Available online at www.sciencedirect.com
ScienceDirect
Methods 44 (2008) 241–249
METHODS
www.elsevier.com/locate/ymeth

Measuring cell death mediated by cytotoxic lymphocytes or their granule effector molecules

Vivien R. Sutton [1], Nigel J. Waterhouse [1], Katherine Baran, Kylie Browne, Ilia Voskoboinik, Joseph A. Trapani *

Cancer Cell Death Laboratory, Peter MacCallum Cancer Centre, Locked Bag 1, A'Beckett Street, Melbourne, Vic. 8006, Australia

Accepted 20 November 2007

Abstract

Cytotoxic lymphocytes (CL) are highly motile cells that utilize granule exocytosis to kill virus-infected or transformed targets. Isolated CL and purified granule proteins have been used to investigate the molecular processes that CL use to kill their targets and to investigate the basis of human disease. We have set out various methods that are routinely used to isolate CL and characterize the cell death pathways they induce. As cell death mediated through TNF-superfamily members and their respective receptors is covered elsewhere, this manuscript will deal specifically with cytotoxic granule-mediated cell death.

Keywords: Cytotoxic lymphocytes; Granule; Granzyme; Apoptosis; Cytolysis

1. Introduction

Assays for CL-induced death generally involve mixing cytotoxic T lymphocytes (CTL) or natural killer (NK) cells with their cognate targets at various ratios. Allogenic CTL recognise and kill major histocompatibility complex (MHC) class I and/or II-mismatched target cells. As such, they are particularly relevant to allograft rejection. To generate these cells, primary splenocytes isolated from a mouse of the same H-2 haplotype as the proposed target cells are used as stimulator cells to raise CTL from MHC mismatched splenic responder T cells. Allogenic CTL can be generated *in vitro* by one way mixed lymphocyte reaction (Section 2.1; [1,2]) or *in vivo* by injecting stimulator cells into the foot pad of responder mice (Section 2.2; [3]).

Transgenic mice that express a single clonotypic T cell receptor can be specifically stimulated to produce T cells that predominantly recognise a single peptide, presented by class I molecules on syngeneic target cells (Section 2.3). For example, CTL generated in C57BL/6.OT1 mice bear the Vα2 and Vβ5 T cell receptor chains that specifically recognise the ovalbumin peptide SIINFEKL presented on H-2K^b [4]. These CTL can be used to examine the death of any peptide-pulsed MHC class I-expressing target cells derived from H-2^b mice. In principle any of the TCR transgenic mouse strains can be used and some TAP-deficient cells such as RMA-S can also be used as target cells if pulsed with exogenous peptide at a permissive temperature [5].

NK cells recognise and kill cells by a non-MHC restricted mechanism and generally do not need to be primed with antigen. Unstimulated splenocytes can be used as a source of NK cells, however they typically comprise only a few percent of resident splenic lymphocytes and very high ET ratios (up to 200:1) may be needed to obtain only modest levels of target cell death. NK cells can be purified from spleen using a two-step process to firstly reduce unwanted T cells and then positively select the NK cells (Section 2.4). These NK cells can be cultured for extended periods (up to 2 weeks) using moderate to high (50–1000 U/ml) doses of IL-2.

CL may kill their targets by various perforin dependent or perforin independent mechanisms [6]. Analysis of cyto-

* Corresponding author. Fax: +61 3 9656 1411.
E-mail address: joe.trapani@petermac.org (J.A. Trapani).
[1] These authors contributed equally to this work.

1046-2023/$ - see front matter
doi:10.1016/j.ymeth.2007.11.011

toxicity of CTL and NK cells mice from mice deficient in perforin, granzymes or death receptors has been integral in elucidating the contribution of the various effector pathways to CL-induced death (Table 1). Alternatively, the contribution of granule exocytosis to CL-mediated cell death can be assessed more generally by blocking this pathway with calcium chelators (such as EGTA) or concanamycin A, which do not block death receptor mediated killing [1,2] but inhibit perforin, whose action is calcium dependent.

Secretory granules of CTL and NK cells contain several cytotoxic proteins, including perforin and granzymes [7–10]. It is therefore problematic to use intact CL to assess the specific action of each granule protein and to determine the functional consequence of mutations in these proteins. The adherent Rat Basophil Leukaemia cell line RBL-2H4 can be used to express and test the activity of murine perforin (Section 3) and to assess the effect of various perforin mutations [10–14]. Human perforin can also be expressed in these cells but is somewhat less active than its mouse counterpart, for reasons not yet fully understood. Cytotoxic proteins may also be isolated from the secretory granules of CTL or NK cells (Section 4). Granzyme B may be purified from human NK tumour lines such as YT and perforin has commonly been purified from the rat cytotoxic cell line, RNK16 [15,16]. Typically, the protocol involves growing large numbers of cells *in vitro* or *in vivo*, gently lysing the cells by nitrogen cavitation to preserve granule integrity, disrupting the granules in high salt buffer and purification of the cytotoxic proteins by ionic exchange chromatography [17,18]. More recently, recombinant perforin has been produced using baculovirus (Section 3.1; [13,14,19,20]) and several granzymes have been produced using baculovirus, bacterial and yeast expression systems [21–25].

When used at high concentrations, perforin has been shown to potently induce target cell lysis and this activity can easily be tested using the sheep red blood cell lysis assay (Section 3.2), however at low concentrations perforin can synergise with granzyme B to induce apoptosis of target cells [3,16,26–28]. Typically, when we treat cells with both perforin and granzyme B, we use a concentration of perforin that, when applied alone, causes no more than 5–10% specific ^{51}Cr release (Section 5.1) or staining with propidium iodide (Section 5.2) in the target cell. Both native and recombinant perforin are inhibited by serum lipids, therefore we usually perform these assays in Hanks Balanced Salt Solution (HBSS) or serum-free medium (SFM) supplemented with 0.1–0.5% BSA (Cohn fraction V). In the absence of purified perforin, alternatively lytic or pore–forming agents have been used to substitute for the action of perforin. These include PLO (pneumolysin), SLO (streptolysin O), LLO (listeriolysin) and replication-deficient adenovirus [29,30].

CL-induced death or death triggered by purified granule proteins is routinely assayed by measuring specific release of ^{51}Cr and/or ^{125}I-deoxyuridine that has been preloaded into target cells or incorporated into target cell DNA, respectively (Section 5.1; [3]). Assaying cytotoxicity by measuring the release of radiolabelled substances requires special handling and regular monitoring of staff and equipment in case of accidental radiation exposure or contamination. These assays can be relatively expensive as radioactive waste must be disposed of safely, and so may not be optimal for large scale studies or in labs that do not specialise in immunological assays. An alternative approach involves using the cytofluorograph and standard assays of apoptosis such as the binding of FITC–annexin V and uptake of PI to track the accumulation of dying target cells (Section 5.2; [27,28,31]). When the death stimulus is delivered by intact CL, it is important to exclude dying effector cells from the analysis. This can be achieved by labelling either the killer or target cells with a specific marker, such as a fluorescent antibody against cell surface proteins or a non-diffusible membrane dye [32]. Several groups have also evaluated methods to substitute for the ^{51}Cr release assay, including granzyme B Elispot (Section 5.3; [33–35]) and fluorolysis (Section 5.4; [36]). CLs possess a range of cytotoxic mechanisms and can kill their targets by apoptosis, lysis or an atypical death that is distinct from both apoptosis and lysis [2,37]. Individual cytotoxic lymphocytes in the same sample may kill their targets by any of these processes but population based assays cannot distinguish between them. We have found that time lapse microscopy is an efficient method to distinguish between the various types of death (Fig. 1) and determine the prevalence of each type of death in a population of dying cells (Section 5.5; [2,37]).

Table 1
Gene-targeted mice helpful in distinguishing target cell death pathways

Mouse strain	Function/use	Reference
Perforin-deficient	Excludes death through all CL granule-dependent pathways	[40]
Individual or collective granzyme-deficient, e.g. GrA$^{-/-}$B$^{-/-}$	Excludes death dependent on the specific granzymes	[41–44]
Gld/lpr	Excludes death dependent on the FasL/Fas interaction—use gld effectors or lpr targets	[45]
TRAIL/TRAIL receptor-deficient	Excludes death dependent on the TRAIL/TRAIL receptor interaction—use TRAIL$^{-/-}$ effectors or DR5-negative targets	[46–48]
TNF/TNF-R1 and TNF-R2-deficient	Excludes death dependent on TNF—use TNF receptor$^{-/-}$ targets	[49–51]
FADD-DN	Dominant negative: expression, under control of *lck* promoter, in T cells. Marked down-regulation of death receptor pathways	[52]