Bio-Plex® suspension array system

tech note 5762

Measurement of Caspase-1-Dependent Cytokine Secretion Using the Bio-Plex Human Cytokine 27-Plex Panel

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Introduction

Mammalian cells use the endoplasmic reticulum/Golgi-dependent pathway to export most proteins. However, some proteins are secreted through unconventional, less-understood mechanisms. These proteins include the proinflammatory cytokine prointerleukin-1β, which requires cleavage by caspase-1 for biological activity and subsequent release.

Several cell types, such as macrophages and keratinocytes, secrete IL-1β in response to stress signals. For example, UV-irradiation of human keratinocytes activates caspase-1 and thereby induces secretion of IL-1β (Feldmeyer et al. 2007). We recently published a report indicating that active caspase-1 regulates the unconventional secretion of many additional proteins from keratinocytes and other cell types (Keller et al. 2008).

In this report, we present an RNAi-based method for analyzing caspase-1-dependent release of various cytokines from UV-irradiated human keratinocytes using the Bio-Plex human cytokine 27-plex panel. The panel includes IL-1β in addition to 26 other cytokines, 21 of which (Table 1) possess a signal peptide. This allowed us to determine whether caspase-1 specifically targets unconventional protein secretion or secretion in general.

Methods

Cultivation and siRNA Transfection of Keratinocytes

Primary human keratinocytes were isolated from the foreskin of a 3-year-old donor as previously described (Rheinwald and Green 1975). Cells were grown as adherent cultures in keratinocyte-SFM growth medium (Gibco Invitrogen Corporation) and supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) provided by the medium supplier.

siRNA transfection has been previously described (Keller et al. 2008). In brief, keratinocytes were seeded into 14 cm dishes (4.5 x 10^6 cells per dish). After one day, the cells were transfected with 21-mer duplex siRNAs (Sigma-Aldrich Co.) specific for caspase-1 or vascular endothelial growth factor (VEGF). Target sequences used for caspase-1 were GGCAGAGAUUUAUCCAAUATT or CCAAUAAUGGACAAGUCAA, and sequences for VEGF were CUGAUGAGAUCGAGUACAUTT or UGUGAAUGCAGACCAAAGATT. INTERFERin (Polyplus-transfection SA) was used as the transfection reagent according to the manufacturer's instructions, and the final transfecting siRNA concentration was 50 nM. Reduction of target gene expression was determined by real-time PCR and western blotting using β-actin as an internal reference (data not shown).

Stimulation of Protein Secretion and Sample Preparation

After siRNA transfection (48 hr, or 24 hr after seeding untransfected cells), cells were washed three times with PBS and 5 ml of fresh medium per dish (without growth supplement) was added. After 1 hr, cells were irradiated with 50 mJ/cm^2 UVB. The supernatant was removed 4 hr after irradiation, and adherent plus pelleted cells from the supernatant were lysed for 10 min in 1 ml of PBS containing 2% Triton X-100 per dish. The lysed cells were diluted to 5 ml in growth medium and cellular debris was removed by low-speed centrifugation. Both cell culture supernatants and cell lysates were snap frozen and stored at –70°C until analysis. Lactate dehydrogenase (LDH) activity was measured by the CytoTox 96 nonradioactive cytotoxicity assay (Promega Corporation).

Multiplex Bead Suspension Array and Data Evaluation

Cytokine concentrations in cell lysates (Lys) and supernatants (SN) were determined using the Bio-Plex multiplex bead-based suspension array system with the human cytokine 27-plex panel (Table 1). Analysis and data processing were performed according to system and panel instructions. Values below the dynamic range of the assay were set to zero and the corresponding cytokines were not included in the figures. Total protein expression levels were calculated using the formula: (Lys + SN). The percent of cytokine release from the cells was calculated according to the formula: SN/(Lys + SN).

Results and Discussion

We first determined which cytokines were secreted by keratinocytes upon UVB irradiation. Supernatants from cells 4 hr after irradiation or from unirradiated cells were collected and analyzed using the human cytokine 27-plex panel. For the UVB-irradiated keratinocytes, 21 out of 27 cytokines measured in the panel were detected in the supernatant (Table 1, Figure 1). Most of these cytokines are known to be produced by keratinocytes. IL-2, IL-4, IL-5, IL-10, macrophage inflammatory protein (MIP)-1β, and RANTES could not be detected in the supernatants. These cytokines, which are normally not produced by keratinocytes, can be used as negative controls to verify the validity of the measurement.

As expected, secretion of IL-1β was enhanced by UV-irradiation (more than 3-fold). The same stimulus also induced the export of IL-7 (nearly 3-fold) and to a lesser extent of eotaxin, platelet-derived growth factor (PDGF), and tumor necrosis factor (TNF)-α. TNF-α expression is known to be induced by IL-1β, and UV-irradiation therefore most likely enhances TNF-α release indirectly via IL-1β.

In order to determine the impact of caspase-1 expression on cytokine secretion, we transfected human primary keratinocytes with siRNA and thereby reduced caspase-1 expression. Supression of VEGF expression served as a control. Knockdown efficiencies were demonstrated in recent publications (Feldmeyer et al. 2007, Keller et al. 2008). Protein expression of caspase-1 was reduced to approximately 20% of its normal expression levels.

UV irradiation leads to some degree of protein release because of cell lysis. In order to quantitate the impact of UV on cell lysis, we measured extracellular versus total activity of the cytoplasmic enzyme LDH (Figure 2). Based on these measurements, the degree of lysis due to irradiation was about 7% (+UV). Cell lysis of caspase-1 siRNA-transfected cells did not differ more than 2% from VEGF siRNA-transfected cells. Therefore, apparent differences in cytokine concentrations in the supernatant are not expected to be due to UV-induced cell lysis.

To analyze the influence of caspase-1 on both cytokine expression and secretion, we analyzed cell lysates and supernatants using the human cytokine 27-plex panel (Figure 3). This allowed us to observe that knockdown of caspase-1 did not change the expression of mo st cytokines (Figure 3, upper panel).

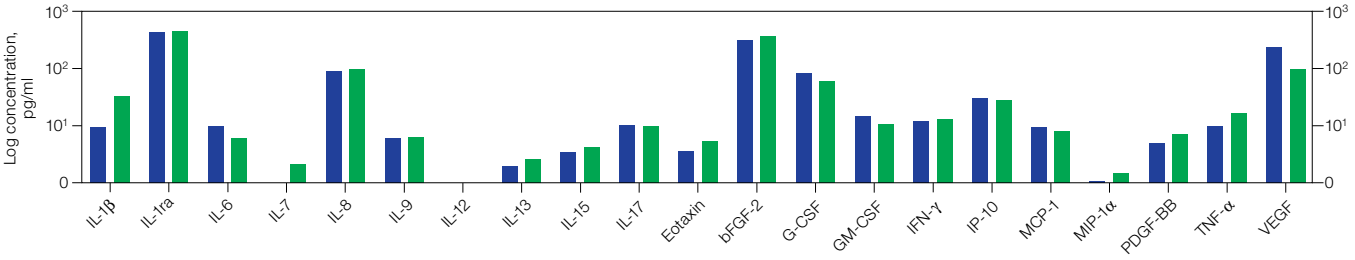

Fig. 1. UVB-induced cytokine secretion from keratinocytes. Cytokines were measured in cell culture supernatants from keratinocytes 4 hr after UVB irradiation (■) or in control cells (■) using the Bio-Plex multiplex array system and human cytokine 27-plex panel. Bars represent values from single measurements. The 21 cytokines found to be secreted are shown.

Table 1. Bio-Plex human cytokine 27-plex panel.

IL-1β*	Basic FGF-2*, (bFGF-2)
IL-1ra*	Eotaxin*
IL-2	G-CSF*
IL-4	GM-CSF*
IL-5	IFN-γ*
IL-6*	IP-10*
IL-7*	MCP-1*
IL-8*	MIP-1α*
IL-9*	MIP-1β
IL-10	PDGF-BB*
IL-12 (p70)*	RANTES
IL-13*	TNF-α*
IL-15*	VEGF*
IL-17*	—

* Cytokine known to be secreted by keratinocytes.

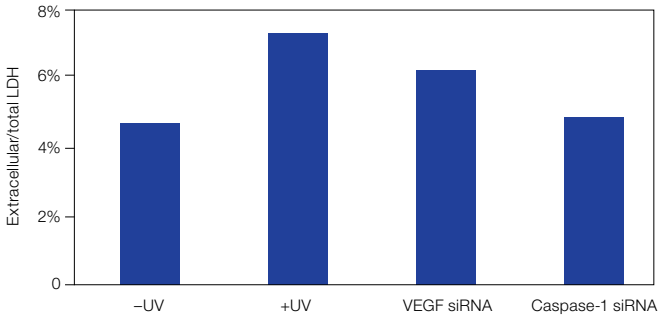

Fig. 2. Assessment of cell lysis by LDH activity measurement. The degree of cell lysis in experiments described in Figures 1 and 3 were determined by measuring extracellular versus total activity of the cytoplasmic enzyme LDH.

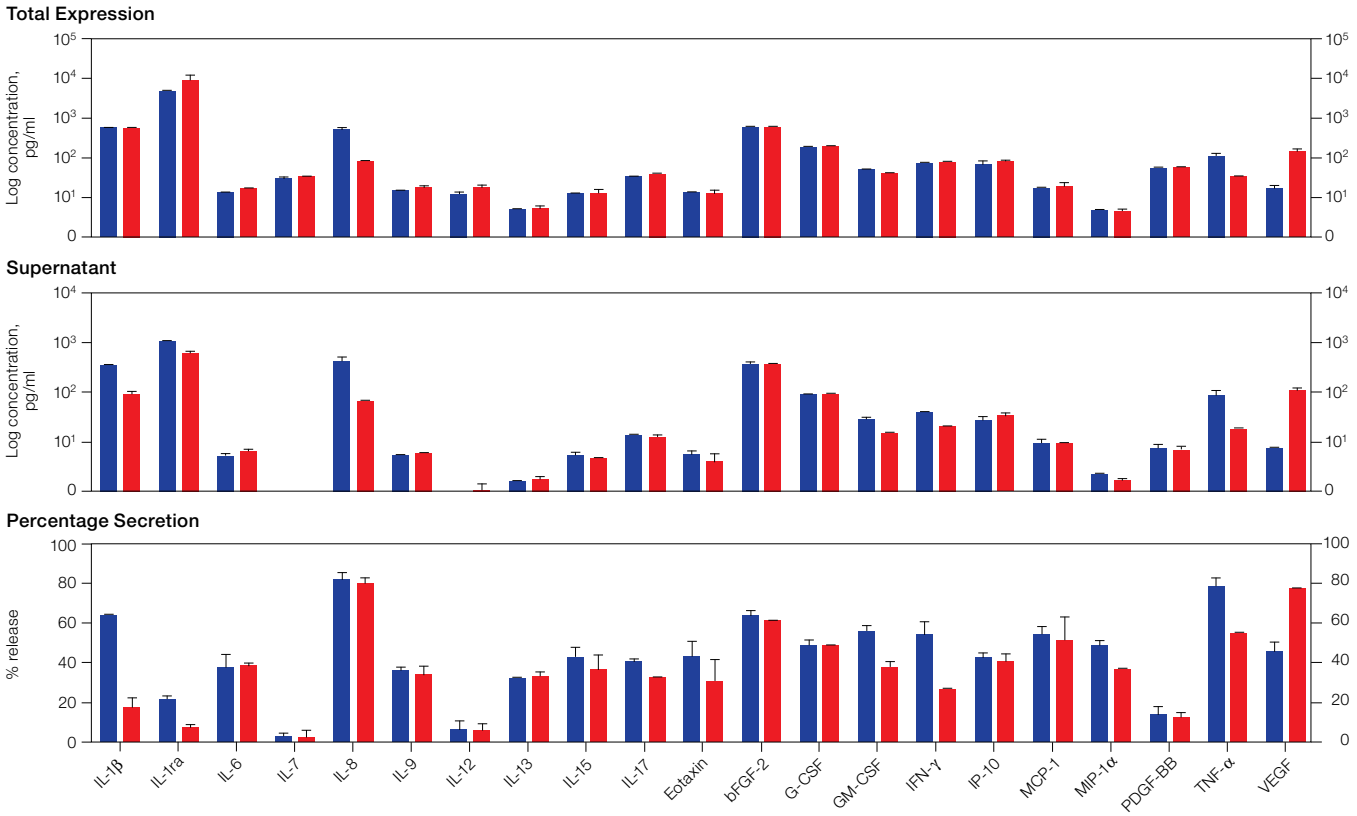

Fig. 3. Caspase-1-dependent cytokine expression and secretion of keratinocytes after UVB irradiation. Cytokines were measured in cell culture supernatants and cell lysates 4 hr after irradiation of keratinocytes transfected with siRNA specific for caspase-1 (■) or VEGF (■) using the Bio-Plex multiplex array system and human cytokine 27-plex panel. Bars represent mean ± standard deviation of values obtained from two different siRNA target sequences per gene.

Cytokine levels from the cell lysate and the supernatant (Figure 3, middle panel) can be used to calculate the percentage of cytokine released by the cells (Figure 3, lower panel). As expected, siRNA-mediated knockdown of caspase-1 leads to a 3-fold reduction of IL-1β secretion after UVB irradiation. Additionally, secretion of IL-1ra, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, MIP-1α, and TNF-α were also reduced by caspase-1 downregulation, however, to a much lower extent. Expression and therefore secretion of several of these proteins are induced indirectly via IL-1β and are therefore only indirectly mediated by caspase-1.

Conclusions

Multiplex cytokine suspension array technology is a powerful tool for identifying cytokines that are secreted from cells under certain conditions. The advantage of this method is the concomitant quantitative measurement of several cytokines from different samples in the same experiment.

Our results show that it is critical to measure cytokine concentrations in both supernatant and cell lysate. This allows the calculations of percentages of secretion, thereby accounting for changes in protein expression.

With our strategy, we could verify that in keratinocytes, caspase-1 only affects unconventional protein secretion and not protein expression in general.

Our approach can be applied to virtually all cell types that can be cultured, and even to tissue cultures. siRNA, specific inhibitors, and knockdown cells can then be used to investigate the impact of genes or cellular processes on protein secretion.

References

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