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Research paper

Granzyme B-mRNA expression by equine lymphokine activated killer (LAK) cells is associated with the induction of apoptosis in target cells

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ABSTRACT

Lymphokine-activated killer (LAK) cells are a subset of cytotoxic cells capable of lysing freshly isolated tumor cells. While LAK activity is typically measured using the ^{51}Cr-release assay, here we used a non-radioactive flow cytometric method to demonstrate equine LAK activity. Equine peripheral blood mononuclear cells (PBMC) were stimulated *in vitro* with recombinant human interleukin 2 (hIL-2) to generate LAK cells. An equine tumor cell line, EqT8888, labeled with carboxyfluorescein succinimidyl ester (CFSE) was used as target cells. Following incubation of the targets with different concentrations of LAK cells, Annexin V was added to identify the early apoptotic cells. With increasing effector to target cell ratios, EqT8888 apoptosis was increased. We also measured interferon-gamma, granzyme B and perforin mRNA expression in the LAK cell cultures as possible surrogate markers for cytotoxic cell activity and found granzyme B mRNA expression correlated best with LAK activity. Also, we found that the reduced LAK activity of young horses was associated with decreased granzyme B mRNA expression. Our results indicate that fluorescence-based detection of LAK cell activity provides a suitable non-radioactive alternative to ^{51}Cr-release assays and mRNA expression of granzyme B can be used as surrogate marker for these cytotoxic cells.

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1. Introduction

Cell-mediated cytotoxicity (CMC) is an important effector mechanism directed against transformed and infected cells. Natural killer (NK), lymphokine activated killer (LAK) and cytotoxic T lymphocytes (CTLs) are examples of these effector cells. The granules of cytotoxic lymphocytes contain potent mediators, which include pore-forming protein (perforin), cytotoxic cytokines, and granzymes (a family of serine esterases). Together these mediators can induce death of the target cells via caspase-dependent and independent apoptotic pathways (Young et al., 1988). While the ^{51}Cr release assay has been widely used to demonstrate CMC (Brunner et al., 1968), this assay has several disadvantages including the use of a radioactive isotope, high levels of spontaneous release by some targets, and a failure to identify the mechanism of cytotoxicity. Since the ^{51}Cr-release assay only detects the late phase of cytotoxicity, it does not distinguish between apoptosis and other mechanisms of cell lysis. Alternative methods using non-radioactive approaches can overcome some of these limitations.

Flow-cytometric methods have been developed which use fluorescent regents to detect target cell killing either as an increase in membrane permeability (Flieger et al., 1995) or by changes in membrane morphology associated with apoptosis (Goldberg et al., 1999). In addition to avoiding the use of radioactive reagents, this approach offers overall greater sensitivity than the ^{51}Cr-release assays (Aubry et al., 1999) and allows for detection of both early and late phases of cell killing (Vermes et al., 1995).

An alternative approach to quantitating CMC is the direct detection of the cytotoxic cell population using antibodies either against specific cell surface proteins (Kim

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et al., 2007) or the effector molecules involved in target cell lysis. While cell surface staining can identify cells with cytolytic potential (*e.g.* NK, $CD8^+$ T cells, etc.), it fails to detect effector function. Detection of specific effector molecules has been used to identify functional cytotoxic cells. Interferon-gamma (IFNγ) has frequently been used in equine research as a surrogate marker for cytotoxic cell activity (Breathnach et al., 2005; Ellis et al., 1997; Paillot et al., 2006). However, studies in other species have shown that IFNγ production does not correlate well with cytotoxic activity (Calarota et al., 2006). Given these findings and the fact that non-cytolytic $CD4^+$ T cells can also produce IFNγ, we believe better surrogate markers for equine cytotoxic effector function are needed. Granzyme B and perforin, two major effector molecules in the granule-mediated cytolytic pathways, are better markers since studies have shown that their expression correlate well with cytotoxic activity in PBMC cultures (Shafer-Weaver et al., 2003; Zuber et al., 2005)

In this report, we use a flow cytometric method for the characterization of equine LAK cell-mediated cytotoxicity using CFSE-labeled target cells and PE-Annexin V to identify target cells in the early apoptotic phase. We also examined three surrogate markers for LAK activity; IFNγ, granzyme B and perforin mRNA expression and found that granzyme B mRNA expression correlates best with cytotoxic activity of LAK cells.

2. Materials and method

2.1. Horses

Five 10 year old horses were used for the initial optimization of the flow cytometric and gene expression assays. Five additional 1 year old mix-breed horses were used to compare LAK activity and gene expression in the different aged horses. All horses were owned by the Department of Veterinary Science, University of Kentucky and handled in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture. All research procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.2. Target cells

Equine tumor cell line, EqT8888 (Hormanski et al., 1992), was used as target cells in the flow cytometric assay. The cells were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM glutamine (Sigma), and 100 units/ml penicillin/streptomycin (Sigma) at 37 °C 5% CO_2 in air. Target cells were harvested in the log phase of growth before CFSE staining.

2.3. LAK cell generation

Heparinized blood was collected via aspetic venipuncture of the jugular vein. Peripheral blood mononuclear cells (PBMC) were isolated using Ficol-Paque Plus™ (Amersham Biosciences, Piscataway, NJ) gradient centrifugation according to the manufacturer's protocol. The PBMC were washed with phosphate-buffered saline (PBS) for three times before re-suspending at 3×10^6 cells/ml in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 2.5% (v/v) fetal equine serum (FES, Sigma, St. Louis, MO), 2 mM glutamine (Sigma), 100 units/ml penicillin/streptomycin (Sigma), and 55 μM 2-mercaptoethanol (GIBCO, Grand Island, NY). The PBMC were incubated up to 5 days in T25 flasks supplemented with different concentration of recombinant human interleukin 2 (hIL-2; R&D Systems, Minneapolis, MN) to generate LAK cells (Hormanski et al., 1992). All cultures were incubated at 37 °C in 5% CO_2 incubator. Each day, the LAK cells were harvested and counted using a ViCell-XR instrument (Beckman Coulter, Miami, FL) before use.

2.4. LAK cell flow cytometric assay

EqT8888 cells were re-suspended at 1.0×10^7 cells/ml in 1 ml PBS followed by the addition of 1 ml solution of 3 μM CFSE (Fluka BioChemika, Buchs, Switzerland). The solution was gently mixed for exactly 8 min followed by the addition of 2 ml of FBS to stop the reaction. The target cells were washed three times with 10% (v/v) FBS in PBS and re-suspended at a concentration of 3×10^5 cells/ml prior to dispensing 100 μl of labeled cells into a 96 well round bottom plate (Corning, Corning, NY). To each well of target cells was added a 100 μl volume of LAK cells at different cell concentrations to yield effector:target (E:T) ratios of 40, 20, 10 and 5:1. Plates were then centrifuged at 200 g for 5 min and incubated at 37 °C in 5% CO_2 incubator. Samples with target cells only were used as spontaneous apoptosis controls. To generate apoptosis positive control samples, EqT8888 cells were incubated for 2 h in a 56 °C water bath. Killing of the cells was confirmed by trypan blue staining. All samples were performed in triplicate.

After 3 h incubation, the cells were transferred to 96 well V bottom plates. The plates were centrifuged at 300 g for 5 min. Cells were washed by PBS for 2 times and re-suspended in 100 μl Annexin V buffer (BD Pharmingen™), and transferred to 12 mm × 75 mm tubes (BD Pharmingen™). Annexin V (PE-conjugated, BD Pharmingen™) was added to each tube and the cells were incubated in the dark at room temperature. After 15 min incubation, all samples were acquired using a FACSCalibur and analyzed by CELL Quest Pro™ (Becton Dickinson).

The target cells were gated using FL1 and 3000 gated events were acquired for each sample. Target cells that underwent apoptosis were both CFSE (FL1) and Annexin V-PE (FL2) positive. Each determination was performed in triplicate. The percentage of cytotoxicity was determined by the equation:

$$\%\ \text{cytotoxicity} = \frac{(\%\text{CFSE}^+\text{Annexin V}^+\ \text{target in test well}) - (\%\text{CFSE}^+\text{Annexin V}^+\ \text{spontaneous release group})}{(\%\text{CFSE}^+\text{Annexin V}^+\ \text{total release group}) - (\%\text{CFSE}^+\text{Annexin V}^+\ \text{spontaneous release group})} \times 100$$

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