The role of oxidants and vitamin C on neutrophil apoptosis and clearance

M.C.M. Vissers1 and M.B. Hampton
Free Radical Research Group, Christchurch School of Medicine and Health Sciences, P.O. Box 4345, Christchurch, New Zealand

Abstract
We have investigated the role of neutrophil oxidants in the surface changes that result in recognition and uptake of neutrophils by macrophages. We have shown that H2O2 produced by stimulated neutrophils is essential for the surface expression of phosphatidylserine. This does not occur in neutrophils from mice with chronic granulomatous disease and may explain the formation of granuloma in this condition. We have also investigated the role of intracellular vitamin C on neutrophil apoptosis. Cells from vitamin C-deficient mice were found to be less likely to undergo both spontaneous and oxidant-induced apoptosis, with eventual necrosis being the most probable outcome.

Introduction
Neutrophil apoptosis is responsible for the removal of aging cells from the circulation, and is accompanied by surface exposure of PS (phosphatidylserine), caspase activation and DNA fragmentation [1,2]. This spontaneous apoptosis occurs over 12–24 h after release into the circulation. In contrast, stimulated neutrophils expose PS within 3 h, and this is not accompanied by caspase activation [2]. As such, the phagocytosing cell does not undergo conventional apoptosis, but is labelled for clearance by macrophages. Failure to undergo either form of apoptosis would result in inappropriate survival of the cells or death by necrosis.

Stimulated neutrophils produce vast amounts of superoxide which leads to the formation of H2O2 and chlorinated oxidants via the action of myeloperoxidase. These oxidants can affect the process of apoptosis. Individuals with CGD (chronic granulomatous disease), whose neutrophils are defective in oxidant production, suffer from recurrent infections and the formation of granuloma, which results in vital organ damage [3]. Whereas the symptoms of CGD have been attributed to the defect in bacterial killing, individuals with myeloperoxidase deficiency, whose neutrophils also show impaired killing, are largely asymptomatic. We have previously shown that neutrophil oxidants are necessary for PS exposure in stimulated neutrophils, and have proposed that in CGD, defective PS exposure could result in impaired clearance of neutrophils and a defect in the resolution of inflammation [4].

Neutrophils are also known to accumulate high concentrations of vitamin C, which is supposed to protect them from products of the oxidative burst [5]. We have previously shown that ascorbate can enhance apoptosis in endothelial cells in the presence of chlorinated oxidants [6]. To determine the role of vitamin C in neutrophil apoptosis, we have used a vitamin C-deficient mouse as a source of neutrophils and have investigated both spontaneous and oxidant-induced apoptosis.

H2O2 is a necessary requirement for PS exposure in stimulated neutrophils
Isolated human neutrophils were stimulated with either phorbol ester or opsonized Staphylococcus aureus and PS exposure was measured by annexin V binding. We showed that this process depends on a functional oxidase, since it was prevented when the cells were treated with DPI (diphenyleneiodonium), and did not occur in neutrophils from an individual with CGD. In contrast, neutrophils from a myeloperoxidase-deficient individual showed normal PS exposure [4]. These results indicate that although myeloperoxidase activity is not necessary for PS exposure, H2O2 generation appears to be an essential requirement.

H2O2 production promotes uptake of neutrophils by macrophages and prevents neutrophil clearance
These results led us to predict that defective oxidant production would prevent recognition by macrophages and result in impaired neutrophil clearance. To test this theory, we monitored the uptake of stimulated neutrophils by cultured macrophages. We found that neutrophils incubated with S. aureus in the presence of DPI were not recognized by monococyte-derived macrophages (Figure 1).

To determine whether a defect in uptake would result in excessive accumulation of neutrophils at an inflammatory focus, we injected CGD and wild-type mice with S. aureus and showed that 3–10 times more neutrophils accumulated in the peritoneal cavities of the CGD mice. These cells also persisted beyond 24 h [4].

Key words: apoptosis, hydrogen peroxide, neutrophil, phosphatidylserine, vitamin C.
Abbreviations used: CGD, chronic granulomatous disease; DPI, diphenyleneiodonium; PS, phosphatidylserine.
1To whom correspondence should be addressed (email margret.vissers@chmeds.ac.nz).
These results are consistent with a clearance defect in CGD that may be a significant contributor to the formation of granuloma in these patients. Our results also shed light on the different clinical manifestations in myeloperoxidase deficiency. Given that these neutrophils produce normal amounts of H₂O₂, we would predict no effect on the clearance of neutrophils, and propose that this accounts for the more benign clinical course of these individuals.

Vitamin C is an essential component for neutrophil apoptosis

Neutrophils contain millimolar concentrations of vitamin C, and this has been postulated to protect the cells against the products of the oxidative burst [5]. We have harvested peritoneal neutrophils from a vitamin C-deficient mouse and from normal littermates after challenge with thioglycollate, and monitored the rates of spontaneous and oxidant-induced PS exposure.

When the mouse neutrophils were stimulated with phorbol ester, PS exposure was decreased and there was an increase in both viable and necrotic cell populations. Similarly, spontaneous apoptosis was considerably inhibited in the vitamin C-deficient cells. Rather, there was an increase in the number of viable and necrotic cells. Morphological examination showed that normal mouse neutrophils took on the appearance of apoptotic cells after 24 h, with condensed nuclei and more intact appearance, whereas vitamin C-deficient cells appeared to be largely necrotic (Figure 2).

From these results, we predict an essential role for vitamin C in the turnover of neutrophils, and propose that the release of hydrolytic enzymes from necrotic neutrophils contributes to the tissue degradation seen in scurvy.

To summarize, these studies have shown that neutrophil oxidants, particularly H₂O₂, play a central role in the clearance of neutrophils from inflammatory sites. Under these circumstances, neutrophils undergo surface changes that result in their recognition by macrophages. This requires a functional oxidative system, and is defective in neutrophils from patients with CGD, possibly explaining the persistence of cells that leads to the formation of granuloma. This system differs from the spontaneous apoptosis that is responsible for the turnover of circulating neutrophils, which is not impaired in CGD.

We have also shown that when neutrophils do not contain vitamin C, apoptosis is impaired in both stimulated and
unstimulated cells, with eventual cell death by necrosis. We are currently investigating the implications of these findings.

References

Received 12 December 2003