Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 306 (2005) 150 – 167

Experimental Cell Research

www.elsevier.com/locate/yexcr

High levels of endogenous nitric oxide produced after burn injury in rats arrest activated T lymphocytes in the first G1 phase of the cell cycle and then induce their apoptosis

Lionel M. Valenti[a,*], Jacques Mathieu[b], Yves Chancerelle[b], Martine De Sousa[a], Maryse Levacher[c], Anh Tuan Dinh-Xuan[a], Irène Florentin[a]

[a]*Laboratoire de Physiologie Respiratoire, Faculté de Médecine Cochin/Port-Royal, Université Paris V, 24 rue du Faubourg Saint Jacques, 75014 Paris, France*

[b]*Unité de Radiobiologie et Inflammation, Centre de Recherche du Service de Santé des Armées (CRSSA), La Tronche, France*

[c]*Département de Pharmacologie, Hôpital Saint Vincent de Paul, Paris, France*

Received 18 August 2004, revised version received 19 December 2004
Available online 23 March 2005

Abstract

Major physical traumas provoke a systemic inflammatory response and immune dysfunction. In a model of thermal injury in rats, we previously showed that an overproduction of nitric oxide (NO) was responsible for the collapse of lymphoproliferative responses. In the present work, we performed a time-course analysis of cell proliferation and cell death parameters in order to establish the sequence of events triggered by the high NO output in Wistar/Han rat splenocytes activated with Con A, 10 days after burn injury. We demonstrate that activated T cells from burned rats never divided whereas normal T cells underwent four division cycles. However, T cells from both burned and normal rat entered the G1 phase as shown by increase of cell size, mitochondria hyperpolarization, and expression of cyclin D1. Burned rat T cells progressed to the late G1 phase as shown by expression of the nuclear Ki-67 antigen, but they never entered the S phase. They underwent apoptosis as shown by morphological parameters, disruption of transmembrane mitochondrial potential, and DNA fragmentation. Persistent accumulation of the p53 protein accompanied these phenomena. NO synthase inhibitors antagonize alterations of cell proliferation and cell death parameters in burned rat T cells and accelerated p53 turnover.

Keywords: Nitric oxide; T lymphocyte; Burn injury; Apoptosis; Cell cycle; Cyclin D1; p53; Ki-67; $\Delta\Psi$m; Flow cytometry

Introduction

Severe traumatic injury induces initially a systemic inflammatory response followed by a long-term immunosuppression affecting mainly cell-mediated immunity, and it predisposes patients to sepsis and multiple organ failure [1,2]. The mechanisms underlying post-traumatic immune dysfunction are far from being elucidated. As we demonstrated before using thermal injury in rats as a model of trauma, an overproduction of nitric oxide (NO) by splenic macrophages is responsible for the collapse of T cell proliferative responses to polyclonal activators without inhibition of interleukin-2 (IL-2) and interferon gamma (IFNγ) production [3,4]. Schwacha et al. [5,6] confirmed and extended our findings as they showed that splenic macrophages from burned C57Bl/6 mice were in a hyperactive state through activation by IFNγ and suppressed T cell proliferation through NO production.

Hoffman et al. [7] showed in 1992 that production of NO by activated macrophages inhibited the proliferative response of normal rat spleen cells to alloantigens in vitro. Since then, data accumulated showed that high concentrations of NO exerted antiproliferative effects on T lymphocyte responses to a variety of stimuli including bacteria, parasites, alloantigens, antigenic peptides, super-

* Corresponding author. Fax: +33 1 58 41 23 45.
E-mail address: valentilio@yahoo.fr (L.M. Valenti).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.02.008

antigens, and mitogens [8,9]. Administration of iNOS inhibitors allowed to demonstrate that in vivo NO could induce suppression of specific immune responses against infectious pathogens and tumors with deleterious effects, but also against alloantigens and some autoantigens with beneficial effects [10,11]. In different experimental models, impairment of T lymphocyte responses to antigens or mitogens has been ascribed either to alterations in cytokine production including IL-2 [12–15], to inhibition of T cell proliferation despite normal IL-2 release [16,17], or to induction of apoptosis in activated T cells [4,18,19]. Identification of molecular targets of NO inhibitory activity within lymphocytes is still limited. Inhibition of IL-2 expression has been linked to *S*-nitrosylation of zinc finger transcription factors by Berendji et al. [20]. Bingisser et al. [21] and Mazzoni et al. [22] reported that NO acted at the level of IL-2 receptor signaling by blocking the phosphorylation and activation of Janus kinases 1 and 3, STAT5, Erk, and Akt. Mahidhara et al. [23] demonstrated that NO inhibited T cell proliferation, at least in part, through the nitrosative inactivation of caspases.

We thought that a better understanding of the mechanisms of how NO inhibits T cell activation may help to predict the complex functions of this mediator during the development of T cell immune responses in normal and pathological situations. Thermal injury in Wistar rats, consisting in a full thickness burn afflicted on 20% of the total body surface area, appears as a suitable model to investigate the mechanisms underlying the immunosuppressive effect of an endogenous and sustained production of NO on naïve T cells. We provided evidence recently that in cultures of burned rat spleen cells stimulated with Con A, the high NO output abrogated the proliferative response by triggering T cell apoptosis through a pathway involving activation of soluble guanylate cyclase and protein kinase G [24].

Antigenic or mitogenic stimulation of resting T lymphocytes resulted in transition from the G0 to the G1 phase of the cell cycle and led to the secretion of IL-2. In conjunction with other growth signals, IL-2 induces D-type cyclin synthesis during the G1 interval. D-type cyclins form active complexes with the cyclin-dependent kinase Cdk4 or Cdk6 that phosphorylate the retinoblastoma protein (pRb). This cancels the growth-inhibitory effects of pRb and allows cells to progress to the so-called “restriction point” in the mid-to-late G1 phase [25]. After the restriction point, cells become irreversibly committed to enter the S phase, and this is mainly due to the activation of the cyclin E–Cdk2 complex, which completes and maintains the neutralizing phosphorylation of pRb. The action of cyclin–Cdk complexes can be counteracted by cyclin-dependent kinase inhibitors (CKI) including $p21^{Waf\text{-}1}$ and $p27^{Kip1}$, which antagonize phosphorylation of pRb by Cdks. Under conditions of cellular stress including genotoxic stress, the tumor suppressor and transcription factor p53 accumulates and induces cell growth arrest in G1 phase through induction of $p21^{Waf\text{-}1}$ gene transcription and/or promotes apoptosis through transcription-dependent and -independent mechanisms [26]. Alteration of the expression and activity of p53 by NO has been implicated in its antiproliferative and proapoptotic effects on various types of cells, more often cell lines exposed to NO donor molecules [27].

The aim of the present work was to establish the sequence of events triggered by high concentrations of endogenous NO on activated T lymphocytes as regards cell cycle alteration and cell death, and we addressed the question of specific cell cycle sensitivity to the proapoptotic effect of NO. Spleen cells from normal and burned rats were analyzed by multivariate cell cytometry, over a 72-h time course after Con A stimulation, for cell cycle progression by measurement of DNA content and of expression of the nuclear antigen Ki-67 and cyclin D1 as indicative of the exit from the G0 phase and of the first step in progression through the G1 phase of the cell cycle, respectively [28,29]. Expression of apoptosis and necrosis hallmarks was also followed in function of time by flow cytometric determination of cell shrinkage, disruption of the mitochondrial transmembrane potential ($\Delta\Psi m$), loss of plasma membrane asymmetry with subsequent externalization of phosphatidylserine (PS) residues, DNA fragmentation leading to a sub-G1 DNA content, and loss of plasma membrane integrity. We investigated time-related alterations in p53 expression in support of the hypothesis that p53 orchestrated cell cycle arrest and apoptotic process in an orderly sequence of events induced by the high and sustained NO production. To validate the role of NO, we looked at the ability of the *S*-methylisothiourea (SMTU), a potent NO synthase inhibitor [30] to antagonize alterations of cell proliferation and cell death parameters in activated burned rat T cells.

Animals, materials and methods

Animals and burn injury

Seven-week-old male Wistar/Han rats were obtained from Charles River Laboratories, France. They were housed individually in a protected environment and left to adapt to the new surroundings for 1 week. A full thickness skin burn covering 20% of the total body surface area was provoked by immersing the shaved dorsum of anesthetized rats in a 90°C water bath for 10 s. Third-degree burns are painless because the nerve endings in the skin are destroyed. Control animals were shaved and anesthetized. They are referred to as normal rats.

Materials

RPMI 1640 culture medium, phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+} unless otherwise stated, 1 M HEPES buffer, fetal calf serum (FCS) and horse serum (HS), 2-mercaptoethanol (2-ME), bovine serum albumin (30%