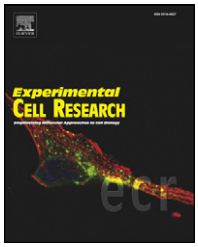
EXPERIMENTAL CELL RESEARCH 317 (2011) 1437–1453

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

At the core of survival: Autophagy delays the onset of both apoptotic and necrotic cell death in a model of ischemic cell injury

B. Loos[a,*], *S. Genade*[b,2], *B. Ellis*[a,1], *A. Lochner*[b,2], *A.-M. Engelbrecht*[a,1]

[a]*Department of Physiological Sciences, Stellenbosch University, Mike de Vries Building, Merriman Street, Stellenbosch, 7600, South Africa*
[b]*Department of Biomedical Sciences, Faculty of Health Sciences, University of Stellenbosch, Tygerberg, South Africa*

ARTICLE INFORMATION

Article Chronology:
Received 3 November 2010
Revised version received 9 March 2011
Accepted 10 March 2011
Available online 21 March 2011

Keywords:
Autophagy
Apoptosis
Necrosis
Life Cell Imaging
Fluorescence Resonance Energy
Transfer-FRET
Ischemic injury

ABSTRACT

Ischemic cell injury leads to cell death. Three main morphologies have been described: apoptosis, cell death with autophagy and necrosis. Their inherent dynamic nature, a point of no return (PONR) and molecular overlap have been stressed. The relationship between a defined cell death type and the severity of injury remains unclear. The functional role of autophagy and its effects on cell death onset is largely unknown. In this study we report a differential induction of cell death, which is dependent on the severity and duration of an ischemic insult. We show that mild ischemia leads to the induction of autophagy and apoptosis, while moderate or severe ischemia induces both apoptotic and necrotic cell death without increased autophagy. The autophagic response during mild injury was associated with an ATP surge. Real-time imaging and Fluorescence Resonance Energy Transfer (FRET) revealed that increased autophagy delays the PONR of both apoptosis and necrosis significantly. Blocking autophagy shifted PONR to an earlier point in time. Our results suggest that autophagic activity directly alters intracellular metabolic parameters, responsible for maintaining mitochondrial membrane potential and cellular membrane integrity. A similar treatment also improved functional recovery in the perfused rat heart. Taken together, we demonstrate a novel finding: autophagy is implicated only in mild injury and positions the PONR in cell death.

Introduction

The concept of damage control in the myocardium was introduced for the first time by Braunwald in 1974: "… just because myocardial tissue lies within the distribution of a recently occluded artery does not mean that it is necessarily condemned to death" [1]. The dynamic nature of cell death was implicated at the same time. However, despite the development of various interventions to provide cardiac protection and to reduce the morbidity and mortality from myocardial infarction and heart failure, the residual death rate remains significant [2].

Three main morphologies of cell death have been described in the diseased myocardium: type I, better known as apoptotic cell death, which is characterized by cell shrinkage and chromatin condensation; type II, or cell death with autophagy, which presents a morphology with intracellular accumulation of autophagic vacuoles; and

* *Corresponding author.* Fax: +27 21 808 3145.
E-mail address: bloos@sun.ac.za (B. Loos).
[1] Fax: +27 21 808 3145.
[2] Fax: +27 21 938 9476.

0014-4827/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2011.03.011

type III, better known as necrosis, which is characterized by cellular swelling and rapid loss in cellular membrane integrity [3]. Their accurate distinction is crucial, because of the association with cell loss in human pathologies [4–6]. In the clinical setting of acute myocardial infarction there is a tremendous need for cell death control [7]. However, the relationship between a defined mode of cell death and the severity and time frame of injury remains unclear. In addition, recent advances in reporting real-time data of dying cells suggest that cell death is a much more dynamic and molecularly overlapping event than previously described [8–10]. Although reports suggest that the mode of cell death is governed by the type of insult, its duration and severity, precise details are still emerging [5,11].

It has been shown that local metabolic conditions and the intensity of the initial insult determine the prevalence of either apoptosis or necrosis [12]. However, there is evidence that this decision is controlled on both the mitochondrial and caspase activation levels [13]. A common theme of these regulatory networks is a perturbation in ATP and mitochondrial function, linking stress signal transduction pathways to regulators which control characteristic [14] and often progressively developing [4] morphological changes. Importantly, ATP availability plays a crucial role in determining the onset of necrosis, and has been described as a switch from apoptosis to necrosis [15].

Recent studies have demonstrated that autophagy occurs in the hypertrophied, failing and hibernating myocardium [16]. In failing human hearts the three cell death modes act in parallel to varying degrees [17] with the incidence of autophagic cardiomyocytes being greater than the incidence of apoptotic cells [18]. In experimental models for myocardial infarction it has been shown that particularly in the first 2–4 h of coronary artery occlusion, most of the cell death manifests with apoptotic morphology [19], with TUNEL positive cells being increased at the borders of infarcts [18]. In the central zone of the infarct, however, primarily necrotic cells are found. Necrosis is a major contributor to heart failure associated with cardiac pathologies such as ischemia/reperfusion injury [20]. However, although in many conditions a distinct morphology of one of the above types of cell death may be encountered, multiple pathological conditions present themselves with morphological overlap [5,18]. The existence of "grey zones" between cell death modes and the existence of the point of no return (PONR) within these have recently been revealed [21]; and mitochondrial membrane permeabilization or the dissipation of mitochondrial transmembrane potential $\Delta\Psi m$ being considered a PONR in programmed cell death [22–24].

The above findings strongly demand the integration of concepts that describe the cellular death process completely and dynamically, so as to better locate and control the onset of cell death. Therefore, we firstly characterized the contribution of each cell death type in context of the severity and duration of an ischemic insult. Secondly, we determined whether manipulation of the autophagic pathway affects this contribution and translates into protection of the heart. By employing rodent-derived cardiac cells, we aimed to assess the temporal onset of apoptosis and necrosis when inducing or inhibiting the autophagic pathway.

Our results strongly indicate a differential induction of cell death, which is dependent on the severity and duration of the ischemic insult. Mild ischemia led to the induction of autophagy and apoptosis, while moderate or severe ischemia induced both apoptotic and necrotic cell death without an indication of autophagy. Furthermore, only mild but not moderate and severe ischemic injury was associated with an ATP surge. Our real-time imaging data provide direct evidence that increased autophagy delays the onset of loss in cellular membrane integrity and delays the onset of caspase-3 activation, as well as mitochondrial depolarization.

Materials and methods

Reagents

Antibodies against PARP and pAMPK were obtained from Cell Signaling Technology (MA, USA). 3 Methyladenine (3MA) and rapamycin were purchased from Sigma. Tissue culture tubes from Greiner Bio-One (Germany) were used. Sterile serological pipettes were obtained from LP Italiana SPA and Sterilin Ltd. Pipette tips were purchased from Greiner Bio-One. Culture medium, Dulbecco's modified Eagle's medium (DMEM) was obtained from Highveld (South Africa), trypsin and phosphate buffered saline (PBS) from SIGMA-ALDRICH (South Africa) and tissue culture flasks and dishes, cell scraper and microcentrifuge tubes were purchased from Greiner Bio-One (Germany). Chambered coverglass slides from Nunc Lab-Tek (Lab-Tek, 155411, USA) were used. Syringe millipore filter, albumine bovine serum (BSA) and Bradford reagent were obtained from SIGMA-ALDRICH (South Africa). For the gel electrophoresis a Mini-Protean BIO RAD system was used. Ammonium persulfate was purchased from SIGMA-ALDRICH (South Africa), Acrylamide/bis-Acrylamide from Promega (South Africa) and *N,N,N,N′*-Tetramethylethylendiamin from MERCK (Germany). The CFP-DEVD-YFP plasmid was kindly provided by Dr Tavaré (University of Bristol, UK).

Cell culture

H9c-2 rat heart myoblasts (European Collection of Cell Cultures – ECACC) were seeded at 12,000/cm^2, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4% glutamine and 1% penicillin/streptomycin in a humidified atmosphere, 37 °C, in the presence of 5% CO_2. Cells were washed with 0.01 M sterile phosphate-buffered saline, (PBS), trypsinized (0.25% Trypsin-EDTA) and centrifuged for 3 min at 6000*g*. Seeding took place as follows: 3×10^5 myoblasts per 25 cm² tissue culture flask, 1×10^5 myoblasts per culture dish in six-well plates and 2×10^4 myoblasts per 8 chamber slide. Growth medium was changed every 48 h.

Cell death experiments

Control cells were kept under normoxic conditions and 5% CO_2. The simulated ischemia lasted for 2 and 8 h. Cells were washed with sterile PBS and incubated with a filtered sterilized modified ischemic buffer [25], pH 6.4, containing (in mM): 137 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 20 Hepes, 20 2-deoxy-D-glucose (2DG, Sigma, D-8375), or 0.5 sodium dithionite (SDT, Fluka, BioChemika, 71699), or 2DG and SDT under hypoxic conditions in a humidified environment containing 0.1% O_2, 5% CO_2 and the balance N_2. Modified ischemic buffer with either 2-deoxy-D-glucose [26], sodium dithionate [27], or both [28] was employed, to create an ischemic insult of mild (mild SI), moderate (moderate SI) and severe (severe SI) character. Temperature was maintained at 37 °C.

The loss of membrane integrity was assessed by using the live cell Propidium Iodide (PI) exclusion technique, coupled with fluorescence