-dependently increased the number of cells with phosphatidylserine exposed on outer side of plasma membranes. Phosphatidylserine is recognized by macrophages for phagocytosis (Fadok et al., 1992; Verhoven et al., 1995). Therefore, the exposure to H_2O_2 at micromolar concentrations induces a change in membranes which is lethal to the cells although the cells are still alive. Further increases in the concentration of H_2O_2 (1 to 10 mM) dose-dependently increased the number of dead cells (Fig. 2B). H_2O_2 at millimolar concentrations seems to induce necrosis because of large blebs on membranes in some cells (not shown in this study).

After a short exposure (15 min) to 300 μM H_2O_2, most (> 80%) cells incubated with Tyrode's solution without H_2O_2 for next 165 min became annexin V-positive, although H_2O_2 did not increase the number of annexin V-positive live cells for 15 min after the start of H_2O_2 application (Fig. 2A). Even the short exposure to H_2O_2 initiated a process of change in the membrane surface. H_2O_2 at 30 μM or more produced an increase in $[Ca^{2+}]_i$ (Fig. 3B). Abnormal increases in $[Ca^{2+}]_i$ are linked to subsequent cell injury or death (Nicotera et al., 1988; Mirabelli et al., 1989; Orrenius et al., 1989). The threshold concentration (30 μM) of H_2O_2 needed to increase the $[Ca^{2+}]_i$ (Fig. 3A) was similar to that needed to increase the number of annexin V-positive live cells (Fig. 2B). The H_2O_2-induced change in plasma membrane surface was greatly suppressed under Ca^{2+}-free condition (Fig. 3B). Furthermore, ionomycin increased the number of annexin V-positive live cells. It can be concluded that the H_2O_2-induced increase in the number of annexin V-positive live cells is dependent on the $[Ca^{2+}]_i$ increased by H_2O_2.

4.3. Protective actions of deferoxamine and quercetin

H_2O_2 and the superoxide anion produce hydroxyl radicals in the presence of Fe^{2+} by the Fenton reaction. Deferoxamine reduced the concentration of Fe^{2+}, resulting in a reduced production of hydroxyl radical. Deferoxamine partly attenuated the H_2O_2-induced changes in cell populations (Fig. 4A). Therefore, it seems that the hydroxyl radical is also involved in the H_2O_2-induced toxicity. Quercetin at a concentration of 10 μM greatly suppressed

the H_2O_2-induced increase in number of annexin V-positive live cells when the concentration of H_2O_2 was 300 μM (Fig. 4B). Quercetin also decreased the number of dead cells increased by 3 mM H_2O_2 (Fig. 4B). Quercetin shows no scavenging action on hydroxyl radicals (Hanasaki et al., 1994). Therefore, the quercetin-induced suppression of H_2O_2-induced changes in the cell populations seems to have partly resulted from a direct inhibitory action of quercetin on the H_2O_2-induced events. Furthermore, quercetin blocks phosphatidylinositol conversion to inositol triphosphate by inhibiting 1-phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase, respectively (Prajda et al., 1995; Shen and Weber, 1997), resulting in suppression of the inositol triphosphate-mediated intracellular Ca^{2+} signal transduction system. The production of inositol triphosphate in cultured retinal cells is increased dose-dependently by oxidative stress (Rego et al., 1996). Thus, there is a possibility that the inhibition of 1-phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase by quercetin contributes partly to the protective action of quercetin on living cells suffering oxidative stress.

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