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Research Paper

Apoptosis-dependent acute pulmonary injury after intratracheal instillation of angiotensin II

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Abstract: To test the hypothesis that exogenous purified angiotensin II (ANG) might cause apoptosis of alveolar epithelial cells (AECs) and acute lung injury, male Wistar rats were intratracheally instilled with purified ANG (10 μmol/L), ANG plus the caspase inhibitor ZVAD-fmk (60 μmol/L), ANG plus the ANG receptor AT1 antagonist losartan (LOS, 100 μmol/L) or sterile phosphate-buffered saline (PBS) vehicle alone. Six or 20 h later, the lungs were lavaged *in situ* for determination of bronchoalveolar lavage (BAL) fluid content of hemoglobin (Hb) and fluorescent (BODIPY)-albumin, a bolus of which was injected intravenously 15 min prior to BAL. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) revealed that instillation of ANG, but not PBS alone, increased labeling of fragmented DNA in bronchiolar epithelial cells and in AECs (P<0.05) at 6 h post-ANG. Increased TUNEL was abrogated by concurrent instillation of ZVAD-fmk or LOS. Significant increased numbers of caspase-positive cells were observed by anti-caspase 3 immunolabeling after instillation of ANG (P<0.01); the same doses of LOS or ZVAD-fmk that blocked TUNEL also blocked the activation of caspase 3 (P<0.01). Intratracheal instillation of ANG also remarkably increased BAL BODIPY-albumin (P< 0.01) and Hb (P<0.05), both of which were eliminated by ZVAD-fmk or LOS. These data indicate that exposure of AECs to ANG *in vivo* is sufficient to induce apoptosis and alveolar epithelial barrier injury mediated by ANG receptor AT1.

Key words: apoptosis; alveolar epithelial cell; acute pulmonary injury; type II pneumocyte

经气管灌注血管紧张素 II 导致凋亡性急性肺损伤

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摘　要：为研究外源性血管紧张素 II (angiotensin II, ANG)在急性肺损伤和肺泡上皮细胞凋亡中的作用，经气管分别给雄性 Wistar 大鼠(175~200 g)灌注 ANG、ANG 加 caspase 抑制剂 ZVAD-fmk、ANG 加 ANG 受体 1 阻断剂 losartan 和仅灌注磷酸盐缓冲溶液(PBS)。6 或 20 h 后在体灌洗动物肺脏，测定灌洗液中血红蛋白(hemoglobin, Hb)和荧光物质(BODIPY)标定的白蛋白含量(在灌洗前 15 min 静脉注入 BODIPY- 白蛋白)。TUNEL 测定显示，灌注 ANG 6 h 后，支气管和肺泡上皮细胞内标定的 DNA 片断显著增加(P<0.05)；ANG 所致的 DNA 片断增加可被同时灌注 ZVAD-fmk 或 losartan 阻断。灌注 ANG 后免疫标定 caspase 3 阳性细胞数量显著增多(P<0.01)，ZVAD-fmk 或 losartan 同样显著减少 caspase 3 阳性细胞的数量。灌注 ANG 显著增加肺泡灌洗液中荧光标定的白蛋白 (P<0.01)和 Hb 的含量(P<0.05)；ZVAD-fmk 或 losartan 亦显著抑制荧光白蛋白和 Hb 含量的变化。结果表明，肺泡上皮细胞在体暴露于外源性 ANG 足以引起 ANG 受体 1 介导的上皮细胞凋亡和肺泡屏障损伤。

关键词：凋亡；肺泡上皮细胞；急性肺损伤；II 型肺泡细胞
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Evidence from animal models suggests that cellular apoptosis within the alveolar epithelial barrier is an important factor in the pathogenesis of acute injury and alveolar edema formation [1]. As summarized by West [2], interstitial edemas resulting from increased pulmonary capillary permeability will not progress to alveolar flooding unless the alveolar epithelial barrier is also breached. Although the factors that lead to the collapse of the capillary endothelial barrier are well studied, those which lead to collapse of the alveolar epithelium, a barrier that is normally far "tighter" than the endothelial barrier [3], are less well understood.

Previous studies of primary cultures of type II alveolar epithelial cells (AECs) showed that angiotensin II (ANG) induces apoptosis *in vitro* in a dose-dependent manner with maximal stimulation at 10 μmol/L ANG [4]. In subsequent work we found that apoptosis of AECs in response to either Fas activation or to tumor necrosis factor α (TNF-α) requires autocrine synthesis of ANG *de novo* by the target cell [5,6], and that apoptosis of AECs evoked by purified ANG could be abrogated by the ANG receptor subtype AT1 selective antagonist losartan (LOS), at least *in vitro* [7].

Investigations of ANG function in the heart and eye have demonstrated a high degree of compartmentation of ANG production and tissue distribution [8]. Although similar studies of ANG tissue concentrations in the lung have not been performed, available evidence suggests that circulating ANG might reach a proapoptotic concentration for AECs in lung injuries such as acute respiratory distress syndrome (ARDS) [9,10]. On the other hand, a variety of investigations of primary AECs in culture have suggested that some of the behaviors of these cells *in vitro* can be attributed to alterations caused by the stress of cell isolation [11, 12]. Indeed, some authors have suggested that freshly isolated AECs act like injured cells, and may not reflect a "normal" phenotype [13]. The possibility thus exists that the expression by AECs of an ANG receptor-mediated signaling pathway for apoptosis [2] is an artifact of cell isolation.

Early studies [14] reported the contradictory results that the intravenous administration of high dose of ANG to rabbits produced no signs of hemodynamic pulmonary edema accompanied with electron-microscopical finding of alveolar epithelial lesions. However, the mechanism of the pulmonary edema remains unclear until now.

For all these reasons, it is of interest to determine if exposure of AECs to ANG *in vivo* might induce apoptosis detectable *in situ*.

1 MATERIALS AND METHODS

1.1 *Reagents and materials*

Purified ANG was obtained from Sigma Chemical Corp., Saint Louis, MO. The peptide inhibitor of ICE-family caspase, ZVAD-fmk (N-benzylcarboxy-Val-Ala-Asp-[O-Me]-CH2F), was obtained from Kamiya Biomedical, Seattle, WA. Caspase 3 antibody was obtained from R&D Systems, Minneapolis, MN. LOS was a gift from Merck Company, West Point, PA. Fluorescent (BODIPY) bovine serum albumin was purchased from Molecular Probes, Eugene, OR. Reagents for detection of fragmented DNA (apoTACS kit) were obtained from R&D Systems, Minneapolis, MN. Aqueous mounting media was purchased from Southern Biotechnology, Birmingham, AL.

1.2 *Animals and treatments*

Sixty adult male Wistar rats, 175–200 g, were housed in a satellite facility of University Laboratory Animal Medicine, Michigan State University, and randomly divided into 5 groups. Each group of animals were intratracheally (i.t.) instilled with either 300 μL solution of purified ANG, ANG plus the caspase inhibitor ZVAD-fmk (60 μmol/L), ANG plus the AT1 antagonist LOS (100 μmol/L) or an equal volume of PBS vehicle alone for sham animals. The concentration of ANG in the 300 μL instillate was 10 μmol/L; prior checks confirmed that all instillate solutions were at neutral pH 7.3. Control animals received the same anesthesia and injections as being described below, but no i.t. instillations.

1.3 *Induction of acute pulmonary injury and sampling*

A single i.t. instillation was performed according to a published protocol [15] under pentobarbital anesthesia performed by a single intraperitoneal injection at a dose of 45 mg/kg body weight, but without surgery, through a 1.5 mm curved intratracheal cannula introduced via the mouth. The animal breathed freely through the cannula until the actual instillation, which was timed to occur at end-expiration and required no more than 3–4 s. The liquid was followed immediately by 3.0 mL of air to ensure liquid dispersion into the distal airways. Fifteen minutes before animal sacrifice and bronchoalveolar lavage (BAL), all animals were injected intravenously with a 400 μL solution of sterile PBS containing 2×10^5 mFU fluorescent (BODIPY) bovine serum albumin (~50 μg protein). Six or 20 h after the instillation, a tracheal cannula was installed surgically 10 min prior to BAL for collection of bronchoalveolar lavage fluid (BALF). Two lavages of 6.0 mL each in sterile PBS were gently instilled and collected by gravity. The recovered amount of BALF was always over 90% of the injected fluid volume.

The BALF was immediately centrifuged to collect cells for lysis and assay of hemoglobin (Hb) spectrophotometrically at 415 nm [16]. The supernatant was stored at –20 °C until required for assay of BODIPY fluorescence. Following BAL, all animals were sacrificed and the lungs were carefully excised and were immediately weighed to calculate the lung index expressed as the percentage of lung wet weight (mg) *versus* body weight (g), then were fixed by i.t. infusion of 4% paraformaldehyde in PBS at 20 cm H_2O constant pressure for 5 min, the trachea was ligated just distal to the cannula to retain fixative, and the lungs were then immersed in the same fixative for 2 h followed by storage at 70% ethanol. The fixed tissues were washed with PBS three times for 15 min and were then embedded in paraffin. Five micron sections of the lungs were processed by standard methods [15].

Induction of inflammation by ANG was evaluated by hematoxylin-eosin staining of the sections through counting the numbers of leukocytes, which was performed by randomly selecting eight 400× microscopic fields per slide from each of at least 6 animals of the control and ANG-treatment groups. Quantitation of inflammatory cells in lung tissue was expressed as numbers of cells per 100 square micrometers.

1.4 *Detection of apoptotic cells in lung section samples*

Terminal deoxynucleotidyl transferase-mediated Biotin-dUTP nick-end labeling (TUNEL) assay of fragmented DNA was applied to lung tissue samples, which acted as a catalyst to aid in the covalent adhesion of a Biotin-dUTP nucleotide with DNA that was fragmented due to apoptosis. Chromogen-avidin interacted with the nucleotide, which caused blue end-labeling of the nuclei. TUNEL was performed with a detection kit according to the manufacturer's instructions. Briefly, the lung section samples were deparaffinized by passing through a series of xylene concentrations, a series of 3 concentrations of ethanol. Following deparaffinization, the sections were covered with proteinase K solution at 22 °C for 20 min and subsequently immersed the slides in quenching solution for 5 min at 22 °C. After the slides were incubated with labeling reaction mixture for 1 h at 37 °C in a humidified chamber, they were incubated in anti-BrdU for 1 h at 37 °C, and then incubated in a streptavidin-HRP solution for 10 min at 22 °C. Finally the slides were covered with TACS blue labeling solution for 10 min at 22 °C. A faint blue coloration of the nuclei may be observed under microscope. The lung sections were counterstained with eosin, and then mounted with aqueous mounting media. The prepared sections were photographed under transmitted light on an Olympus BH2 epifluorescence microscope with automatic photographic equipment.

For quantitation of apoptotic epithelial cells, the number of positive nuclei was counted in a minimum of six randomly selected 400× microscopic fields per sample. The counts of positive nuclei per field were expressed as the percentage of the total number of nuclei in the same field; this analysis was applied to each of at least 6 animals per treatment group.

1.5 *Immunohistochemistry (IHC) for activated caspase 3*

Assay of caspase 3 activity was performed with an antibody that recognizes only the active form of caspase 3. Deparaffinized lung sections were blocked with a solution of 3% bovine serum albumin in PBS for 1 h at 22 °C. Following the deparaffinization, the primary antibody was then applied and incubated for 45 min at 37 °C. After washing in PBS, the antibody was detected with a biotin-conjugated and avidin-linked chromogen system. The active form of caspase 3 appeared purple colour under microscope. The method for quantitation of TUNEL-positive cells was used for quantitation of caspase 3-positive cells.

1.6 *Determination of alveolar epithelial barrier injury and statistical analysis*

Lung permeability and cellular injury after i.t. instillation of ANG was assessed by analysis of the flux of fluorescent (BODIPY)-albumin, administered i.v. in the tail vein, from the vascular to alveolar compartment. After collection of BALF and centrifugation to remove cells, the entire supernatant was lyophilized and resuspended at ×50 dilution in water, and the BODIPY fluorescence was determined on a Biotek FL600 fluorescence plate reader (Winooski, VT, USA). To determine whether the labelled albumin was crossing the alveolar-capillary barrier intact, the reconstituted BAL supernatants were then passed through PD-10 desalting columns (Pharmacia). Over 90% of the BAL BODIPY fluorescence was eluted in the PD-10 eluant fractions, corresponding to protein rather than free BODIPY (data not shown).

The amount of Hb in BALF was measured as another indicator of lung permeability and epithelial injury. After centrifugation of BALF, the cell pellet was resuspended in 500 μL of distilled water to lyse erythrocytes, and a 50 μL of aliquot was taken and Hb concentration in the lysate was determined at A_{415}. Systolic blood pressure was determined by the tail cuff method with the RTBP2000 system (Kent Scientific, Litchfield, CT). Statistical comparisons were performed by ANOVA followed by Student-Newman-Keul's *post hoc* analysis.

2 RESULTS

2.1 TUNEL of fragmented DNA

The single concentration of 10 μmol/L ANG was tested for the ability to induce AEC apoptosis *in vivo* after i.t. instillation. Detection of fragmented DNA by TUNEL revealed significant increased numbers of TUNEL-positive cells, particularly near alveolar ducts, 6 h after i.t. ANG (Fig. 1*B*). At higher magnification (Fig. 1*C*), many of the labeled cells were found in location consistent with alveolar epithelial type II cells (arrows). Foci of labeled cells were often observed in close association with intra-alveolar erythrocytes (Fig. 1*B* and *C*, arrowheads).

Figure 2 displays quantitation of the TUNEL-positive cells. Intratracheal instillation of the vehicle (PBS) alone did not induce detectable apoptosis, but 10 μmol/L ANG increased the apoptotic index of alveolar wall by 4.5-fold when measured at 6 h after the instillation. More than 70% of TUNEL-positive cells were localized within the surface of alveolar walls. By 20 h after delivery of ANG, TUNEL had returned to the control value, presumably by a process involving epithelial repair.

2.2 The active form of caspase 3

Six or 20 h later, immunohistochemistry for the active form of caspase 3 was performed on lung sections and was quantitated (Fig. 1*D–F*). Control uninstilled lungs (Fig. 1*D*) revealed occasionally positive cells. Six hours after i.t. instillation of ANG, significant increased numbers of caspase-positive cells (Fig. 1*E* and *F*, arrowheads) were observed. At higher magnification, many labeled cells were found in "corners" of the surface of alveolar walls (Fig. 1*E* and *F*, arrowheads), consistent with the location of alveolar epithelial type II cells. Over 90% of caspase-positive cells were observed inside the alveolar walls.

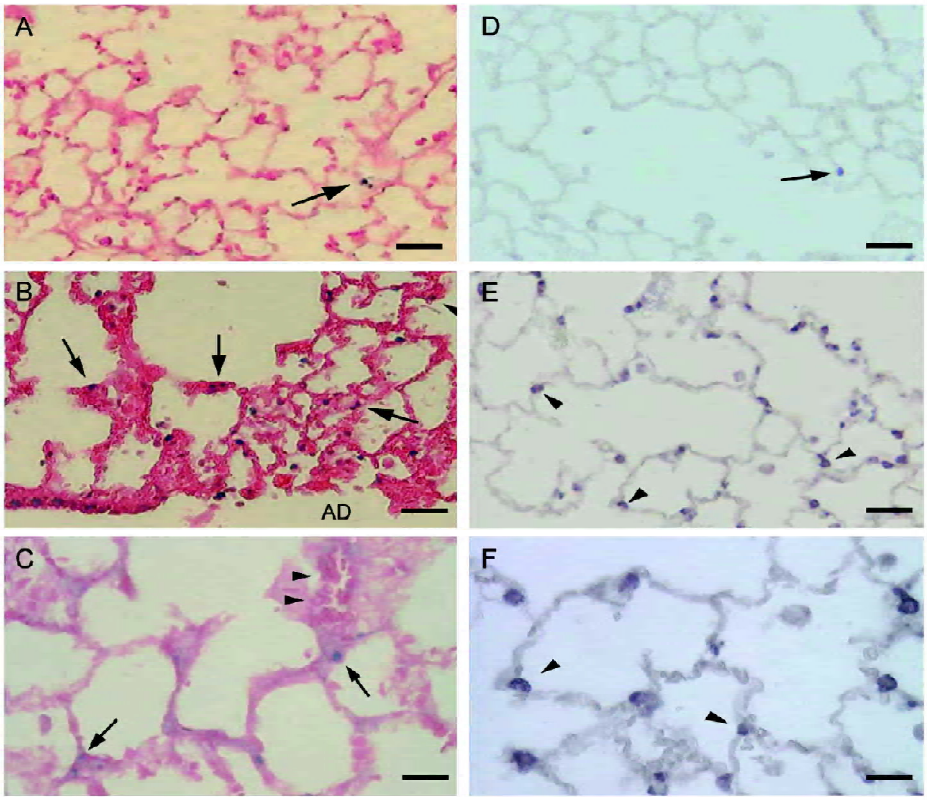

Fig. 1. Histology and detection of apoptosis *in situ* after intratracheal (i.t.) instillation of angiotensin II (ANG). At the indicated times after instillation, 5 μm lung sections were subjected to TUNEL and eosin counterstain (*A-C*), and immunohistochemistry for the active form of caspase 3 (*D-F*). Control uninstilled lungs (*A* and *D*) revealed occasional positive cells. Six hours after i.t. instillation of ANG (*B* and *E*), significant increased numbers of TUNEL-positive (*B*, arrows) and caspase 3-positive cells (*E*, arrowheads) were observed, especially in regions adjacent to alveolar ducts (AD). At higher magnification (*C* and *F*), many labeled cells were found in "corners" of the surface of alveolar walls (arrows in *C* and arrowheads in *F*), consistent with the location of alveolar epithelial type II cells. The TUNEL-positive alveolar wall cells were often found in close association with focal hemorrhage, revealed by intra-alveolar erythrocytes (arrowheads in *C*). Scale bar: *A* and *D*=100 μm; *B* and *E*=50 μm; *C* and *F*=25 μm.

2.3 Effects of losartan and ZVAD-fmk on apoptosis

Coadministration of ANG, and either the broad spectrum caspase inhibitor ZVAD-fmk or the angiotensin receptor subtype AT1-selective antagonist LOS eliminated the TUNEL results (Fig. 2*A*) in both airway epithelial and alveolar wall cells at 6 h post-ANG. Caspase 3 activity (Fig. 2*B*) *in vivo* was also blocked by the same concentration of ZVAD-fmk or LOS.

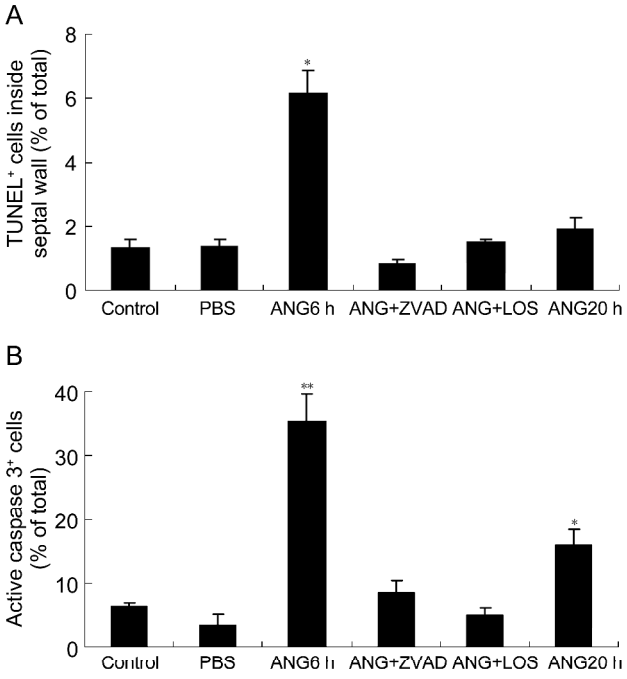

Fig. 2. Blockade of ANG-induced TUNEL and caspase 3 activity *in vivo* by a caspase inhibitor or by an ANG receptor antagonist. TUNEL (*A*) or immunohistochemistry for the active form of caspase 3 (*B*) was performed on lung sections from rats treated with the indicated compounds and was quantitated. PBS: phosphate buffered saline, the instillation vehicle; ANG: angiotensin II (10 μmol/L in PBS); ZVAD: broad spectrum caspase inhibitor *N*-benzylcarboxy-Val-Ala-Asp-[O-Me]-CH2F (60 μmol/L); LOS: selective ANG receptor AT1 antagonist losartan (100 μmol/L). See Figure 1 for images of labeling. Bars are the mean±SEM of at least 6 observations; $^{*}P<0.05$ *vs* PBS and ANG6 h, $^{**}P<0.01$ *vs* PBS.

2.4 Quantitation of fluorescent (BODIPY)-albumin in BALF

To assess the possibility that ANG-induced apoptosis might lead to alveolar edema, rats i.t. instilled with ANG were injected intravenously with fluorescent (BODIPY)-albumin 15 min prior to the 6 h post-instillation time point. At 6 h, the lungs were lavaged *in situ* for determination of BODIPY-albumin flux from the vascular to alveolar compartments. As shown in Fig. 3*A*, i.t. instillation of ANG significantly increased BODIPY-albumin recoverable in the lavage ($P<0.01$), but instillation of the PBS vehicle alone did not. By 20 h after instillation of ANG, BODIPY-albumin level had returned to the control. Gel filtration analyses showed that the fluorescence recovered in the lavage was bound to protein rather than free BODIPY (data not shown).

2.5 The relative amount of erythrocytes in BALF

As an additional measure of acute lung injury, the relative amount of erythrocytes entering the alveolar compartment was estimated by spectrophotometric assay of lavage Hb content obtained from lysed BAL cells. In Fig. 3*B*, instillation of ANG, but not PBS alone, significantly increased lavage Hb content ($P<0.05$). At 20 h post-ANG, Hb content had returned to the control level.

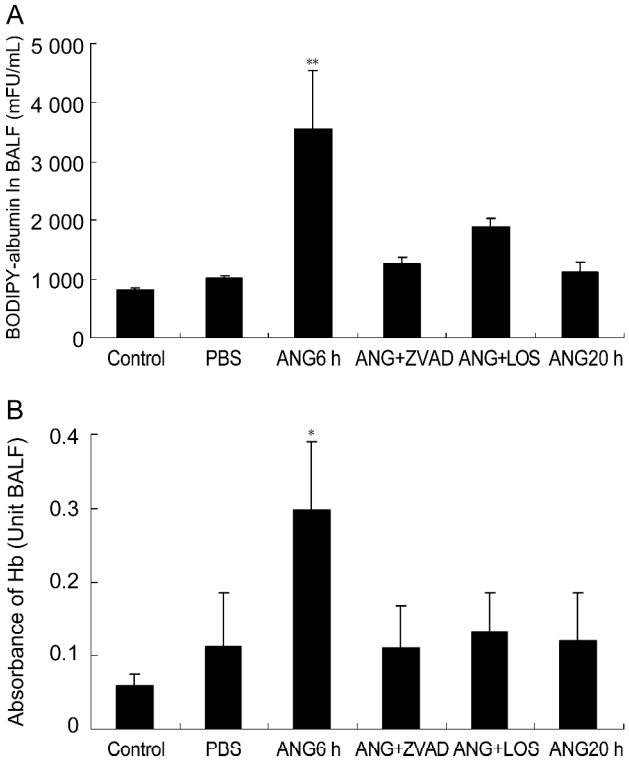

Fig. 3. Induction of alveolar edema and microhemorrhage by i.t. instillation of ANG and blockade by a caspase inhibitor ZVAD-fmk or by an ANG receptor antagonist losartan. At the indicated times after instillation of a 300 μL solution of the indicated compounds, the lungs were lavaged *in situ*. *A*: The lavage content of BODIPY-albumin (in fluorescence units or mFU). *B*: The lavage content of hemoglobin (Hb) determined by absorbance at 415 nm after hemolysis. BALF, bronchoalveolar lavage fluid. See legend to Figure 2 for definitions of other abbreviations. Bars are the mean±SEM of at least 6 observations. $^{*}P<0.05$, $^{**}P<0.01$ *vs* PBS.

2.6 Blockade of contents in BALF by LOS and ZVAD-fmk

Coadministration of the inhibitor of caspase (ZVAD-fmk) or ANG receptor antagonist LOS, which blocked ANG-induced apoptosis, and ANG reduced the increases in lavage BODIPY-albumin (Fig. 3*A*) and lavage Hb (Fig. 3*B*) caused by ANG to a value not significantly different from the control. Neither ANG nor ZVAD-fmk had any measurable effect on systolic blood pressure measured 1 h after i.t. instillation (data not shown).

2.7 Morphological observation

Gross examination of the whole lungs for both ANG-treated animals and the controls did not appear apparent difference visible to the naked eye, including the colour and focal lesions. Although the lung indexes of ANG administered rats showed an increasing tendency, there was no statistical significance between the experimental group (4.58 ± 0.20) and control group (6.12 ± 0.21). At the light-microscopic level we did not recognize remarkable thicker alveolar septa in the ANG-treated sections than that in the control samples. Higher magnification revealed that the only inflammatory cells in alveolar airspace were macrophages, and neutrophils and lymphocytes were in alveolar walls. No statistical differences were proved in the each number of macrophages, neutrophils and lymphocytes between the ANG-treated lung tissue samples and the controls (data not shown).

3 DISCUSSION

In the normal lung, AECs are likely not in direct contact with ANG produced within the vasculature due to the extremely short half-life of ANG in the plasma (approximately 30 s) [17] and the intact alveolar-capillary barrier [18]. Although studies of primary cultures of type II pneumocyte showed that ANG induces apoptosis *in vitro* in a dose dependent manner [4], it has been speculated that primary isolated AECs behave like 'injured' cells, and can exhibit artifactual behaviour *in vitro* resulting from the stress of cell isolation. On these bases it was hypothesized that purified ANG, administered intratracheally, might induce AECs apoptosis and concomitant damage to the alveolar epithelial barrier. This expectation depends, however, on the presumption that AECs *in vivo* constitutively express the same ANG-dependent apoptosis signaling pathway expressed by primary cultures of AECs *in vitro* [4, 7].

Although Gil *et al.* [14] found that ANG-induced pulmonary edema accompanied with electron-microscopical finding of alveolar epithelial lesions, there is no report to our knowledge, on the mechanism or the relationship between lung injury and apoptosis of AECs, especially in signaling of alveolar epithelial apoptosis.

Significant increased numbers of TUNEL-positive cells and cells containing the active form of caspase 3 were revealed 6 h after i.t. instillation of the single concentration of 10 μmol/L ANG. The data of Fig. 1 and 2 are consistent with the hypothesis that direct exposure of AECs to exogenous ANG is sufficient to induce apoptosis inhibitable by blockade of caspase (by ZVAD-fmk). It has been determined that there are both of the two major subtypes of the ANG receptor, AT1 and AT2 in AECs by analyses of total RNA using RT-PCR technique [4]. In agreement with Papp's report that apoptosis of cultured AECs in response to ANG is mediated by receptor subtype AT1 but not AT2 *in vitro* [7], we found that the ANG receptor AT1-selective antagonist LOS abrogated ANG-induced apoptosis of AECs as well as the increase in the BALF contents 6 h post ANG administration, which indicates that the mechanism of the induction of alveolar cell apoptosis by ANG *in vivo* just as it can be *in vitro* [7]. Exogenous ANG directly acts on AECs which are directly towards the airspace.

The majority of TUNEL-positive and caspase 3-positive cells were localized within the surface of the alveolar walls, and often found in the corners of the walls, a location consistent with alveolar type II pneumocyte [2]; furthermore, foci of TUNEL-positive cells were often observed with intra-alveolar erythrocytes. These findings imply that apoptosis of AECs is involved in pulmonary epithelial barrier injury. The real permeability barrier in pulmonary alveolar wall is alveolar epithelial layer [2,3]. AECs have critical roles in homeostasis of lung epithelial barrier and in the repair of epithelial damage after lung injury [19]. Despite being a few of the AEC population, the alveolar type II cell is a reparative cell and rapidly proliferates following epithelial cell injury. Excessive induction of cell suicide in the epithelium would be likely to cause the destruction of lung barrier integrity associated with permeability edema [19]. Apoptosis detectable at 6 h post instillation was resolved by 20 h, a time sufficient for epithelial repair by cell proliferation [20]; this finding may reflect the short biological half-life of the ANG peptide [8]. Regardless, the induction of the apoptosis by 6 h is consistent with the work of Hagimoto *et al.* [21], who found that apoptosis induced by i.t. instillation of bleomycin also was detectable by 6 h post instillation.

More importantly, we discovered that the epithelial

apoptosis induced by i.t. instillation of ANG was associated with increased passage of BODIPY-albumin and leakage of erythrocytes from the capillary to the alveolar compartments, and focal microscopical hemorrhage, which could consistently be blocked by inhibition of ANG-induced apoptosis. These results are consistent with the hypothesis that apoptosis of AECs may be sufficient to promote disruption of alveolar epithelial barrier function, which means the leakage of the vascular content to the alveolar space caused by the injury is secondary to the apoptotic death of certain lung cell types; the increased albumin flux and erythrocytes leakage were due, at least in part, to apoptosis of AECs. Together with the investigation that high dose of ANG evoked no signs of hemodynamic pulmonary edema but with the findings of alveolar epithelial cell lesions, the finding that induction by i.t. instillation of ANG could lead to alveolar permeability edema supports the premise that induction of apoptosis of AECs by ANG is sufficient to cause lung epithelial barrier collapse, at least transiently. In other words, we could say the apoptosis of AECs resulted in the destruction of the barrier integrity, which in turn caused hyperpermeability, in an apoptosis-dependent manner, in the alveolar septa; it is theoretically reasonable that the relationship between alveolar epithelial apoptosis and lung barrier injury should be a kind of causality. That i.t. instillation of ANG did not significantly alter the recovery rate of BALF and no apparent appearances of pathological edema were observed under the light-microscopic level suggests that i.t. administration of 300 μL solution of ANG (10 μmol/L) might cause apoptosis-dependent alveolar micro-edema without remarkable flooding.

In other system such as the gut epithelium, barrier function is surprisingly resilient in the face of ongoing apoptosis [22]. However, the data in Fig. 3 indicated that the apoptosis induced by i.t. instillation of ANG was associated with alveolar epithelial barrier injury and focal micro-hemorrhage, both of which could be eliminated by inhibition of ANG-induced apoptosis. Although the normal plasma level of ANG is much lower than the concentration (10 μmol/L) used in this study [23], the plasma ANG level is known to rise significantly in ARDS [9]. Moreover, we have shown that physiologically relevant concentration of Fas ligand or TNF-α cause the alveolar epithelial cell itself to produce ANG, to which it responds in an autocrine fashion [5, 6].

That light-microscopic observation of the HE-stained sections did not show induction of inflammation by ANG implies a key role for AEC apoptosis in the integrity of alveolar wall barrier function and ANG, at least the dose in this study, not to induce severe inflammatory infiltrates like bleomycin [16].

Together, these data support the theory that apoptosis of AECs within the epithelial barrier *in vivo*, mediated by exogenous ANG, is sufficient to result in pulmonary epithelial barrier lesions and leakage of vascular contents into the alveolar compartment. Successful blockade of this leakage by the caspase inhibitor ZVAD-fmk supports the contention that the apoptosis is a causal factor in the micro-edema formation. Further, the blockade of albumin and erythrocyte flux into the alveolar compartment by the AT1-selective antagonist LOS suggests that ANG-induced apoptosis of AECs is mediated by ANG receptor subtype AT1 *in vivo* as it is *in vitro* [8].

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