Cytoprotective Response of A1, a Bcl-2 Homologue Expressed in Mature Human Neutrophils and Promyelocytic HL-60 Cells, to Oxidant Stress-Induced Cell Death

Chien-Ying Liu a Peter I. Chuang b Chun-Liang Chou a Shu-Min Lin a Hao-Cheng Chen a Paichien Chou a Yun-Hen Liu c Chih-Ten Yu a Chun-Hua Wang a Horng-Chyuan Lin a Han-Pin Kuo a

Departments of aThoracic Medicine II and cThoracic Surgery, Chang-Gung Memorial Hospital, Taipei, Taiwan; bDepartment of Medicine, University of Washington, Seattle, Wash., USA

Key Words
A1 • Reactive oxygen intermediate • Neutrophils • HL-60 cells • Apoptosis • Cell death

Abstract
The ability to generate reactive oxidative intermediates is one of the quintessential properties of mature human neutrophils. Endogenously generated oxidants have been shown to be an important mechanism underlying neutrophil cell death. In acute lung inflammation, newly recruited neutrophils further encounter external oxidants, including reactive oxygen and nitrogen intermediates. In our present study, we showed that A1, a constitutive and inducible Bcl-2 homologue expressed in mature circulating human neutrophils, might confer the protection from hydrogen peroxide (H₂O₂)- and peroxynitrite (ONOO⁻)-induced cell death. Utilizing the myeloid precursor cell line, HL-60, we further examined the hypothesis that A1 was capable of conferring cytoprotective activity against these oxidative stresses. Whereas the control-transfected HL-60 cells expressed small amounts of A1 and were sensitive to the biologically relevant, cell death-inducing oxidants, H₂O₂ and ONOO⁻, the stable transfectants that overexpressed A1 were significantly more tolerant. Furthermore, there was a correlation between the level of A1 expression and the anti-apoptotic activity. Thus, our results suggest a cytoprotective role of A1 in mature human neutrophils under oxidant stresses in host defense and inflammation.

Introduction
A turnover of nearly a billion neutrophils each day has been estimated in an average human adult [9]. When circulating mature human neutrophils were aged in vitro, they died spontaneously [64]. Recently, it has been shown that endogenously generated oxygen and nitrogen species, including hydrogen peroxide (H₂O₂) and nitric oxide (NO), were one of the major mechanisms accounting for the spontaneous demise of isolated circulating neutrophils in vitro [29, 55, 56, 61]. In a variety of inflammatory...
lung diseases, H₂O₂ and NO concentrations in the expired air were significantly elevated [10, 12, 45, 77], suggesting that recruited inflammatory cells encountered an increased oxidant burden. During the respiratory burst, activated neutrophils not only released hydrogen peroxide, superoxide, and other reactive oxygen intermediates (ROI), but also upregulated inducible NO synthase and produced NO in vivo [70, 81]. Recent evidence suggests that peroxynitrite (ONOO⁻), a reactive nitrogen intermediate (RNI), is a reaction product of superoxide and NO [4, 60]. It can induce neutrophil cell death in vitro [5, 17, 84]. Furthermore, nitrotyrosine, which is among the by-products of ONOO, is present in the inflamed lung [33, 62], heart [32], and gastrointestinal tract [24, 67], supporting the biological relevance of ONOO in vivo. Intriguingly, nitrotyrosine is present around ingested bacteria in activated neutrophils in vitro [15], and neutrophils ingesting bacteria actively undergoing apoptosis [79]. Thus, when neutrophils are recruited into a site of active inflammation, they encounter an oxidant-rich environment that contains ROI and RNI generated by resident cells and previously recruited inflammatory cells. Accordingly, the protection of these newly recruited neutrophils from the exogenous and endogenous oxidant stresses is important so that they can survive long enough to carry out their functions of host defense [73].

Bcl-2 and Bcl-x are antiapoptotic proteins that have been shown to protect cells from ROI- and RNI-induced cell death in vitro [3, 16, 21, 49, 57, 58, 75]. Although there are conflicting reports [23, 80], the majority of researchers have reported that neither Bcl-2 nor Bcl-x is present in circulating mature human neutrophils [26, 53, 54, 63]. Chuang et al. [8] previously showed that mature circulating human neutrophils constitutionally expressed the Bcl-2 homologue, A1. Furthermore, A1 was the first antiapoptotic Bcl-2 homologue that was shown to be inducible by proinflammatory cytokines [27]. Additionally, Chuang et al. [8] showed that A1 gene expression in neutrophils was upregulated by antiapoptotic agonists such as lipopolysaccharides, granulocyte-colony-stimulating factor (G-CSF), and granulocyte-macrophage-colony-stimulating factor (GM-CSF). Significantly, G-CSF and GM-CSF have been suggested to play important roles in modulating neutrophil survival in the lungs of patients with acute respiratory distress syndrome [47]. Correspondingly, survival of neutrophils from mutant mice deficient in both G-CSFR and A1 was shortened [20, 43].

Emerging evidence suggests that Bcl-2 homologues confer differential cytoprotective capacity against certain cell death stimuli [1, 28, 40, 66]. In human promyelocytic HL-60 cells, it has been reported that ROI and RNI, including H₂O₂ and ONOO⁻, have an important role in modulating cell death [44, 76, 84] and Bcl-2 could block apoptosis induced by reactive oxygen species [42]. Using G-CSF-primed or antisense oligodeoxyribonucleotide (ODN)-pretreated neutrophils and the neutrophil precursor HL-60 cells as a model, we sought to determine whether A1 could be conferred to demonstrate cytoprotection against H₂O₂ and ONOO⁻, oxidant stresses that are pertinent to neutrophils in inflammation.

**Materials and Methods**

**Cells and Reagents**

The solutions for cell culture EMEM, RPMI 1640, Opti-MEM, FCS (10x), nonessential amino acids (100x), sodium pyruvate (100x), and penicillin-streptomycin-L-glutamine (100x) were purchased from Life Technologies (Grand Island, N.Y., USA). The human promyelocytic HL-60 cells (ATCC, Rockville, Md., USA) were maintained in EMEM with the additives mentioned above. Recombinant G-CSF was obtained from R & D Research (Minneapolis, Minn., USA). The A1 antisense ODN was obtained from Isis Pharmaceuticals (Carlsbad, Calif., USA) [1]. Hydrogen peroxide was purchased from Sigma (St. Louis, Mo., USA) and peroxynitrite from Oxis International (Portland, Ore., USA). Hydrogen peroxide was prepared fresh for each experiment. Particular attention was paid to the handling and monitoring of the peroxynitrite stock with every use as described [41]. The general caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethylketone (Z-VAD.fmk) was obtained from Calbiochem (La Jolla, Calif., USA). Gentamicin and G418 were purchased from Life Technologies (St. Louis, Mo., USA).

**Human Neutrophil Preparation**

Human neutrophils were isolated and purified from freshly drawn heparinized blood obtained from healthy adult volunteers as previously described [14]. In brief, the leukocyte-rich fraction was separated from erythrocytes using dextran sedimentation and the neutrophils were separated from mononuclear cells by centrifugation (450 g, 25°C, 30 min) on a Ficoll-Paque gradient (density: 1.077; Amersham Pharmacia Biotech, Piscataway, N.J., USA). The contaminating erythrocytes were lysed with 0.9% buffered ammonium chloride. For G-CSF, H₂O₂ and ONOO⁻ treatments, purified neutrophils were resuspended at a concentration of 2 × 10⁶ per milliliter in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM of L-glutamine, 10 U/ml penicillin, and 50 μg/ml streptomycin. For the antisense ODN treatment, the neutrophils were resuspended in Opti-MEM medium at the same concentration. Cell purity was determined by examining centrifuged preparations (fixed in methanol and stained with Diff-Quick) and viability assessed by trypan blue exclusion. The neutrophils were consistently >98.5% pure and >99.5% viable.

**Assessment of Neutrophil Apoptosis by Annexin V Binding**

Apoptosis of neutrophils was performed by flow cytometry using fluorescein isothiocyanate-labeled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic A1 Confers Tolerance to Oxidant Stresses