Analysis of cytotoxicity induced by proinflammatory cytokines in the human alveolar epithelial cell line A549

Mitsuaki Muroya, Kyungho Chang*, Kanji Uchida, Masahiko Bougaki, Yoshitsugu Yamada

Department of Anesthesiology, Faculty of Medicine, The University of Tokyo, Tokyo, Japan.

Summary

Epithelial cell injury under hyperinflammatory conditions is critical in the development of septic acute lung injury (ALI). The aim of the present study is to analyze the cytotoxic effects of a mixture of proinflammatory cytokines in the human alveolar epithelial cell line A549. The cytotoxicity of proinflammatory cytokines were assessed in A549 cells by measuring lactate dehydrogenase released into the culture medium and by crystal violet staining of surviving cells. Activation of the caspase-dependent apoptotic pathway was evaluated by monitoring cleavage of cytokeratin 18 by caspases using enzyme-linked immunosorbent assay (ELISA). To estimate the cytotoxic signaling pathways responsible for epithelial injury, agents with antiinflammatory or antioxidative properties were extensively screened for cytoprotective effects in the inflammation-associated epithelial injury model. The present study revealed that inflammatory cytokines exerted cytotoxicity in A549 cells. A mixture of interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), designated as cytomix, augmented cytotoxicity compared with each individual cytokine. Treatment with glucocorticoid (dexamethasone), tetracycline-derived antiinflammatory antibiotics (minocycline or doxycycline), angiotensin II receptor blockers (losartan or telmisartan), or antioxidants (dimethyl sulfoxide, catalase) attenuated cytomix-induced cytotoxicity, including caspase activation. These results implied that inflammatory cytokines alone could cause alveolar epithelial injury in the pathophysiology of septic ALI. Caspase-dependent apoptosis was speculated to be one mechanism responsible for the cytokine-induced cytotoxicity. Agents with antiinflammatory or antioxidative properties such as glucocorticoid, tetracycline-derived antibiotics, angiotensin II receptor blockers, or direct antioxidants showed substantial effect in attenuating cytokine-induced cytotoxicity and may be candidates for treatment options.

Keywords: Acute lung injury, sepsis, proinflammatory cytokine, epithelial cell injury, apoptosis

1. Introduction

Despite the broad repertoire of potent antibiotics and progress in intensive patient care, sepsis remains a life-threatening condition (1). Multiple organ dysfunctions are responsible for the high mortality rate of septic patients. Pulmonary, renal, cardiovascular and coagulation systems are susceptible to acute injury during septic sequelae and therefore resolution of vital organ dysfunctions is pivotal in management of patients with sepsis (2). Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by hypoxemia and diffuse bilateral infiltrates in the lung (3). Although protective ventilatory strategies have been reported to improve patient survival (4), core information leading to decisive therapeutic intervention is still lacking. The pathophysiological basis of ALI consists of excessive and protracted alveolar inflammation accompanied by alveolar epithelial injury, including epithelial cell death (5-7). Thus, it is critical to clarify
how the hyperinflammatory condition damages alveolar epithelial cells and to explore what pharmacological agents have potential to protect lung tissue.

In this study, we evaluated the cytotoxicity induced by a mixture of proinflammatory cytokines (IL-1β/TNF-α/IFN-γ), which have been suggested to play major roles in sepsis or ALI (3,8-13), in the human alveolar epithelial cell line A549. Then, to clarify the cytotoxic signaling pathways responsible for alveolar epithelial cell injury, various agents with antiinflammatory or antioxidative properties were screened for cytoprotective effects in an in vitro acute lung injury model (9,14-17).

2. Materials and Methods

2.1. Cell culture and reagents

Human lung carcinoma type II epithelium-like A549 cells, purchased from Riken BioResource Center Cell Bank (Tsukuba, Ibaraki, Japan), were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin in 10 cm dishes at 37°C in a humidified atmosphere of 5% CO₂.

Reagents were purchased commercially as follows; human IL-1β, human high-mobility group box 1 (Humanzyme, Chicago, IL, USA); human TNF-α, human interleukin-6 (IL-6) (Prospec, Rehovot, Israel), human IFN-γ (Peprotech, Rocky Hill, NJ, USA); dexamethasone, minocycline, doxycycline, human Fas ligand (Enzo, Plymouth Meeting, PA, USA); losartan, telmisartan (LKT Laboratories, St. Paul, MN, USA); dimethyl sulfoxide (DMSO), catalase-polyethylene glycol (PEG), crystal violet (Sigma, St. Louis, MO, USA).

2.2. Drug treatment

A549 cells were seeded in 24-well tissue culture plates at 5 × 10⁴ cells/well overnight. After cell attachment, the content of FBS in medium was decreased to 2% by medium change. Cells were pretreated for 1 h with agents which were expected to confer cytoprotection and then stimulated with proinflammatory cytokines. To recapitulate the severe inflammatory condition in vitro experiments, stimulation of cultured cells by combination of proinflammatory cytokines, such as IL-1β/TNF-α/IFN-γ (designated as cytokmix for convenience) was employed (15-17). The concentration of these cytokines was mainly 10 ng/mL in the present study. This value was adopted with reference to other reports (15-17) and to our preliminary experiments. In fact, at this concentration, either IL-1β or TNF-α exerted substantial cytotoxicity in A549 cells. Control (ctr) cells were treated with the corresponding vehicle alone. The concentration of vehicle DMSO or ethanol in the medium was kept ≤ 0.2% to minimize the effect of solvents.

2.3. Cytotoxicity of cytokines against A549 cells

The cytotoxicity of cytokines against A549 cells was evaluated after 24 h-60 h of cytokine stimulation depending on the purpose of the experiments. Cell morphologies were observed and photographed under a light microscope. Cytotoxicity was evaluated quantitatively by monitoring lactate dehydrogenase (LDH) concentration in culture medium and by crystal violet staining (18,19). LDH is abundant in the cytoplasm and is released into the culture medium accompanying damage of the cell membrane. Crystal violet solution can promptly fix and stain living cells on culture plates. Thus, LDH concentration reflects the total amount of damaged cells, while crystal violet staining reflects the total amount of surviving cells.

2.3.1. LDH determination

LDH concentration in culture medium was determined using a Cytotoxicity Detection Kit Plus (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The samples were measured spectrophotometrically at a wavelength of 490 nm (with a reference wavelength of 620 nm). After subtracting the background control value (medium only), each sample value was represented in relation to the untreated control value.

2.3.2. Crystal violet staining

After aspiration of culture medium, surviving cells were fixed and stained with 0.5% crystal violet in 95% ethanol for 5 min and then washed with tap water several times. After photographs were taken, 1% sodium dodecylsulfate (SDS) solution was added to each well to elute the blue dye and the absorbance at 595 nm of the eluted samples was measured spectrophotometrically for quantitative evaluation. The values of samples stimulated with cytokmix in the presence of pretreatment drugs were calibrated relative to that of the corresponding control with pretreatment drug only.

2.3.3. Evaluation of caspase activity

To explore the contribution of apoptosis to the cytokine-induced cytotoxicity of A549 cells, caspase activity was investigated. To this purpose, the levels of soluble caspase-cleaved cytokeratin 18 fragments were measured by M30 cyto/death ELISA (Peviva, Bromma, Sweden) according to the manufacturer's instructions. The M30 antibody recognizes a neo-epitope (Asp396) exposed after cleavage of cytokeratin 18 by effector caspase (caspase 3, 6, and 7) in human, monkey, and
bovine epithelial cells (20,21). After subtracting the background control value (empty blank), each sample value was represented in relation to the untreated control value.

2.4. Statistical analysis
Experiments were carried out in triplicate and repeated at least three times. Data are expressed as means ± standard error of mean (S.E.M.). Statistical significance of differences between means was determined with either by Student's t test or by analysis of variance followed by post hoc Tukey test for multiple comparisons. All statistical procedures were performed using Excel 2004 (Microsoft, USA) with the add-in software Statmate™ 2008 (ATMS, Japan). In all analyses, p < 0.05 was taken to indicate statistical significance.

3. Results
3.1. Mixture of proinflammatory cytokines exerted cytotoxicity synergistically in A549 cells
The cytotoxic effects of proinflammatory cytokines were investigated in A549 cells. Cytomix (a mixture of IL-1β/TNF-α/IFN-γ) induced cell death, a decrease in cell number, and morphological changes in A549 cells in a dose dependent manner (Figures 1A and 1B). The cytotoxicity became prominent 48 hours after cytomix challenge (Figure 1C). IL-1β decreased cell survival (Figures 2A and 2B) and significantly induced cell damage (Figure 2C). TNF-α showed similar cytotoxicity, although to a lesser extent than IL-1β at the same concentration. IFN-γ alone failed to show cytotoxicity at this concentration. However, adding IFN-γ to IL-1β/TNF-α significantly augmented the cytotoxicity compared to IL-1β/TNF-α alone. This cytotoxic synergism was thought to reflect the clinical observations in sepsis where multiple cytokines are responsible for biological toxic effects in cooperation (22).

To estimate the role of apoptosis in cytokine-induced cytotoxicity, the activity of caspases was evaluated by measurement of cleaved cytokeratin 18 released into the culture medium. Cytokeratin 18 is a cytoskeleton protein distributed in the cytoplasm of human epithelial cells. It is a substrate of activated effector caspases (caspase 3, 6, 7), and the amount of cleaved form in culture medium

Figure 1. Cytomix induced cell death in A549 cells in a dose-dependent manner. (A) Light microscopic images of A549 cells stimulated with cytomix (CM: a mixture of IL-1β/TNF-α/IFN-γ) for 48 h. Dose dependent decrease in cell number and changes in morphology were observed. The original magnification of the images was 100×. (B) A549 cells were stimulated with cytomix at various concentrations. 1× CM stands for 1 ng/mL each of IL-1β, TNF-α, and IFN-γ. Cell damage was evaluated by monitoring the concentration of LDH in culture medium at 48 hours after cytomix challenge. Cytomix induced cell damage in a dose dependent manner in A549 cells. (C) A549 cells were stimulated with 10× CM. Cell damage was evaluated by LDH measurement at the indicated times after cytomix challenge. The cytotoxicity induced by cytomix became remarkable 48 h after cytomix challenge. Control cells were treated with the corresponding vehicle alone. Bar graph shows means ± S.E.M. * p < 0.01 vs. the corresponding control, † p < 0.01 vs. 24 h, ‡ p < 0.01 vs. 32 h.
is thought to reflect the extent of apoptotic cell death, as explained in the "Materials and Methods". Similar to LDH (Figure 2C), the amount of cleaved cytokeratin 18 was increased significantly by IL-1β or TNF-α but not by IFN-γ alone (Figure 2D). In addition, cytomic showed the most prominent increase in level of cleaved cytokeratin 18.

In addition to IFN-γ, to explore a more appropriate combination of inflammatory mediators, cytotoxicity of other inflammatory mediators, such as Fas ligand, IL-6, or high-mobility group box 1 (HMGB1), was also investigated because these mediators have been reported to play significant roles in sepsis (14,21,23). With contrast to IFN-γ, however, Fas ligand, IL-6, or HMGB1 failed to show cytotoxicity in A549 cells, either alone or in combination with IL-1β/TNFα, up to 100 ng/mL (Figure S1) (http://www.biosciencetrends.com/getabstract.php?id=532).

3.2. Pharmacological attenuation of cytomic-induced cytotoxicity in A549 cells

Next, to search for cytoprotective agents that can suppress the cytotoxicity induced by cytomic, various agents with anti-inflammatory or antioxidative properties were extensively screened using LDH measurements. Preliminary experiments revealed that treatment with glucocorticoid (dexamethasone), tetracycline-derived anti-inflammatory antibiotics (minocycline or doxycycline), angiotensin II receptor blockers (losartan or telmisartan), or antioxidants (dimethyl sulfoxide, catalase) attenuated cytomic-induced cytotoxicity in a dose dependent manner (data not shown and Table S1 (http://www.biosciencetrends.com/getabstract.php?id=532)). On the other hand, pharmacological agents, which failed to attenuate cytokine-induced cytotoxicity in A549 cells, are listed in Table S2 (http://www.biosciencetrends.com/getabstract.php?id=532).

Dexamethasone is one of the most widely used drugs for various inflammatory diseases. For ALI, it has been repeatedly reappraised (24,25) although its clinical validity has not been established. Dexamethasone improved cell survival after cytomic stimulation in A549 cells (Figure 3A). Dexamethasone also markedly attenuated LDH leakage (Figure 3B) and cleavage of cytokeratin 18 (Figure 3C) induced by cytomic stimulation.

Tetracycline-derived antibiotics, such as minocycline
or doxycycline, are known to have pleiotropic effects other than inhibiting growth of microorganisms, including antiinflammatory and cytoprotective properties (17,26,27). Minocycline or doxycycline was shown to attenuate the cytotoxicity induced by cytomix in A549 cells similar to dexamethasone (Figure 3).

Angiotensin II receptor blockade has been shown to confer cytoprotection in cardiovascular tissues subjected to inflammation or ischemia/reperfusion injury (28,29). Some reports have argued that it is also a promising candidate for alleviating the pathophysiology of ALI (30,31). Losartan, the first angiotensin II receptor blocker (ARB) clinically applied, improved cell survival after cytomix stimulation in A549 cells (Figure 5A). In addition, it attenuated LDH leakage and cleavage of cytokeratin 18 induced by cytomix stimulation (Figure 5B).
Figures 5B and 5C). Telmisartan, another established ARB, showed similar cytoprotective effects in cytomix-stimulated A549 cells (data not shown).

Oxidative stress is potentially cytotoxic and has been implicated in the mechanisms of various diseases, including acute disease such as systemic inflammation or ischemia-reperfusion injury (32,33). There are a number of antioxidants with respective spectrum for neutralizing reactive oxygen species. DMSO, an important polar aprotic solvent, has antioxidant properties such as reducing lipid peroxidation (34). Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen (35). These antioxidants attenuated the cytotoxicity induced by cytomix in A549 cells, in terms of cell survival, cell damage, or cleavage of cytokeratin 18 (Figure 6).

3.3. Dexamethasone or losartan still exerted cytoprotection with delayed treatment after challenge of cytomix in A549 cells

Cytoprotective effects of dexamethasone or losartan were tested as therapeutic agents, meaning that drug administration began after cytomix challenge in A549 cells. Dexamethasone (Figures 7A and 7B) or losartan (Figures 7C and 7D) still conferred cytoprotection significantly with delayed administration up to 12 hours after cytomix challenge, although the degree of cytoprotection decreased compared to pretreatment.

4. Discussion

The main findings of the present study can be summarized as follows:

1. A mixture of proinflammatory cytokines (IL-1β/TNF-α/IFN-γ) exerted synergistic cytotoxic effects in the human alveolar epithelial cell line A549.

2. Cytokine-induced cytotoxicity was mediated at least partially by the caspase-dependent apoptotic signaling pathway.

3. Anti-inflammatory agents, such as glucocorticoid (dexamethasone), tetracycline-derived antibiotics (minocycline, doxycycline), angiotensin II receptor blockers (losartan, telmisartan), or antioxidants (DMSO, catalase) showed prominent cytoprotective effects against the cytotoxicity induced by proinflammatory cytokines.

4. Dexamethasone or losartan still exerted cytoprotection following late treatment after challenge of cytomix in A549 cells.

IL-1β and TNF-α are postulated to be the two main proinflammatory cytokines involved in systemic inflammation (7,11). Either of these cytokines alone can induce clinical manifestations, such as hypotension, high fever, chills, and organ dysfunction, representative of sepsis (22). In the present study, these cytokines showed cytotoxicity in A549 cells. Although IFN-γ has not yet been regarded as essential in pathophysiology of sepsis like IL-1β or TNF-α, this study clearly showed that it enhanced the cytotoxicity of IL-1β and TNF-α. In fact, the importance of IFN-γ in the native
immune system has recently attracted a great deal of attention (36). For example, IFN-γ promotes innate immune response by activating macrophage, which plays major roles in eliminating infectious pathogens. IFN-γ enhances immune cell responsiveness to other inflammatory stimuli, such as toll-like receptor ligands and TNF. This phenomenon, termed as “priming”, greatly augments toll-like receptor-induced expression of inflammatory mediators and immune effectors including multiple cytokines and chemokines, and profoundly affects biological outcomes of inflammation.

In a murine model of sepsis induced by cecal ligation and puncture (CLP), late administration of anti-IFN-γ antibody enhanced survival (37). In fact, high levels of IFN-γ has been demonstrated in some populations with clinical systemic inflammation, especially in virus-associated ALI such as SARS (severe acute respiratory syndrome) (8,10). Multiple inflammatory mediators have been proved in the bloodstream and bronchoalveolar lavage fluid of patients with septic ALI (10-13). The observation that a mediator such as IFN-γ, which has minimal direct toxicity against...
parenchymal cells, can exert synergistic cytotoxicity in combination with other mediators implies the complex pathophysiology of sepsis and suggests one reason for the failure of antiinflammatory strategies targeting a single mediator such as IL-1β or TNF-α (38).

Neutrophil recruitment and epithelial injury play pivotal roles in the development of ALI (5-7,21). A number of inflammatory mediators, such as proinflammatory cytokines, lipid mediators, complements, reactive oxygen species or neutrophil-derived proteases such as elastase (39), are postulated to be responsible for loss of epithelial integrity. However, there is little doubt that proinflammatory cytokines make a substantial contribution in initiating the pathological process of septic ALI. Thus, elucidation of the direct link between proinflammatory cytokines and epithelial injury, focusing on molecular mechanisms, is indispensable to understand the progression of septic ALI. The present study confirmed that human alveolar epithelial cells were damaged after challenge by proinflammatory cytokines. Cell death evoked by cytokine challenge was remarkable, and the increase in level of cleaved cytokeratin 18 strongly suggested the substantial involvement of caspase-dependent apoptosis. These results are compatible with other reports arguing that apoptosis pathways are activated in septic ALI experimentally or clinically (5-7,11,40,41).

As it has been established that hyperinflammatory status is the major pathophysiology of septic ALI, modifying inflammation should be highlighted as one of the central strategies for alleviating septic ALI. Although protective ventilatory strategies have been shown to lessen mortality in ALI patients (4), the way of directly downregulating inflammation itself has not been fully examined. In addition, there is increasing evidence that oxidative stress as well as nitrosative...
stress prevails at sites of inflammation as adaptive responses of the host, such as antimicrobial strategies or stress-evoked signal transduction (32,33). As excessive oxidative stress in turn causes tissue damage of host cells, alleviation of oxidative stress can be a promising alternative to controlling inflammation-associated injury in septic ALI (42,43). In the present study, we screened agents with antiinflammatory or antioxidative properties for cytoprotective effects in the in vitro model mimicking alveolar hyperinflammation. We have demonstrated that antiinflammatory agents dexamethasone, tetracycline-derived antibiotics, or ARBs attenuated cytokine-induced cytotoxicity including caspase activation in A549 cells. In addition, antioxidants such as DMSO or catalase similarly attenuated cytokine-induced cytotoxicity. These agents have been referred to as cytoprotective in inflammation-associated conditions (24-35), although clinical validity has not been fully defined, especially in septic ALI. Further investigations are underway to clarify the mechanism of pharmacological attenuation of cytokine-induced cytotoxicity. It is widely accepted that inflammatory stimuli stimulate NF-kappaB pathway and MAP kinase pathway (44). Simultaneously, oxidative stress contributes to epithelial injury because catalase was effective in attenuating inflammation-associated cell death in the present results. Thus, we are now focusing on NF-kappaB pathways, MAP kinase pathways and sources of oxidative stress (such as mitochondria, NADPH oxidase, xanthine oxidase, inducible nitric oxide synthase, or arachidonic acid cascade), as cytotoxic signal pathways evoked by inflammatory stimuli (Figure 8).

The present study has some limitations. First, A549 is a cancer cell line and its intrinsic cell death pathways may differ from those of physiological human alveolar epithelial cells. Second, cytotoxic mediators employed here were IL-1beta/TNF-alpha/IFN-gamma and it is not clear whether this combination is optimal in simulating in vivo hyperinflammation status. Third, the methods used to detect cell death in our experiments were limited. For example, the apoptotic component was estimated based on the level of cleaved cytokeratin 18 and was not confirmed directly by morphological evidence of apoptosis.

However, it is important to check the signaling pathway using human cells as well as using in vitro animal models. It is practically impossible to include all of inflammatory mediators as stimulants. The combination of IL-1beta/TNF-alpha/IFN-gamma has often been adopted to set up hyperinflammation in vitro and accepted in many medical journals (15-17,45-48). In the present study it exerted sufficient cytotoxicity with clinical relevance as discussed earlier. It is reported that in clinical septic patients, numerous cytokines, irrespective pro-, or anti-inflammatory, appear in an overlapping manner as long as inflammatory process continues (49). Thus, we postulate that IL-1beta/TNF-alpha/IFN-gamma is a minimum and appropriate mixture to simulate the status of clinical cytokine storm in vitro system. Lastly, direct confirmation of apoptosis was not the main objective of this study. Apoptosis is now regarded as just one form of cell death patterns in the recent framework explaining the physiological cell death mechanism (6). Thus, evaluation of overall cytotoxicity is more important when differential demonstration of other cell death patterns, such as autophagy, necroptosis, or caspase-independent other programmed cell death, has not been well established yet.

In conclusion, inflammatory cytokines showed synergistic cytotoxic effects on A549 cells. Caspase-dependent apoptosis was speculated to be one mechanism responsible for the cytokine-induced cytotoxicity. Agents with antiinflammatory or antioxidative properties such as glucocorticoid, tetracycline-derived antibiotics, angiotensin II receptor blockers, or antioxidants showed substantial effect in attenuating cytokine-induced cytotoxicity. Further investigations to clarify the mechanisms of their beneficial effects will contribute to exploring new treatment options for ALI.

Acknowledgements

We thank Dr. Fumito Ichinose (Boston, MA, USA) and Dr. Masaomi Nangaku (Tokyo, Japan) for the critical reading of the paper and insightful suggestions. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21592304 to K.C.).

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