Role of oxidative stress and antioxidant enzymes in Crohn's disease

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Abstract

There is increasing interest in oxidative stress being a potential aetiological factor and/or a triggering factor in Crohn's disease, rather than a concomitant occurrence during the pathogenesis of the disease. Recent research has shown that the immune mononuclear cells of Crohn's disease patients are induced to produce hydrogen peroxide (H_2O_2). Similarly, the regulation of antioxidant enzymes during disease in these cells has been unravelled, showing that SOD (superoxide dismutase) activity and GPx (glutathione peroxidase) activity is increased during active disease and returns to normal in remission phases. However, catalase remains constantly inhibited which supports the idea that catalase is not a redox-sensitive enzyme, but a regulator of cellular processes. ROS (reactive oxygen species) can be produced under the stimulus of different cytokines such as TNFα (tumour necrosis factor α). It has been shown in different experimental models that they are also able to regulate apoptosis and other cellular processes. The status of oxidative stress elements in Crohn's disease and their possible implications in regulating cellular processes are reviewed in the present paper.

Introduction

The cause of Crohn's disease remains unclear and the specific pathways leading to mucosal damage are not completely understood. In recent years, Crohn's disease pathogenesis has been proposed to be a mixture of an exaggerated immune response, genetic susceptibility and environmental/microbiota factors [1]. The role of oxidative stress as a potential aetiological and/or triggering factor for IBD (inflammatory bowel disease) has been a subject of increasing interest [2]. The immune cells that reach the mucosa in Crohn's disease release a number of ROS (reactive oxygen species) that are potentially detrimental [3]. The main pro-oxidant agents are ROS formed by unstable forms of oxygen [superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$)]. In contrast, antioxidant agents include molecules such as glutathione or ascorbic acid and AOEs (antioxidant enzymes) such as catalase, SOD (superoxide dismutase) or GPx (glutathione peroxidase). Only when the antioxidant capacity of the cell is overwhelmed, can ROS exert their damaging potential [4].

Nowadays, with regard to ROS generation and function, there is enough scientific evidence showing that, before reaching deleterious effects, the ROS can exert a signalling function inside the cells which regulates growth, differentiation, cell death and inflammatory processes. The mechanisms and specific signalling events of ROS have not been properly characterized. Similarly, the role of AOEs is now interpreted in the context of fine-tuning of ROS levels in the redox regulation of the cell cycle and of programmed cell death [5,6].

Studies are accumulating growing evidence for the role of oxidative stress in the pathogenesis of IBD. Oxidative damage has been detected not only in the intestinal mucosa of IBD patients [7], but also in peripheral blood leucocytes [8]. Similarly, plasmatic antioxidant defences are diminished in Crohn's disease [9]. However, there has been no clear study analysing which ROS are produced during Crohn's disease, where they are produced and how the AOEs are expressed or modified. Equally, no work has clarified whether the oxidative stress appears only when the disease is active or whether it is also present in remission.

Oxidative stress in Crohn's disease

Our group has characterized the oxidative stress generated in the mononuclear cells from the peripheral blood of Crohn's disease patients and the impact of the oxidative stress on tissue damage. In summary, we have shown that the cells present a significant increase in the ROS H_2O_2 in both activity and remission phases of the disease (Figure 1). Levels of other ROS, such as nitric oxide (NO) or O_2^-, are not different from control subjects. However, owing to the fact that H_2O_2 is a product of O_2^- metabolism, an increase in O_2^- could lead to an increase in H_2O_2. An increase in O_2^- has been found indirectly, showing a clear increase in SOD activity

Key words: apoptosis, catalase, Crohn's disease, oxidative stress, superoxide dismutase, tumour necrosis factor α (TNFα).

Abbreviations used: AOE, antioxidant enzyme; GPx, glutathione peroxidase; IBD, inflammatory bowel disease; IL, interleukin; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumour necrosis factor α.

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Figure 1 | Stimulated production of H_2O_2 by TBH (t-butyl-hydroperoxide) in lymphocytes from control subjects and from active Crohn's disease (CD) patients

$^*P < 0.01$ (TBH compared with basal production in controls); $\#P < 0.01$ (TBH compared with basal production in active Crohn's disease patients); $+P < 0.01$ (basal production in controls compared with active Crohn's disease patients); $//P < 001$ (TBH in controls compared with TBH in active Crohn's disease patients) ($n = 25$). DCFH, 2′,7′-dichlorodihydrofluorescein.

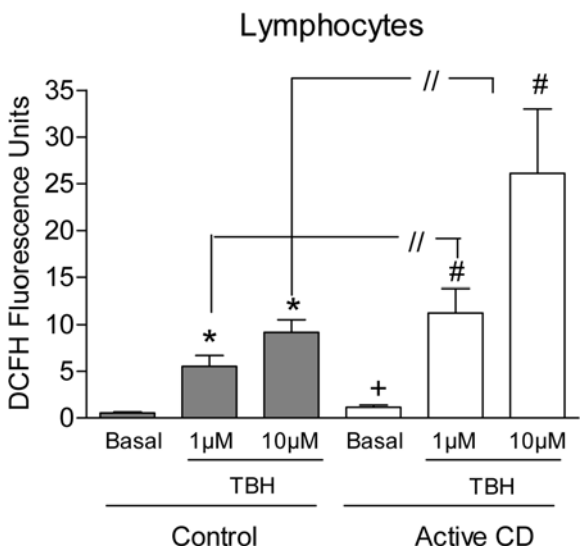
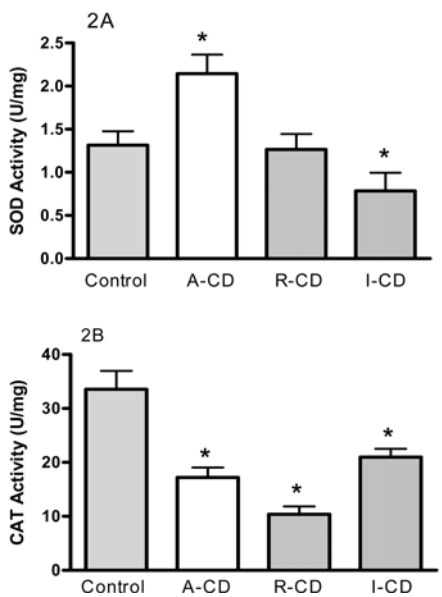


Figure 2 | AOE capacity

SOD activity (**A**) and catalase (CAT) activity (**B**) in different groups assayed ($n = 20$): controls, active Crohn's disease at onset (A-CD), Crohn's disease patients in remission (R-CD), and inactive Crohn's disease patients (I-CD). $^*P < 0.01$. The group of remission (R-CD) are patients who have achieved remission after first flare of the disease. The group of inactive patients are the ones in a long-lasting remission. U, units.



in cells from Crohn's disease patients. Metabolism must be immediate and quick, so that the ROS is not detected except when an inhibitor of SOD is added. Furthermore, the mitochondria of the cells from Crohn's disease patients show a significant inhibition of their function, which can be measured by showing an inhibition of the mitochondrial membrane potential (40% inhibition). This could be due to a direct effect of H_2O_2 in the mitochondria, or could be itself a mechanism to initiate the formation of ROS [10].

The increase in O_2^- is accompanied by an increase in SOD activity in order to eliminate a potentially damaging radical. In the same way, an increase in H_2O_2 may be associated with an increase in the activity of enzymes implicated in H_2O_2 detoxification. One such enzyme could be catalase. We measured catalase activity, and, in contrast with expectations, we observed that the enzyme was significantly inhibited in lymphocyte cells from Crohn's disease patients [10] (Figure 2). Other studies [8] have detected an increase in GPx in patients with Crohn's disease. This increase in GPx must help to detoxify H_2O_2; however, it is not enough because we detect a significantly increased concentration of H_2O_2 in cells from Crohn's disease patients. Finally, we observed that the oxidative stress generated in Crohn's disease patients produced oxidative damage demonstrated by an increase in lipid peroxidation [MDA (malondialdehyde) formation] and an increase in DNA oxidation (detection of 8-oxoguanosine in the DNA) [10].

Experiments carried out in lymphocytes from the same patients, but when they were in remission, showed that, although SOD activity had returned to normal levels, catalase activity continued to be inhibited and H_2O_2 levels continued to be elevated, independently of Crohn's disease activity (Figure 2). Also contrary to expectations, we showed that the oxidative damage detected in active Crohn's disease samples was also permanently present (MDA and 8-oxoguanosine) [10]. Catalase may be participating in the regulation of some cellular processes as described above. It has also to be noted that in Crohn's disease, ANCAs (anti-neutrophil cytoplasmic antibodies) have been described against catalase [11]. All of these findings help to explain why, even with a wide range of drugs against the disease, we have been unable to change the natural progression of the disease. There must be permanent pathogenic events that the actual therapy cannot reverse [12].

Immunity and oxidative stress relation in Crohn's disease

In Crohn's disease, it is known that the intestinal mucosa accumulates $CD4^+$ T-lymphocytes in the lamina propria with an immunological response of the Th1/Th17 type. These lymphocytes are resistant to apoptosis [13]. It is not known whether this difficulty in exerting the apoptotic programme is primary in the cells or secondary to changes acquired during the activity of the disease (i.e. a consequence of cytokine action on the cells). Independently of the origin of

the resistance to apoptosis, it perpetuates the inflammatory responses in the intestinal epithelium.

Cytokines, small proteins that allow communication between different immune cells, not only regulate the differentiation and proliferation of different immune cells, but also can control apoptosis processes in these cells for appropriate immune homoeostasis. Resistance of immune cells, such as T-cells, to apoptosis clearance plays an important role in mucosal inflammation in IBD. Beneficial effects of antibodies against pro-inflammatory cytokines, such as TNFα (tumour necrosis factor α), IL (interleukin)-12 or IL-6, in IBD patients have shown the importance of cytokines in regulating the immune cells' fate, survival or apoptosis.

TNFα is a cytokine that plays a pivotal role in the pathogenesis of Crohn's disease. TNFα concentration is increased in the faeces and in the serum of Crohn's disease patients [14,15] and the most recent treatments developed for the disease are targeted to block TNFα [16]. Although infliximab is an anti-TNFα drug, its mechanism of action is not completely understood. Some studies have shown that its administration provokes an increase in the apoptosis of the mononuclear cells from the intestinal mucosa [16] and from peripheral blood [17]. Many apoptotic stimulators, similar to TNFα, are able to induce the generation of ROS by interaction with the respiratory chain [18], and these ROS are thought to be mediators in the apoptotic pathways [19].

Unravelling the meaning of AOE status in Crohn's disease

The status of particular ROS production and AOE activities in immune cells of Crohn's disease patients has not been previously assessed in detail [10] (see above and Figures). Similarly, the permanent inhibition of catalase activity in Crohn's disease patients has been an original observation which deserves investigation. A review of the literature on what is known regarding AOEs and ROS regulation from experimental knowledge not related to Crohn's disease (see below) will clarify the possible importance of our findings and the need for further research.

Among the AOEs, MnSOD has been characterized by many studies as an anti-apoptotic enzyme [20]. Increased MnSOD activity has been shown to prevent cell death via the receptor-mediated apoptotic pathway as well as cell death via the mitochondrial pathway [21]. If MnSOD fulfils an anti-apoptotic function, the first interpretation would be that either O_2^- is involved in apoptosis or that O_2^- inhibits survival pathways. However, it should be considered that one reaction product from the SOD reaction is H_2O_2, a ROS also implicated in the regulation of cell proliferation and cell death (see below). This consideration sheds light on the balance between MnSOD activity and the activity of the H_2O_2-degrading enzymes catalase and GPx. In fact, there is evidence that an increase in GPx activity occurs in MnSOD-overexpresssing cells as a compensatory response to the increased H_2O_2 level. However, a compensatory increase in catalase activity has not been reported [22], and, in contrast with expectations, catalase expression and activity are diminished in tumoral cells resistant to apoptosis [23].

Some studies seem to indicate that catalase function would not be the detoxification of H_2O_2, but the regulation of apoptosis [24]. In this sense, the *CAT* (catalase) gene is insensitive to oxidant stress and it is considered to be a non-regulated housekeeper gene [25]. The literature on the influence of catalase activity on apoptosis is less consistent than the respective literature on the effects of MnSOD. Two assumptions might be generated with the results of many different studies: (i) some studies [26] show that catalase overexpression, and its consequent diminution, protects against apoptosis, whereas (ii) other studies [9] show that low catalase activity confers resistance to apoptotic stimuli. This could be due to the requirement of H_2O_2 for a survival pathway or an inhibitory action of H_2O_2 on a step within the apoptotic programme.

The mechanisms by which catalase influences apoptosis may be ascribed to direct alteration of the conformation and activity of kinases, phosphatases or redox-sensitive proteins by ROS [6,27,28], and it would be regulated depending on the amount of H_2O_2 that catalase activity could produce in the cells. In fact, it has been shown *in vitro* that H_2O_2 can inhibit caspase 3 and caspase 8 with an IC_{50} of $\sim$20 μM [29]. Many apoptotic stimulators, similar to TNFα, are able to induce the generation of ROS by interaction with the respiratory chain [18], and ROS are thought to be mediators in the apoptotic pathways [19]. Mitochondria are a main source of ROS generation, including H_2O_2, and an important target for interaction with H_2O_2. This ROS can induce mitochondrial permeability transition and decrease the mitochondrial membrane potential [30] and ATP formation, which can also limit the capacity of the cell to exert apoptosis. It has been shown that the H_2O_2 generated via the induction of MnSOD confers on the cells resistance to the cytotoxic effect of TNFα. The same resistance capacity can be generated by being given the catalase inhibitor AZT (3-aminotriazole) [31]. Finally, an analysis of gene expression in cells resistant to the effect of TNFα showed that none of the AOEs was overexpressed, whereas the expression of two enzymes (catalase and CuSOD) was down-regulated [23]. All of these observations indicate that H_2O_2, and thus catalase activity, are key regulators of the sensitivity that cells have to the effects of TNFα.

In vivo studies carried out in animals showed that the exposure of cells to TNFα induces an inhibition of catalase activity [32] and a down-regulation of *CAT* mRNA levels [33]. Catalase regulation is wider than genetic expression modification. It is regulated post-transcriptionally by some protein kinases (protein kinase A and protein kinase C) and phosphatases [34,35]. Thus there is an important amount of possible pharmacological manipulation which will have an impact on apoptosis. Different catalase mutations have been reported in the literature [36] and they are related to some diseases such as diabetes mellitus [37], vitiligo [38] or hypertension [39]. However, not all of the alterations of

catalase activity depend on a mutation, but on regulatory mechanisms not completely understood [40]. A previous study has found that a deficiency in telomerase induces an inhibition of catalase activity and a consequent decrease in ROS [41]. These ROS stimulate the synthesis of profibrotic cytokines. This could be one of the mechanisms by which catalase is regulated.

Occurences of acatalasaemia have been described in clinic, which can show as a homozygotic or heterozygotic condition. Acatalasaemia is a benign syndrome described by Takahara (Takahara's disease) which is classically manifested as oral ulcerations [42] and in which the bacterial flora, the hygienic conditions of the mouth and other environmental factors have a role. All of the genetic defects of catalase and all of the possible clinical manifestation have been reviewed in [43].

In summary, regarding catalase, we can summarize that it is an AOE implicated more in the regulation of some cellular processes than in determining the concentration of H_2O_2 in the cell. Its regulation is exerted both transcriptionally and post-transcriptionally. TNFα is a factor with the capacity to modify catalase activity and expression. Finally, catalase mutations or inhibition of activity can have clinical repercussions among which effects on the gastrointestinal mucosa can be found.

Conclusions

Our results show that immune cells of Crohn's disease patients are induced to produce H_2O_2 which is significantly increased in the cells. The immune cells also have a persistent inhibition of catalase activity and a permanent oxidative damage. Crohn's disease is a disease in which TNFα plays a pivotal role and in which resistance to apoptosis has been described. Biological anti-TNFα drugs exert their effect by increasing the apoptosis of the immune cells. The inhibition of catalase has been observed previously in tumoral cell lines in which it regulates apoptosis, but our finding is the first *in vivo*. The regulation of catalase and its impact on the apoptotic capacities of cells should be explored in immune cells from Crohn's disease patients. Together, these observations provide a sensible hypothesis that catalase could be regulating cellular processes such as apoptosis and could offer a new therapeutic manipulation for Crohn's disease.

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