Free Rad. Res., Vol. 28, pp. 485–495
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Biochemical Properties of Human Oral Polymorphonuclear Leukocytes

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Accepted by Prof. N. Taniguchi

(Received 27 November 1997; In revised form 7 December 1997)

Polymorphonuclear leukocytes (PMN) isolated from the oral cavity of healthy human volunteers, spontaneously generated superoxide, nitric oxide (NO) and other reactive oxygen species (ROS) which exhibited strong luminol chemiluminescence (LCL). To understand the physiological roles of oral PMN (OPMN), biochemical properties of the cells were analyzed. Biochemical analysis revealed that OPMN were already primed under physiological conditions. Western blot analysis revealed that they strongly expressed the inducible type of NO synthase (NOS II) and exhibited the activity to catalyze tyrosine phosphorylation of various proteins including a 115 kDa protein (cbl product). OPMN also generated H_2O_2 and $^{\bullet}OH$ by some superoxide dismutase (SOD)-sensitive mechanism and released myeloperoxidase (MPO). Kinetic analysis using specific inhibitors revealed that OCl^- generated by OPMN was predominantly responsible for the enhanced LCL. During the incubation under standard culture conditions, OPMN underwent apoptosis which proceeded more rapidly than that of the circulating PMN (CPMN). Immunochemical analysis revealed that expression of apoptosis-related gene products, such as Bcl-2, Bcl-xL and Bax, was below detectable levels with both cell types. However, caspase-3 but not caspase-1 was markedly activated in OPMN. These results indicate that the primed OPMN spontaneously generate ROS and play an important role in the defense mechanism in the oral cavity and that the generated ROS activate caspase-3 thereby inducing apoptosis of the cells.

Keywords: Apoptosis; Bcl-2; caspase-3; NOS II; oral polymorphonuclear leukocyte; reactive oxygen species; tyrosine phosphorylation

Abbreviations: AMC, 7-amino-4-methyl-coumarin; CPMN, circulating PMN; DETAPAC, diethylenetriaminepenta-acetic acid; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DMPO/$^{\bullet}$OH, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; Cyt. c, ferricytochrome c; FMLP, formyl-methionyl-leucyl-phenylalanine; G-CSF, granulocyte colony stimulating factor; ICE, interleukin-1β-converting enzyme; KRP, Krebs–Ringer-phosphate buffer; LCL, luminol chemiluminescence; MCA, 4-methyl-coumaryl-7-amide; MCLA, 2-methyl-6-[para-methoxyphenol]-3,7-dihydroimidazo[1,2-α]pyrazine-3-one; MPO, myeloperoxidase; OPMN, oral PMN; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; NO, nitric oxide; NOS II,

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inducible NO synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

INTRODUCTION

Neutrophils play important roles in protecting hosts against infection and in the modulation of an inflammatory process.[1] CPMN in healthy human subjects are relatively inactive and exhibit a minimal response to various stimuli, such as formyl-methionyl-leucyl-phenylalanine (FMLP) and opsonized zymosan.[2] Low concentrations of cytokines and endotoxin do not trigger the respiratory burst in CPMN but activate tyrosine kinase[3] and induce the metabolic condition called "priming" by which PMN can readily react strongly to various stimuli.[2,4,5] Under physiological conditions, substantial numbers of CPMN migrate into the oral cavity, gastrointestinal lumen, respiratory tract, glandular lacrimalis, and milk. Although biochemical properties of CPMN have been well documented, only limited information is available for PMN from other sources. OPMN which migrate into the oral cavity are fully primed and spontaneously release ROS.[6,7] Furthermore, when stimulated by various ligands, OPMN also rapidly generate substantial amounts of ROS.

Because PMN undergo apoptosis soon after differentiation from promyelocytic cells, their life-time is fairly short (~16 h).[8] When treated with either retinoic acid or dimethylsulfoxide, human myelocytic leukemia cells (HL-60) differentiate to PMN-like cells. Apoptosis of the differentiated HL-60 cells is associated with the down-regulation of their Bcl-2.[9] A number of apoptosis-related gene products have been identified, such as the Bcl-2 family[10,11] and the interleukin-1β converting cysteine protease family (ICE).[12,13] Apoptosis of various types of cells is also suppressed by Bcl-xL[14] which also counters cell death by Bax.[15] ICE is synthesized as an inactive proenzyme which requires proteolytic cleavage to generate the active form of heterodimeric enzyme.[16] Overexpression of the ICE family (caspases) induces apoptosis of various cells, suggesting their role in the pathway leading to apoptosis.[12] Caspases comprise three subfamilies, caspase-1 (ICE), caspase-2 (ICH-1), and caspase-3 (CPP32).[17] The present work describes biochemical properties of OPMN and the mechanism for their priming and apoptosis.

MATERIALS AND METHODS

Chemicals

Diethylenetriaminepenta-aceticacid (DETAPAC), ferricytochrome c (Cyt. c), FMLP, horseradish peroxidase, MPO, phorbol myristate acetate (PMA), sodium arachidonate, scopoletin, superoxide dismutase (SOD) and catalase were purchased from Sigma Co. (St. Louis, Mo.). 2-Methyl-6-[para-methoxyphenol]-3,7-dihydroimidazo [1,2-α]pyrazine-3-one (MCLA) was obtained from Tokyokasei (Tokyo, Japan). Polyclonal antibodies against Bcl-2, Bcl-xL and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA). Antiphosphotyrosine and anti-NOS II monoclonal antibodies were purchased from ICN Biomedical (Costa Mesa, CA) and Transduction Laboratories (Lexington, KY), respectively. Fluorogenic tetrapeptide substrates, acetyl-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA for caspase-1) and acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA for caspase-3) were obtained from the Peptide Institute (Osaka, Japan). 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was obtained from Dojindo Laboratory (Kumamoto, Japan). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan). FMLP and PMA were dissolved in ethanol, and the final concentration of ethanol in the reaction mixtures was less than 0.5%.

Cells

CPMN were isolated from venous blood of healthy human subjects by the Ficoll/Hypaque gradient method.[7] OPMN were obtained from six healthy volunteers having no sign of periodontal inflammation as described previously.[7] Briefly, one hour after brushing the teeth without toothpaste, the oral cavity is thoroughly washed for 60 s with 20 ml of Krebs–Ringer-phosphate buffer (KRP). After repeating this washing 5 times, the combined solution (500–600 ml) was passed through a nylon filter (300 mesh) to eliminate epithelial cells and cell debris. The filtrate was centrifuged at $250 \times g$ for 5 min at 4°C. The sedimented cells were resuspended in 10 ml KRP and layered on Polymorphoprep (Nycomed, Oslo, Norway). After centrifugation at $450 \times g$ for 30 min, the cells collected in the interface between KRP and Polymorphoprep were washed twice with KRP and kept on ice until used for the experiments. After sedimentation with Ficoll/Hypaque gradient, OPMN (about 5×10^5 cells) were obtained from each individual. The purity and viability of OPMN were higher than 90% and 80%, respectively. PMN were stimulated either by 0.2 µM FMLP, 0.1 nM PMA, or 30 µM sodium arachidonate at 37°C. Biochemical properties of OPMN samples from different healthy subjects are similar with each other.[7]

HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2/95% air. Cells in a logarithmic growth phase were used for assays. Cell viability was routinely determined by the trypan blue dye exclusion method.

Measurement of Superoxide Generation

Cellular generation of $O_2^{\bullet -}$ was assayed by the Cyt. c method using a dual beam spectrophotometer (Shimadzu UV 3000) equipped with a water-jacketed cell holder and magnetic stirrer.[2,3] Briefly, the reaction was started by adding PMN at 37°C in KRP containing 10 mM glucose, 20 µM Cyt. c, and 1 mM $CaCl_2$ in the presence or absence of various reagents. Changes in the absorbancy at 550–540 nm ($A_{550-540}$) were monitored continuously.

Measurement of H_2O_2 Generation

Production of H_2O_2 was assayed at 37°C in a fluorospectrophotometer (Hitachi 650-10 LC) as described previously.[18] Cells (10^5/ml) were suspended in KRP containing 10 mM glucose, 1 mM $CaCl_2$, 50 nM horseradish peroxidase and 4 µM scopoletin. Change in fluorescence intensity was measured at 450 nm with excitation at 360 nm.

Measurement of Luminol Chemiluminescence

Chemiluminescence experiment were performed using a Luminescence Reader (Aloka BRL-201) or a calcium analyzer (Jasco CAF 100).[18] The reaction mixture contained in a final volume of 1 ml KRP containing 10 mM glucose and 1 mM $CaCl_2$, 100 µM luminol, 1×10^5 cells, and other additions. The intensity of luminol chemiluminescence (LCL) was recorded for 10–15 min.

Assay for Myeloperoxidase Activity

MPO activity was measured by using MCLA.[19] Briefly, OPMN (5×10^5 cells/ml) and CPMN (5×10^6 cells/ml) were incubated in KRP containing 1 mM $CaCl_2$ and 10 mM glucose for 5 min at 37°C. The supernatant fraction was obtained by centrifugation at $250 \times g$ for 5 min. Aliquots of 50 µl of the supernatant were added to 1 ml of the assay medium (0.1 M acetate buffer, pH 4.5, 10 µM MCLA, 500 µM KBr, 500 µM H_2O_2, 20 µM desferrioxamin, and 10 U/ml SOD). In the presence or absence of 10 µM NaN_3, chemiluminescence intensity was measured at 25°C by using luminescence Reader (Aloka BRL-210).

Electron Paramagnetic Resonance (EPR) Analysis

OPMN (5×10^6 cells/ml) were incubated in KRP containing 950 mM DMPO in the presence or absence of 1 mM DETAPAC. EPR spectrum for 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl adduct (DMPO/$^{\bullet}$OH) was determined by using an EPR spectrometer (JEOL JES FE-IX) with 100-kHz field modulation at 8 mW, magnetic field of 334.7 ± 5.0 mT, and sweeping time of 30 s or 4 min.[20]

Nitric Oxide Production

OPMN were cultured in RPMI 1640 medium in 24-well cell culture plates (1×10^6 cells/ml/well) in a CO_2 incubator. After incubation, the amounts of NO_2^- released into the culture medium were determined by the Griess method.[21] Briefly, 100 μl of culture medium were mixed with 150 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/5% H_3PO_4). After incubation at 25°C for 10 min, absorbancy at 535 nm was determined in a Hitachi U-2000 spectrophotometer.

Analysis of DNA Fragmentation

The extent of DNA fragmentation was determined spectrophotometrically using diphenylamine.[22] The cells were lysed at 4°C in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 0.5% Triton X-100) for 10 min. The lysate was centrifuged at $20{,}000 \times g$ at 4°C for 20 min to separate intact and fragmented chromatin. Both the pellet and the supernatant fractions were incubated with 6% perchloric acid at 4°C for 30 min. The incubated samples were centrifuged at $20{,}000 \times g$ for 20 min at 4°C. The DNA samples thus obtained were heated at 70°C for 20 min in 50 μl of 6% perchloric acid and mixed with 100 μl of diphenylamine solution (1.5% diphenylamine, 1.5% sulfuric acid, and 0.01% acetaldehyde in glacial acetic acid). After incubation in the dark at 30°C for overnight, optical density of the samples was measured at 600 nm. The extent of DNA fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA.[23,24]

Western Blot Analysis

Cell lysates were prepared as described elsewhere.[5,7,24] Cells (2×10^6) were dissolved in SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and boiled at 100°C for 5 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis. After transfer of proteins in the gel to an Immobilon-P membrane (Millipore Co.), the membrane was incubated with primary antibody (1 : 1000 dilution) and then with horseradish peroxidase-linked secondary antibody (1 : 4000 dilution) and analyzed by using an enhanced chemiluminescence kit (Amaersham Co.). Proteinconcentrations were determined by the method of Lowry *et al.*[25] using bovine serum albumin as a standard.

Assay for Caspase Activity

The cells (5×10^5) were lysed in 50 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 0.5 mM EDTA and 150 mM NaCl) at 4°C for 30 min. The lysates were then centrifuged at $20{,}000 \times g$ for 10 min. Caspase activity of the supernatant was determined in 20 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl and 5 mM DTT at 37°C using 10 μM of either Ac-YVAD-MCA for caspase-1 or Ac-DEVD-MCA for caspase-3 as described previously.[24] The fluorescence of released 7-amino-4-methyl-coumarin (AMC) was measured by a fluorospectrophotometer (Hitachi 650-10 LC). The wavelengths for excitation and emission were 355 and 460 nm, respectively. One unit of the enzyme is defined as the amount activity that liberates 1 nmol of AMC during one hour.

Statistical Analysis

At least 3 independent experiments were performed, except where indicated. Results were presented as mean ± S.D. from 5 separate experiments.

RESULTS

Tyrosine Phosphorylation of 115 kDa Protein

We previously reported that, when primed by either tumor necrosis factor-α (TNF-α) or granulocyte colony stimulating factor (G-CSF), tyrosine residues of a 115 kDa protein in CPMN (c-cbl protooncogene product, c-Cbl) was phosphorylated.[5] Kinetic analysis revealed that, in the absence of any stimulants, tyrosine phosphorylation of a 115 kDa protein was apparent with OPMN but not with nonprimed CPMN (Figure 1).

Expression of NOS II and Production of NO_2^-

Western blot and immunocytochemical analysis revealed that NOS II was expressed in OPMN but not in CPMN (Figure 2). Coincident with this expression of NOS II, a significantly large amount of NO_2^-, a metabolite of NO, was released from OPMN into the medium whereas that release from CPMN was very small (Figure 2).

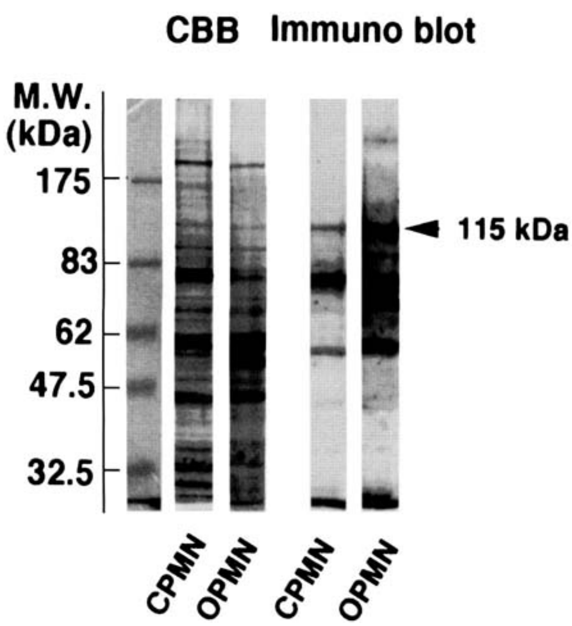

FIGURE 1 Tyrosyl phosphorylation of a 115 kDa protein in PMN. Proteins extracted from PMN were subjected to SDS-PAGE. They were transferred to an Immobilon-P transfer membrane and stained with antiphosphotyrosine antibody (PY-20) and peroxidase-conjugated anti-mouse IgG antibody as described in the text. CBB; proteins stained with CBB; Immuno blot; immuno-staining; CPMN, circulating PMN; OPMN, oral PMN.

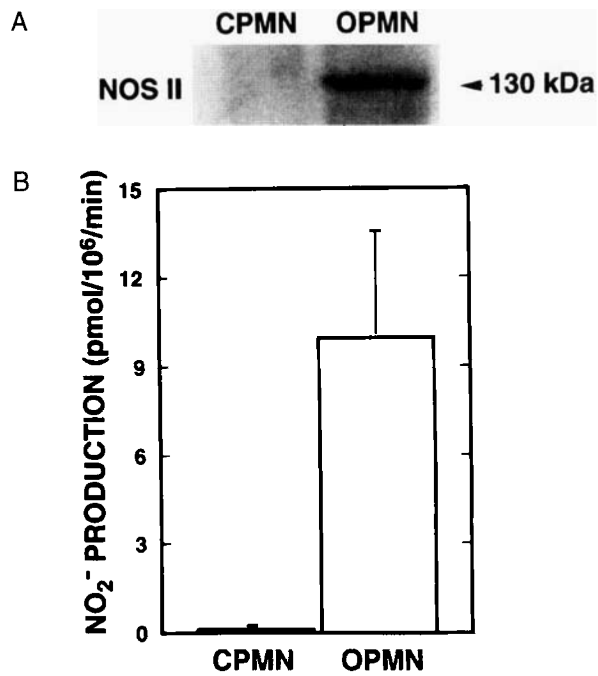

FIGURE 2 Expression of NOS II and generation of NO_2^- in PMN. (A) After SDS-PAGE followed by transfer to an Immobilon-P transfer membrane, NOS II was detected by anti-NOS II antibody. (B) The amounts of NO_2^- released from CPMN and OPMN were measured by the method of Griess. Closed column, CPMN; open column, OPMN. Data were expressed as mean $\pm$ SD from 5 experiments.

Effect of Various Drugs on LCL

Figure 3 shows LCL of OPMN and CPMN. In the absence of any stimulants, strong and spontaneous LCL is apparent with OPMN but not with CPMN. LCL of OPMN was strongly inhibited by azide, an inhibitor of MPO and slightly by SOD and catalase but not by uric acid, a scavenger for $^{\bullet}OH$ and $^1O_2$. LCL intensity was also suppressed by a membrane stabilizer cetylamine and by genistein and staurosporin, inhibitors of tyrosine kinase and protein kinase C, respectively.

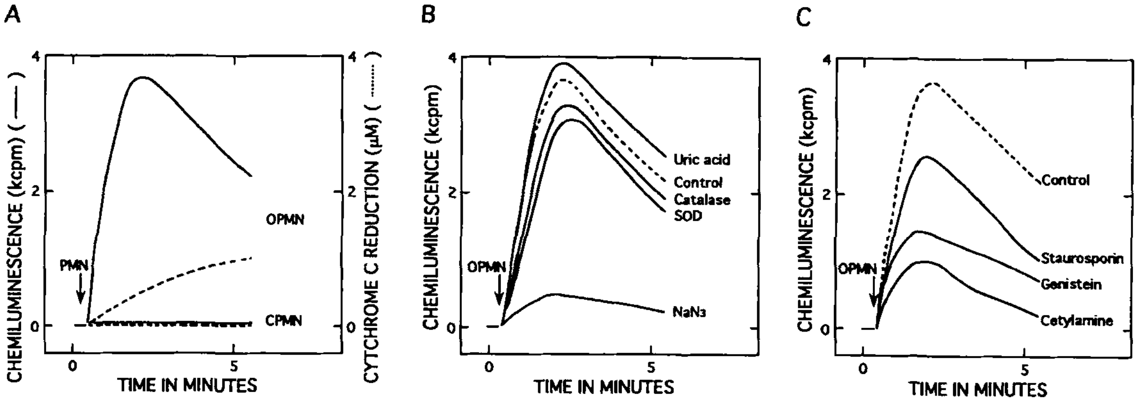

FIGURE 3 Effect of various reagents on the LCL of unstimulated PMN. (A) Reaction mixture contained in a total volume of 1∼2 ml of KRP, 10 mM glucose, 1 mM $CaCl_2$, 20 μM Cyt. c or 100 μM luminol and CPMN (5×10^5 cells/ml) or OPMN (1×10^5 cells/ml). The reaction was started by adding PMN at 37°C. In the presence of various reagents (B and C), LCL was determined with OPMN. Concentrations of NaN_3, uric acid, genistein, cetylamine and staurosporin were 5, 5, 10, and 10 μM and 10 nM, respectively. Activities of SOD and catalase were 10 and 100 U/ml, respectively.

Effect of Various Agents on OPMN-Generated ROS

To obtain further insight into chemical nature of spontaneously generated ROS, EPR analysis was carried out with OPMN in the presence of DMPO (Figure 4). EPR spectra of OPMN exhibited a signal characteristic of DMPO/•OH adduct. The intensity of the signal was decreased strongly by the presence of either uric acid, SOD, or catalase but increased by azide (Figure 4). Under identical conditions, the signal was not observed with CPMN (data not shown).

Generation of H_2O_2 and Release of Myeloperoxidase

Generation of H_2O_2 by PMN was measured by means of the increase in fluorescence intensity of scopoletin. Under nonstimulating conditions, about 0.162 and 0.013 nmoles of $H_2O_2/10^6$ cells/min were generated by OPMN and CPMN, respectively. Consistent with this result, MPO was released from OPMN but not from CPMN. Although total activity of MPO in OPMN and CPMN were 1.0 and 1.6 U/10^6 cells, respectively, 6.2 and 1.2 mU/min of the enzyme were released from 10^6 of the former and the latter cells into the medium, respectively (Figure 5).

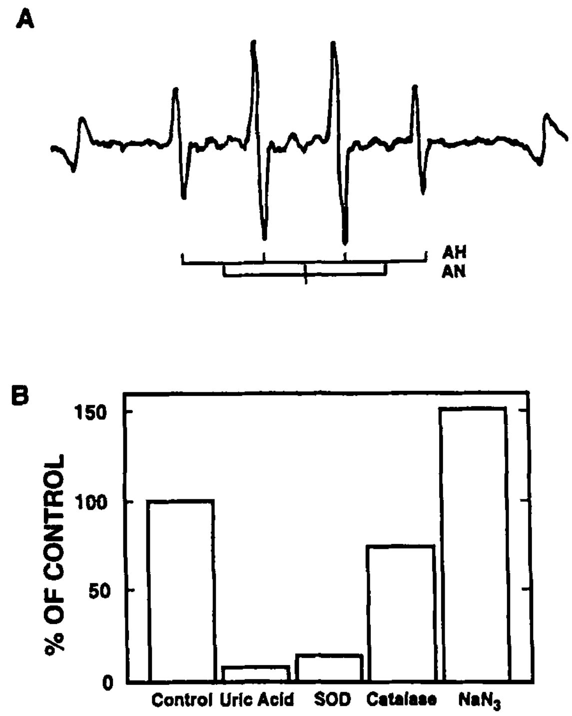

FIGURE 4 Effect of various reagents on EPR spectra. (A) Reaction mixture contained, in a final volume of 0.2 ml of KRP solution, 1 mM DETAPAC, 950 mM DMPO and 10^6 cells of OPMN. (B) Effect of various reagents on the signal intensity of DMPO/•OH. Data were expressed by % of control. Concentrations of NaN_3, uric acid, catalase, SOD were 5 and 5 μM and 50 and 10 U/ml, respectively.

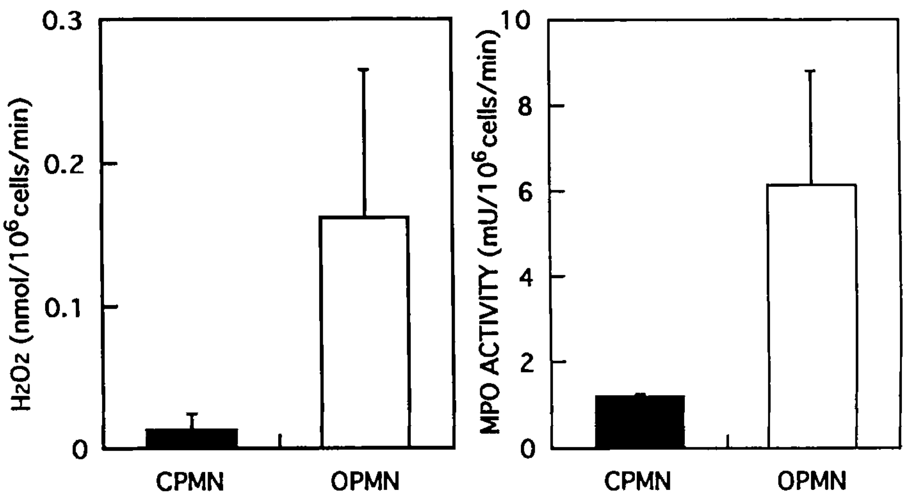

FIGURE 5 Spontaneous release of myeloperoxidase and generation of H_2O_2. Activity of MPO and production of H_2O_2 were monitored by MCLA method and fluorescence change of scopoletin, respectively. Released MPO activity and the amount of generated H_2O_2 were expressed by mU/10^6 cells/min and nmoles/10^6 cells/min, respectively. Closed column, CPMN; open column, OPMN. Data were expressed as mean ± SD from 5 experiments.

Stimulation-Dependent Generation of Superoxide

Figure 6 shows the effects of various stimulants and inhibitors on the generation of $O_2^{\bullet-}$ by OPMN. FMLP enhanced the generation of $O_2^{\bullet-}$ by a mechanism which was inhibited by genistein, a tyrosine kinase inhibitor.[26] The genistein-inhibited generation of $O_2^{\bullet-}$ was reversed by PMA. However, $O_2^{\bullet-}$ generation was again inhibited by staurosporin, an inhibitor of protein kinase C.[27] The staurosporin-inhibited generation of $O_2^{\bullet-}$ was reversed by sodium arachidonate by a mechanism which was inhibited by cetylamine, a membrane stabilizer. The effects of these stimulants and inhibitors on $O_2^{\bullet-}$ generation by OPMN were quite similar to those seen with CPMN.[2] PMA is known to stimulate the generation of $O_2^{\bullet-}$ by nonprimed CPMN.[2,3]

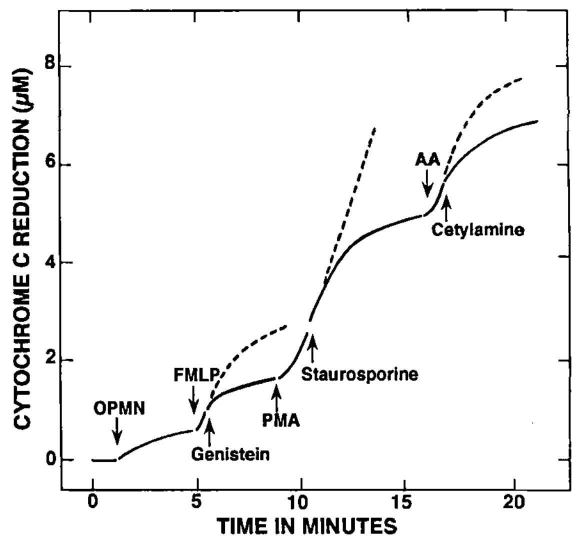

FIGURE 6 Effects of various reagents on superoxide generation by OPMN. Reaction mixture contained in a final volume of 2 ml KRP (pH 7.4), 10 mM glucose, 1 mM $CaCl_2$ and 2×10^5 cells (OPMN). Cyt. c reduction was measured spectrophotometrically at 37°C. The broken lines show the control experiment in the absence of inhibitors. Final concentrations of FMLP, sodium arachidonate, and PMA were 0.2 and 30 μM and 0.1 nM, respectively. The concentrations of genistein, cetylamine and staurosporin were 10 and 10 μM and 10 nM, respectively.

Expression of Bcl-2 in PMN

Bcl-2 is expressed in a variety of cells, such as the early myeloid precursor cells and HL-60 cells.[28] This protein has been postulated to inhibit

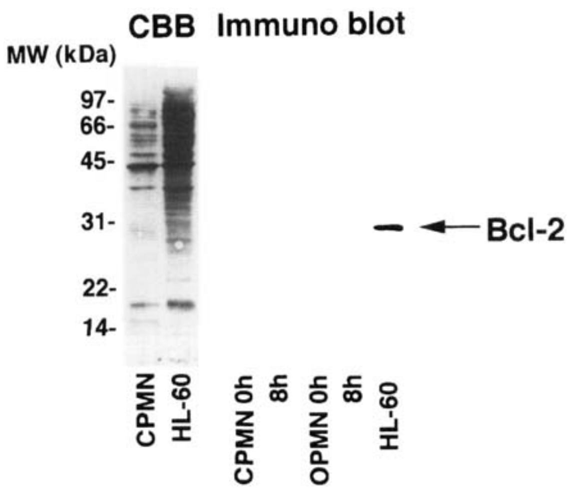

FIGURE 7 Expression of Bcl-2 in PMN and HL-60 cells. Cells (10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2/95% air. After 8 h, 10^6 cells were solubilized in 30 µl SDS-sample buffer. The cell lysates (19 µg in protein) were subjected to SDS-PAGE, followed by western blotting with anti-Bcl-2 antibody. CBB, proteins stained with CBB; Immuno blot, immuno-staining.

apoptotic cell death.[10,11,14,28] Western blot analysis revealed that Bcl-2 was not expressed in CPMN and OPMN (Figure 7). Neither Bcl-xL nor Bax was expressed in the two cell types (data not shown).

Fragmentation of PMN DNA

After differentiation from myeloid precursor cells, PMN die during a fairly short period. To investigate the possible occurrence of apoptosis, nuclear DNA samples were isolated from cultured OPMN and CPMN and analyzed by the diphenylamine method. Although DNA fragmentation occurred in both types of PMN, the extent of fragmentation was more marked with OPMN than with CPMN (Figure 8).

Caspase Activity of PMN

Because PMN underwent apoptosis, possible involvement of caspases was investigated with

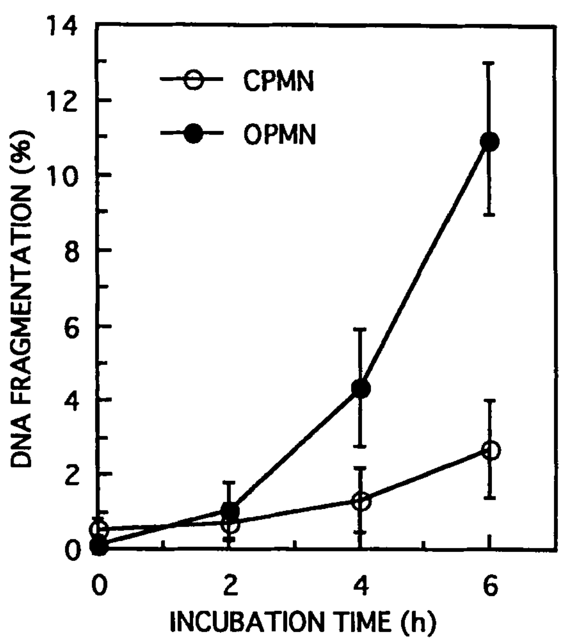

FIGURE 8 DNA fragmentation in cultured PMN. (A) PMN were cultured in RPMI 1640 medium (10^6 cells/ml). At the indicated times, the amount of fragmented DNA was determined by the diphenylamine method. Closed circles, OPMN; Open circles, CPMN. Data show mean ± SD derived from 5 separate experiments.

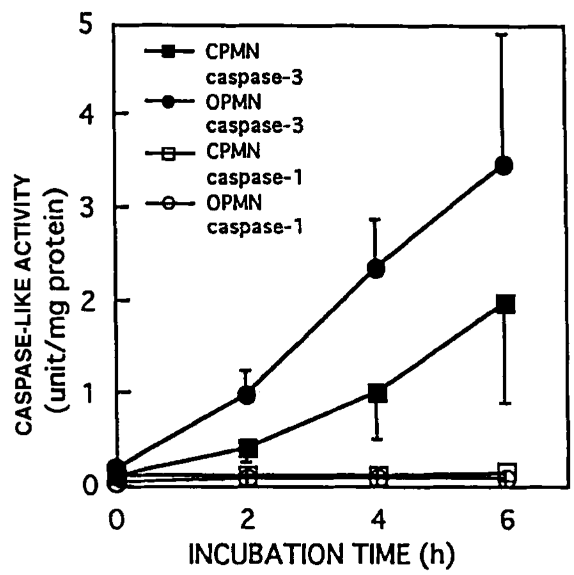

FIGURE 9 Caspase-3 activity in PMN. PMN were cultured in RPMI 1640 medium at 37°C. At the indicated times, cell lysates were incubated at 37°C for 1 h with 10 µM of Ac-YVAD-MCA for caspase-1 or Ac-DEVD-MCA for caspase-3. Fluorescence intensity of the mixture was measured at 355 and 460 nm for excitation and emission, respectively. Data show mean ± SD derived from 5 separate experiments.

cultured PMN. Biochemical analysis revealed that the activity of caspase-3 but not caspase-1 was markedly higher with OPMN than with CPMN (Figure 9). The activities of both enzymes in HL-60 cells were below detectable levels.

DISCUSSION

The present work shows that OPMN from healthy human subjects are fully primed *in situ* and generate spontaneously various ROS. Although OPMN generated various ROS, kinetic analysis using specific inhibitors suggested that hypochloride was principally responsible for the strong chemiluminescence elicited by unstimulated cells.[6,18,30–33] Consistent with this notion, both secretion of MPO and generation of H_2O_2 were significantly higher with OPMN than with CPMN. Another characteristic feature of OPMN is the generation of NO. Although PMN have been postulated to express only NOS III,[40] the present work demonstrates that OPMN express NOS II.

Intracellular mechanism for triggering oxygen burst in PMN involves at least two pathways; one is a PMA-stimulated and staurosporin-inhibitable protein kinase C pathway[34–36] and the other is an FMLP- and opsonized zymosan-stimulated and genistein-inhibitable tyrosine kinase pathway.[2,3,5] Another mechanism for triggering oxygen burst may involve an SDS- and arachidonic acid-stimulated and cetylamine-inhibitable pathway.[36,37] Because OPMN-dependent LCL was inhibited by either genistein, staurosporin, or cetylamine, generation of ROS might be triggered through some pathway similar to that of pathways of stimulation-dependent generation of ROS. The properties of $O_2^{\bullet -}$ generation by stimulated OPMN monitored by the Cyt. c reduction method were similar to those observed with ligand-stimulated CPMN. Thus, mechanism for the generation of $O_2^{\bullet -}$ in OPMN might be identical to that of CPMN.

We previously reported that both G-CSF and TNF-α induced the priming of PMN with concomitant enhancement of tyrosyl phosphorylation of a 115 kDa protein and suggested the involvement of the phosphorylation of this protein in the mechanism for triggering PMN priming.[2,3,5] Consistent with this notion, the present work demonstrates that tyrosyl phosphorylation of 115 kDa protein(s) was substantially enhanced in OPMN. Recent studies[38] revealed that the 115 kDa protein in PMN was identical to c-Cbl, an oncogene product which binds to phosphatidylinositol-3-kinase; tyrosyl phosphorylation of this protein plays an important role in cellular signal transduction.[39] Thus, the 115 kDa protein might play a role in the signal transduction for triggering the priming of OPMN.

The present work shows that OPMN underwent apoptosis more rapidly than did CPMN. Bcl-2 and proteins of the ICE family have been postulated to play important roles in the process of apoptosis.[28,29,41–43] Thus lack of Bcl-2 and the activation of caspase-3 would have enhanced the process of apoptosis in OPMN. Recent studies revealed that ROS might be involved in the mechanism leading to apoptosis.[44–48] Thus, the mechanism by which OPMN rapidly undergo apoptosis might also involve oxygen stress induced by endogenously generated ROS.

Acknowledgments

The authors are grateful to Dr. Alan A. Horton for his suggestion and helpful discussion during the preparation of the manuscript. This work was supported in part by grants from the Japan Keirine Association and the Supirulina Res. Co. Ltd, Japan. We also thank Miss Hirono Tabuchi for excellent technical assistance.

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