ADAR1 Is Involved in the Development of Microvascular Lung Injury

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Abstract—Deamination of adenosine on pre-mRNA to inosine is a recently discovered process of posttranscription modification of pre-mRNA, termed A-to-I RNA editing, which results in the production of proteins not inherent in the genome. The present study aimed to identify a role for A-to-I RNA editing in the development of microvascular lung injury. To that end, the pulmonary expression and activity of the RNA editase ADAR1 were evaluated in a mouse model of endotoxin (15 mg/kg IP)–induced microvascular lung injury (n=5) as well as in cultured alveolar macrophages stimulated with endotoxin, live bacteria, or interferon. ADAR1 expression and activity were identified in sham lungs that were upregulated in lungs from endotoxin-treated mice (at 2 hours). Expression was localized to polymorphonuclear and monocyctic cells. These events preceded the development of pulmonary edema and leukocyte accumulation in lung tissue and followed the local production of interferon-γ, a known inducer of ADAR1 in other cell systems. ADAR1 was found to be upregulated in alveolar macrophages (MH-S cells) stimulated with endotoxin (1 to 100 μg/mL), live Escherichia coli (5×10^7 colony-forming units), or interferon-γ (1000 U/mL). Taken together, these data suggest that ADAR1 may play a role in the pathogenesis of microvascular lung injury possibly through induction by interferon. (Circ Res. 2001;88:1066-1071.)

Key Words: ADAR1 ■ adult respiratory distress syndrome ■ endotoxin ■ interferon ■ RNA editing

The adult respiratory distress syndrome (ARDS) is a known complication in critically ill patients leading to acute respiratory failure and mortality.1 This pulmonary condition emanates from an intense, localized inflammatory response, which includes the production of multiple inflammatory proteins such as cytokines, chemokines, adhesion molecules, NO, prostaglandins, and platelet aggregating factor (for review see Reference 2). Thus, protein production in the lung plays a key role in the development of ARDS, and additional insights into the mechanisms of peptide expression can enhance our understanding of the pathogenesis of this grave syndrome.

According to the traditional paradigm of protein production, nucleus-derived mRNA (pre-mRNA) is further processed in the cytoplasm by capping, splicing, and polyadenylation to become mature mRNA, which is then translated into specific proteins. Recently, it has been shown that pre-mRNA is also subjected to posttranscription modification by deletion, addition, or modification of nucleotides.3,4 This process, termed RNA editing, can lead to the production of protein isomers not encoded in the genome or to the suppression of some functional proteins. Such downstream actions of RNA editing could have a profound impact on cell function5,6 and on protein-mediated inflammatory processes such as ARDS.

One of the few editing events reported so far involves site-selective deamination of adenosine to inosine in cellular pre-mRNA with the resultant production of I-mRNA.7–9 Several editases including double-stranded (ds) RNA–dependent adenosine deaminase (ADAR1),10 ADAR2,11 ADAR3,12 and ADAR13 have been shown to mediate this process; of these, ADAR1 has been emphasized the most. ADAR1 is known to be inducible by the inflammatory mediators interferon (IFN)–γ14 and IFN-α.14 To catalyze the editing reaction, ADAR1 requires a dsRNA structure, which engulfs the “to-be-edited” mRNA.15–17

To date, the functional consequences of RNA editing have been reported only in neuronal cells. In these cells, site-specific A-to-I editing of the glutamate receptor subunit B mRNA, which codes for glutamate receptors in the central nervous system, altered calcium influx, and electrical properties.4,18–24 No information is available regarding the function of RNA editing in inflammation. The present study aimed to investigate whether A-to-I RNA editing plays a role in the inflammatory events that lead to ARDS. Specifically, the expression and activity of the RNA editase ADAR1 was monitored in lungs from sham and endotoxin-infused animals as well as in cultured alveolar macrophages stimulated with endotoxin, live bacteria, or IFN.
Materials and Methods

Materials

Endotoxin
Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma) was used as a fresh solution in 0.9% NaCl.

*Escherichia coli*

*E. coli* strain DH5α was incubated on Luria Broth Base medium for 16 to 24 hours at 37°C on a rotary shaker (200 revolutions/mL), and the optical density of bacterial cell suspension was monitored with a spectrophotometer at 600 nm. Incubation was terminated when the optical density600 of the bacterial cells reached 1.0, which reflected 5×10⁸ colony-forming units (CFU). Serial dilutions were made from the bacterial suspension, and 50 μL of each dilution were plated in triplicates on Luria Broth Agar plates. The number of colonies was counted after incubation of these plates for 12 to 16 hours at 37°C and the total number of viable bacterial cells calculated.

Mouse Alveolar Macrophages

Mouse alveolar macrophages (MH-S cell line, American Type Culture Collection) were cultured in 100-mm-diameter tissue culture dishes with RPMI 1640 containing 10% FBS and 0.5 μmol/L mercaptoethanol. Cells were subcultured 8 hours before stimulation, typically with 60% of confluence.

Animal Experiments

**Animals**

All experiments and animal care procedures were approved by the Yale Animal Resource Center and were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

C57BL/6 mice (20 to 30 g) were purchased from The Jackson Laboratory (Bar Harbor, Me). All animals were housed until the time of experiments in standard cages, with access to food and water ad libitum in a temperature-controlled room (22°C) with a 12-hour dark/light cycle.

**Experimental Design**

Conscious mice were injected with LPS (15 mg/kg IP), and lungs were removed at 1, 2, 4, 6, 8, 16, or 20 hours (n=5) and stored at −70°C for evaluation of lung myeloperoxidase activity, ADAR1 mRNA, ADAR1 activity, and tissue IFN level or were immediately taken for determination of wet lung weight (see below).

In Vitro Experiments

**Endotoxin- or IFN-Induced ADAR1 Expression in Mouse Alveolar Macrophages**

Mouse alveolar macrophages (MH-S cell line, American Type Culture Collection) were cultured as described above. When cell growth reached 70% confluence, cells were challenged with LPS at 1, 10, or 100 μg/mL and harvested at 0, 2, 4, 8, or 16 hours after stimulation. In another experiment, cells were stimulated with IFN-γ (1000 U/mL) and harvested at 10, 20, 30, 40, 50, and 60 minutes. Total RNA was reversed transcribed using oligo(dT) as the primer and Superscript (Life Technologies) at 42°C.

**E. coli–Induced ADAR1 Expression in Mouse Alveolar Macrophages**

Mouse alveolar macrophages (MH-S cell line) and *E. coli* cells were cultured as described above. When MH-S cells reached 70% confluence, the culture medium was replaced with medium containing serial dilutions of *E. coli* (1×10⁷, 5×10⁶, 1×10⁶, 1×10⁵, and 1×10⁴ CFU) prepared from suspension of optical density600 of 1.0 (5×10⁸ CFU). After coincubation for 2 hours at 37°C, the culture medium was removed and each dish was rinsed 3 times with PBS. One milliliter of TRIzol solution was added to each dish, and the cell lysates were transferred to 1.5-mL Eppendorf tubes for total-cell RNA purification. In another set of experiments, MH-S cells were incubated with *E. coli* (5×10⁷ CFU) for 0.25, 0.5, 0.75, 1.0, or 1.5 hours. Thereafter, total-cell RNA was purified and reverse transcriptase–polymerase chain reaction (RT-PCR) was performed to quantify the transcription products of mouse ADAR1. The transcription expression of the mouse GAPDH gene was measured as internal control in all experiments.

**Pulmonary Water Content**

This was determined as previously described.

**Lung Myeloperoxidase Activity Assay**

This was determined as previously described.

**Northern Blot**

Removed tissues were put into liquid nitrogen and total RNA and mRNA isolated according to the manufacturer’s protocol (Qiagen). After quantification by UV spectrum and agarose gel, equal amounts of mRNA (~4 μg) were resolved on denatured agarose gel and transferred to nitrocellulose membrane. ADAR1 mRNA was detected by hybridization of the membrane with a synthetic probe (1305-1265, GenBank accession No. AF291050). The membrane was hybridized at 65°C overnight and washed with 0.1× SSC at 55°C for 10 minutes. β-Actin was used as an internal control to normalize ADAR1 mRNA level.

**Quantitative RT-PCR**

Two micrograms of total RNA was used for reverse transcription using poly(dT)12-18 as primer. Partial gene of ADAR1 from exons 5 to 8 was amplified by PCR (primers, 1975-2003 and 2436-2408; GenBank accession No. AF291050). Samples were taken at 18, 20, 22, 24, and 26 cycles; analyzed on agarose gel; and semi-quantified by scanning. The relative expression level of ADAR1 mRNA in comparison with β-actin was calculated and used to determine the induction of ADAR1 during inflammation.

**Lung ADAR1 Activity Assay**

**Whole-Cell Extract Preparation**

Lungs were removed from mice at 0, 2, 4, 8, and 16 hours after endotoxin stimulation and immediately frozen in liquid nitrogen. The frozen lungs were transferred to a mortar and reduced to powder in liquid nitrogen. All further procedures were performed at 0°C, as follows. The powdered tissue was transferred to an Eppendorf tube and 2 volumes of hypotonic buffer (containing, in mmol/L, Tris-HCl 10, MgCl₂ 1.5, KCl 10, DTT 0.5, and PMSF 0.2, and 0.6% Nonidet P-40) was added. The sample was broken up with ultrasonic cell disruptor (Versonic 475, VirTis Co), and a similar volume of high-salt buffer (containing, in mmol/L, Tris-HCl [pH 7.8] 20, MgCl₂ 1.5, PMSF 0.2, DTT 0.5; KCl 1200; and 25% glycerol) was added. The sample was sonicated again and centrifuged at 8000 rpm for 8 minutes, and the supernatant was dialyzed against dialysis buffer (containing, in mmol/L, HEPES [pH 7.8] 10, KCl 80, and EDTA 50, as well as 15% glycerol). Aliquots were stored at −80°C.

**Editing Activity**

Synthetic dsRNA substrate was prepared by in vitro transcription using pBluescriptSK(+) vectors containing a gene of α-tropomyosin. The plasmids were linearized with either EcoRI or HindIII and then transcribed with T7 RNA polymerase, respectively, resulting in complementary transcripts, which were purified through a Sephadex G25 column. The RNA transcripts generated with T7 polymerase were labeled with [α-32P]ATP (3000 Ci/mmol, Amer sham). The [32P]-labeled dsRNA substrate was formed by annealing complementary single-stranded transcripts in a TNE buffer (containing, in mmol/L, Tris-HCl [pH 7.4] 10, NaCl 100, and EDTA 1) by heating at 94°C for 3 minutes and then slowly cooling to room temperature.

One microliter of [32P]ATP-labeled dsRNA was mixed with 10 μL of whole-cell extract (100 μg protein), 20 units of RNase inhibitor, and 8.5 μL of dialysis buffer with a total reaction volume of 20 μL. After incubation at 30°C for 1 hour, an equal volume of proteinase K solution (300 mmol/L NaCl, 1% SDS, and 20 μg of proteinase K)
was added to stop the reaction. The dsRNA substrate was extracted with phenol:chloroform followed by ethanol precipitation. Precipitated RNA was suspended in 10 μL of nuclease P1 buffer (25 mmol/L sodium acetate, pH 5.3, with 1 unit of nuclease P1; Sigma) and digested for 2 hours at 37°C. 5'-IMP and 5'-AMP were resolved from each other by thin-layer chromatography (TLC) on a cellulose plate (Aldrich-Sigma) in a solvent consisting of saturated (NH₄)SO₄, 100 mmol/L sodium acetate (pH 6.0) and propanol (79:19:2). Autoradiography was performed for 10 to 16 hours at 2°C with an intensify screen. Quantification was performed by measuring the radioactivity of excised TLC spots using a liquid scintillation system (1900TR, Packard Instrument Company).

Lung IFN-γ ELISA
Lung IFN-γ was measured using a “sandwich” ELISA as previously described. 24

In Situ Hybridization
This was performed as previously described. 26

Data Analysis
Data in text and figures are mean±SEM. One-way ANOVA followed by Student-Newman-Keuls test was used for multiple comparisons among groups. Chi-square test was used for analysis of mortality data. A P value of <0.05 was considered significant for both tests.

Results

Lung Injury
The intraperitoneal administration of endotoxin induced pulmonary edema, which peaked at 6 hours after injection (Figure 1A). This was preceded by leukocyte accumulation in lung tissue at 1 hour (Figure 1B) and by local production of IFN-γ at 30 minutes (Figure 2). All responses persisted for the entire 20-hour observation period.

Upregulation of Lung ADAR1 mRNA In Vivo
ADAR1 expression was observed in control animals. Endotoxin administration upregulated lung ADAR1 expression as

Figure 1. Endotoxin-induced pulmonary edema (A) and leukosequestration (B). Data are mean±SEM (n=5). *P<0.05 vs sham controls. MPO indicates myeloperoxidase.

Figure 2. Endotoxin-induced production of lung IFN. Data are mean±SEM (n=5). *P<0.05 vs baseline value.

Figure 3. Upregulation of ADAR1 in lungs from endotoxin-treated mice. Animals (n=6) were given endotoxin bolus (15 mg/kg IP) and lungs were harvested at 1, 2, 4, 6, 8, 16, and 20 hours. Northern blots were performed on 5 μg brain mRNA (B); 15 μg total lung RNA (T); 5 μg lung mRNA from sham control (C); and lung mRNA at 2, 4, 8, and 16 hours after LPS injection. Note the 2 transcripts of ADAR1, the presence of which is in agreement with a previous report in rat lungs, and the pronounced induction of ADAR1 mRNA as early as 2 hours after endotoxin administration. β-Actin was used as an internal control.

Figure 4. Upregulation of ADAR1 activity in lungs from endotoxin-treated mice. Animals (n=6) were given endotoxin bolus (15 mg/kg IP) and lungs harvested at 1, 2, 4, 6, 8, 16, and 20 hours. Lungs were extracted (100 μg protein), incubated for 1 hour with 5'-monophosphate adenosine and inosine generated from synthetic oligoribonucleotide duplex, and analyzed by TLC (A). Percentages of A-to-I conversion calculated from cpm values of recovered IMP and AMP from TLC plate are presented in panel B. Note the increased activity as early as 2 hours after endotoxin administration, which was sustained throughout the experiment.
Upregulation of Lung ADAR1 Activity In Vivo
ADAR1 activity was observed in lungs from control animals. Endotoxin increased lung ADAR1 activity as early as 2 hours after infusion, which was sustained at all tested time points (Figure 4).

Cellular Localization of Lung ADAR1
Using in situ hybridization, no ADAR1 was identified in sham mouse lungs hybridized with sense (Figure 5A) or antisense (Figure 5B) ADAR1 RNA probe. In contrast, a strong positive signal was detected in lung cells from endotoxin-challenged mice hybridized with ADAR1 antisense RNA probe, identified as neutrophils and monocytes (Figure 5D). No ADAR1 signal was observed in lungs hybridized with the negative control ADAR1 sense RNA probe (Figure 5C).

Upregulation of Lung ADAR1 In Vitro

Endotoxin-Induced Expression
ADAR1 expression was detected in nonstimulated cultured alveolar macrophages (Figure 6). Endotoxin at 1 and 10 μg/mL significantly induced further ADAR1 expression at 4 and 2 hours after stimulation, respectively, which persisted at all tested time points. There was also induction of ADAR1 expression after stimulation with 100 μg/mL of endotoxin, although to a lesser degree.

E. coli-Induced Expression
Stimulation of cultured alveolar macrophages with increasing doses of live E. coli yielded a monophasic bell-shaped response, which peaked at 1:100 dilution (Figure 7A). Incubation of cultured alveolar macrophages with E. coli for 15
minutes resulted in ADAR1 upregulation. ADAR1 expression was more prominent after incubation for 30 and 60 minutes (Figure 7B) and was not observed after 90 minutes of incubation time.

**IFN-Induced Expression**

IFN (1000 U/mL) upregulated ADAR1 expression from cultured alveolar macrophages as early as 10 minutes after stimulation (Figure 8).

**Discussion**

The most significant finding of this study is that the expression and activity of the RNA editase ADAR1 is upregulated in lungs subjected to endotoxin-induced microvascular injury and in alveolar macrophages stimulated with endotoxin, live bacteria, or IFN. These findings implicate for the first time RNA editing as a possible inflammatory event. Specifically, the data suggest that A-to-I RNA editing is involved in the pathogenic mechanisms that lead to acute lung injury. Another significant finding is that ADAR1 is expressed in sham mouse lungs, which was previously demonstrated only in the rat.

The sequence of early production of IFN, a known inducer of ADAR1, followed by ADAR1 expression and development of microvascular lung injury, suggests that A-to-I RNA editing may be a proximal event in the inflammatory cascade involved in the pathogenesis of microvascular lung injury. Furthermore, it is conceivable that early induction of pulmonary IFN during the inflammatory process could be the mechanism, which triggers enhanced RNA editing activity. This is supported by the demonstration of IFN-induced ADAR1 expression in alveolar macrophages in vitro (Figure 8), as well as in several other cell systems. For example, IFN stimulation of human amnion U cells has been shown to increase the steady-state level of mRNA encoding the dsRNA-specific adenosine deaminase as measured by Northern blot analysis. A single major dsRNA-specific adenosine deaminase transcript of ≈6.7 kb was detected; the transcript was induced by both IFN-α and IFN-γ. Likewise, Western immunoblot analysis revealed that a 15-kDa protein recognized by antiserum prepared against recombinant dsRNA-specific adenosine deaminase was increased in the human amnion U and neuroblastoma SH-SY5Y cell lines treated with IFN-α or IFN-γ.

The pulmonary expression of ADAR1 was localized by in situ hybridization to inflammatory cells including neutrophils and monocytes. This is the first demonstration in these cells of ADAR1, which has been previously identified only in the cultured U cell line. Of special importance is the expression of ADAR1 in polymorphonuclear cells, which migrate in the circulation into areas of inflammation. Thus, ADAR1 expression could be a generalized phenomenon in inflamed tissues.

The role ADAR1 plays in the development of microvascular lung injury is still obscure. Nevertheless, it is conceivable that on appropriate stimulation upregulated ADAR1 is acting on yet-unknown mRNAs with the resultant modification of inflammatory protein production. Furthermore, as ADAR1 targets and unwinds dsRNA, it is possible that this enzyme regulates other dsRNA-dependent regulatory proteins by controlling intracellular dsRNA levels. One such protein is PKR, a known modulator of both I-kB and eIF2α, which affect transcription and translation, respectively.

In summary, this paper presents preliminary evidence that ADAR1 is involved in the pathogenesis of microvascular lung injury. Additional investigations are needed to further delineate the role of A-to-I RNA editing in this inflammatory stress situation.

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**References**

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