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Cytokine effects on cell survival and death of A549 lung carcinoma cells

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ABSTRACT

Purpose: IL-13, TNF-α and IL-1β have various effects on lung cancer growth and death, but the signaling pathways mediating these effects have not been extensively analyzed. Therefore, the effects of IL-13, TNF-α and IL-1β alone, and in combination with Fas, on cell viability and death as well as major signaling pathways involved in these effects were investigated in A549 lung carcinoma cells.

Results: Using MTT and flow cytometry, IL-13, TNF-α and IL-1β pretreatment decreased Fas-induced cell death. These anti-cell death effects were attenuated by pretreatment with inhibitors of Nuclear factor-κB [NF-κB], Phoshatidylinositole-3 kinase [PI3-K], JNK, p38 and ERK1/2 pathways.

Results: Using Western blot, IL-13, TNF-α and IL-1β treated cells showed time-dependent expression of p-ERK1/2, p-p38, p-JNK, p-Akt and p-IκBα proteins, decreased IκBα protein expression, no cleavage of Caspase-3 and PARP1 proteins and no notable alterations of Fas protein. IL-13 and TNF-α treated cells showed time-dependent increase of $FLIP_L$ expression.

Conclusion: IL-13, TNF-α and IL-1β attenuate the pro-cell death effects of Fas on A549 cells, at least partially, by pathways involving the NF-κB, PI3-K and MAP kinases, but not by alterations of Fas protein expression. The IL-13 and TNF-α cell survival effects may also be due to increased expression of $FLIP_L$ protein.

1. Introduction

The development and progression of cancer cells involves impairement of the mechanisms controlling cell-cycle and death [1–5]. The cell cycle, proliferation and death of cancer cells may be regulated by cytokines which are secreted by tumor cells and/or immune cells of the tumor microenviroment [6–12].

Lung cancer is one of the most commonly diagnosed malignant tumor and one of the most common causes of cancer-related mortality [13]. Interleukin-13 (IL-13), TNF-α and IL-1β are potent immunoregulatory cytokines which may have various effects on growth and death of normal and cancerous lung cells [14–41].

The A549 human lung carcinoma cell line has been used as a model system to examine cytokine influence on cycle growth and death of lung cancer cells [8,16,18–21,25,27–29,31–35,38,42]. Indeed, TNF-α in combination with flavopiridol resulted in rapid induction of apoptosis whereas pretreatment with TNF-α [10 ng/ml for 3 h] inhibited the apoptosis by the following treatment with flavopiridol in A549 cells [28]. TNF-α treatment for 4 h (25 ng/ml) [36] or for 6 h (10 and 50 ng/ml) [28] did not produce notable A549 cell death, as assessed by flow cytometry and Western blot. However, another study reported that after 6 h exposure to TNF-α (20 ng/ml) there is evidence of A549 cell apoptosis since the cleaved Caspase-3 was detected by Western blot [16]. IL-1β pretreatment (50 pg/ml for 24 h) prevents the propagation of the Fas-mediated death signal in A549 cells which did not show notable changes of Caspase-3 activity after treatment with IL-1β alone [21]. Treatment with IL-13 (0.05–1 nM or 5–10 ng/ml) induced apoptosis on A549 cells after long (72 h) incubation, but the IL-13 effects on cell death were not analyzed in shorter incubation times [18]. This may be interesting since TGF-β1 has dual time-dependent effects on Fas-induced apoptosis in A549 cells [42]. Taken together, the above data indicate that a systematic dose

Abbreviations: interleukin, IL; tumor necrosis factor-α, TNF-α; transforming growth factor, TGF β1; FLICE-like inhibitory protein, FLIP; protein kinase B, Akt; inhibitor of apoptosis, IAP; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha, IκBα; TNF receptor-associated factor, TRAF.

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and time-dependent approach is important to gain further insight in the cell survival/death effects of IL-13, TNF-α and IL-1β alone and in combination with Fas on A549 cells.

On the other hand, IL-13, TNF-α and IL-1β may activate signaling pathways involving nuclear factor-κB [NF-κB], Phoshatidylinositole-3 kinase [PI3-K]/Akt and mitogen-activated protein kinases (MAPK) [p38, extracellular signal regulated kinase (ERK1/2) and c-jun NH2-terminal kinase (JNK)] in lung cells, including A549 cells [7,10,14,15,19–21,25–36,43,44]. Moreover, there is evidence that NF-κB and PI3-K/Akt mediate TNF-α and IL-1β cell survival events on A549 cells, respectively [21,28]. However, to the best of our knowledge, the involvement of NF-κB, PI3-K/Akt, and MAPK signaling pathways in the IL-13 effects on cell viability, cycle and death of A549 cells has not been investigated. Moreover, the involvement of MAPK signaling pathways in the TNF-α and IL-1β effects on cell viability and death of A549 has not been extensively analyzed.

Therefore, we analyzed by flow cytometry, MTT (Methylthiazolyldiphenyl-tetrazolium bromide assay) and Western blot a) the (time and dose-dependent) effects of IL-13, TNF-α and IL-1β alone and in combination with the proapoptotic Fas-crosslinking antibody (clone CH11) [45] on cell viability, cycle and death of A549 cells and b) the involvement of NF-κB, PI3-K/Akt, JNK, p38 and ERK1-2 signaling pathways in these IL-13, TNF-α and IL-1β-induced effects. Moreover, we examined whether the IL-13, TNF-α and IL-1β effects on cell viability and death of A549 cells are mediated by alterations in the expression of proteins involved in cell cycle and apoptosis regulation such as Fas, Cyclin D1, Bcl-2 family, FLIP, c-IAP1 and 2.

2. Materials and methods

2.1. Cell culture

A549 human lung adenocarcinoma cells (CCL-185, ATCC), were cultured in a 37 °C, 5% CO_2 humidified incubator in Ham's F12K medium (Gibco 21127-022), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Gibco 10270-106) and 1% antiobiotic–antimycotic (Gibco 15240-062). A549 cells were passaged at 80–90% confluence using 1× Trypsin-EDTA (Gibco 15400-054).

2.2. Treatment with IL-13, TNF-α, IL-1β, anti-Fas crosslinking antibody and inhibitors

A549 cells were plated at 80% confluence in 6-well tissue culture plates for 24 h in complete medium. Cells were then serum-starved for 24 h and growth arrested A549 cells were either untreated (control) or treated with TNF-α, IL-1β, IL-13 (all from Sigma) and anti-Fas crosslinking antibody (IgM CH11 clone; Millipore) alone or in various combinations, with or without pretreatment with inhibitors. Inhibitors of NF-κB (BAY-117082) and ERK1/2 MAPK (UO126) pathways were purchased from Calbiochem, inhibitors of PI3-K (LY-294002) and p38 MAPK (SB203580) pathways were purchased from Sigma, and inhibitor of JNK (SP600125) pathway was purchased from Alexis Biochemicals. The concentrations of the cytokines and the chemical inhibitors used in the present study were chosen on the basis of previous studies [14,15,20,24–28,30,33,40,41,45].

2.3. Flow cytometry

A549 cells were plated in 6-well tissue culture plates in complete culture medium for 24 h and then sustained growth arrest in serum-free culture medium for 24 h. Cells were stimulated with TNF-α, IL-1β, IL-13 and anti-Fas crosslinking antibody CH11 alone or in various combinations. Adherent cells were trypsinised with CMFH-Trypsin-EDTA [(calcium magnesium-free HEPES), 0.13 M NaCl, 5 mM KCl, 0.6 mM $Na_2HPO_4{:}2H_2O$, 1 mM $NaHCO_3$, 5 mM Glucose, 25 mM HEPES], and pooled with nonadherent cells. Cells were then pelleted by spinning at 100*g*, 4 °C for 5 min. Cell pellets were then resuspended in 200 μl of ice-cold PBS. Cells were fixed by adding 4 ml of ice-cold 70% ethanol and stored at 4 °C in this fixation buffer until ready for analysis (no more than 3 days). Fixed cells were then centrifuged and the pellet was resuspended in 1 ml of PBS pH 7.0. Staining procedure was performed using the Cycle Test™ DNA Reagent Kit (Becton Dickinson) and the samples were analyzed by flow cytometry at an excitation wavelength of 488 nm. The PI fluorescence at >600 nm was collected on Becton Dickinson FACScan. Fixation of cells with precipitating fixatives (such as ethanol) causes the leakage of the cleaved low molecular weight DNA fragments that are produced during cell death. Healthy cells generate a typical cell cycle histogram and the sub-G1 fraction represents the percentage of cell death [46]. For evaluation of the results 10,000 events were required. Experiments were performed in triplicate and the results were expressed as mean values with standard deviations and subjected to statistical analysis.

2.4. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) Assay

A549 cells were seeded in 96-well plates at 5×10^3 cells/well in complete culture medium. After 24 h the medium was changed to serum-free F12K medium for another 24 h before cells were treated with the indicated reagent. After treatment MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma Life Sciences) reagent was added to a final concentration of 0.5 mg/ml and cells were incubated for 4 h in the dark at 37 °C. At the end of incubation, the MTT solution was removed and 200 μl of DMSO were added to each well and mixed thoroughly to dissolve the formazone crystals formed. The viable cells proportion was determined by reading the absorbance at 570 nm with a reference wavelength of 630 nm using an absorbance plate reader and each data-point was measured in triplicate wells. The absorption correlates with the number of viable cells and was normalized to that of untreated controls.

2.5. Cell extracts

Following treatment as described above, adherent cells were trypsinised and pelleted at 100*g* for 5 min at 4 °C, supernatants were discarded and the cell pellets were resuspended in 300 μl of RIPA buffer (Tris–Hcl pH 7.4, 50 mM, NaCl, 150 mM, EDTA 20 mM, Sodium Dexycholate 25 mM, SDS 35 mM, Triton X-100 1%), containing 1 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin, and 4 μg/ml pepstatin. Cell lysates were sonicated 5× for 10 s and then vortexed. Samples were then centrifuged at 16,000*g* for 25 min at 4 °C. The resulting supernatant or cell extract analyzed for protein concentration by the Bradford method (Bio-Rad, Hercules, CA) and stored at −80 °C until further use.

2.6. Western blot

Equal amounts of total cell lysates (50–100 μg) were mixed with Laemmli buffer, denatured by boiling and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) as we previously described [47]. Proteins were then transferred to nitrocellulose membranes (Hybond-C Extra membrane, Amersham Biosciences). All membranes were blocked for 2 h in Phosphate Buffered Saline with 0.05% Tween 20 (PBS-T) with 5% non-fat milk at room temperature. Membranes were then incubated in their respective primary antibody.

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