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Interferon-\(\beta\) attenuates lung inflammation following experimental subarachnoid hemorrhage

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Abstract

Introduction: Aneurysmal subarachnoid hemorrhage (SAH) affects relatively young people and carries a poor prognosis with a case fatality rate of 35%. One of the major systemic complications associated with SAH is acute lung injury (ALI) which occurs in up to one-third of the patients and is associated with poor outcome. ALI in SAH may be predisposed by neurogenic pulmonary edema (NPE) and inflammatory mediators. The objective of this study was to assess the immunomodulatory effects of interferon-\(\beta\) (IFN-\(\beta\)) on inflammatory mediators in the lung after experimental SAH.

Methods: Male Wistar rats were subjected to the induction of SAH by means of the endovascular filament method. Sham-animals underwent sham-surgery. Rats received IFN-\(\beta\) for four consecutive days starting at two hours after SAH induction. After seven days, lungs were analyzed for the expression of inflammatory markers.

Results: SAH induced the influx of neutrophils into the lung, and enhanced expression of the pulmonary adhesion molecules E-selectin, inter-cellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 compared to sham-animals. In addition, SAH increased the expression of the chemokines macrophage inflammatory protein (MIP)-1\(\alpha\), MIP-2, and cytokine-induced neutrophil chemoattractant (CINC)-1 in the lung. Finally, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) was significantly increased in lungs from SAH-animals compared to sham-animals. IFN-\(\beta\) effectively abolished the SAH-induced expression of all pro-inflammatory mediators in the lung.

Conclusions: IFN-\(\beta\) strongly reduces lung inflammation after experimental SAH and may therefore be an effective drug to prevent SAH-mediated lung injury.
SAH on inflammatory mediators in secondary organs including the lung are still unknown.

Current therapy is mainly supportive since no effective drugs are available to treat NPE. Mechanical ventilation (MV) is necessary in the majority of patients to assure adequate oxygenation. However, MV can also contribute to lung injury, called ventilation-induced lung injury (VILI) [10]. A staggering 40% of SAH patients on MV develop VILI [11].

Interferon-β (IFN-β) is a small protein with immunomodulatory properties that has been approved for treatment of multiple sclerosis. It has been shown that IFN-β decreases pro-inflammatory cytokines and inhibits the migration of lymphocytes across the blood-brain barrier by decreasing the expression of chemokines and adhesion molecules on endothelial cells [12].

The aim of the present study was to investigate whether experimental SAH contributes to lung inflammation. Moreover, we examined the effects of IFN-β treatment on the expression of inflammatory mediators in the lung associated with brain injury in a rat model of SAH.

Materials and methods

Animals

The experiments were performed in accordance with international guidelines and approved by the experimental animal committee of the Academic Biomedical Center Utrecht. Male Wistar rats (weighing 320 to 350 g) were obtained from Harlan CPB (Horst, The Netherlands) and randomly assigned to the different treatment groups. Both the executers of the experiments and of the statistical analysis were blind for randomization.

Experimental SAH-model

Rats were intubated under gaseous anesthesia (65% air/33% oxygen/2% isoflurane) and mechanically ventilated for a maximal 90 minutes in a pressure controlled time-cycled mode, at a fractional inspired oxygen concentration (FiO₂) of 0.5, inspiration to expiration (I/E) ratio of 1:1 and peak inspiratory pressure of 10 cmH₂O. To maintain normocapnia, the respiratory rate was set at 55 breaths per minute. The left external carotid artery (ECA) was ligated and cut, while the ipsilateral internal carotid artery (ICA) was ligated and temporarily clipped. A sharpened 4.0 prolene suture was introduced through an opening in the ligated left ECA and distally advanced through the ICA until the suture perforated the intracranial bifurcation of the ICA. In sham-animals, the suture was withdrawn prior to perforating the ICA. The presence of subarachnoidal blood was confirmed with magnetic resonance imaging.

Animals were treated for four consecutive days with subcutaneous injections of 1.75 × 10⁶ U/kg IFN-β (U-Cytech, Utrecht, The Netherlands) (SAH-animals: N = 6; sham-animals: N = 4) or saline (SAH-animals: N = 7; sham-animals: N = 4) starting at two hours after SAH. Treatment dosage and time of treatment were based on previous results of Veldhuis et al. where IFN-β proved to be clinically efficacious by reducing the influx of inflammatory cells into the brain and reducing infarct volume in a rat model of ischemic stroke when started up to six hours after the induction of stroke [13]. Since we wanted to initiate treatment as soon as possible, but with respect to a realistic clinical moment at which patients are admitted to a hospital, we decided to start treatment at two hours after the induction of stroke.

Preparation of tissue homogenates

Lungs were removed at seven days post-SAH. Tissues were pulverized using a liquid nitrogen-cooled mortar and pestle and stored at -80°C for further analysis.

Myeloperoxidase (MPO) assay

MPO activity was determined as described previously [14]. Briefly, pulverized tissues were homogenized in 50 mM HEPES buffer (pH 8.0), centrifuged and pellets were rehomogenized in H₂O/0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 μM citrate buffer (pH 5.0)/0.22% CTAC. Substrate solution containing 3 mM 3’,5,5’-tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, Steinheim, Germany), 120 μM resorcinol (Merck) and 2.2 mM H₂O₂ in distilled water was added. Reaction mixtures were incubated for 20 minutes at room temperature and stopped by 4 M H₂SO₄, followed by determination of optical density at 450 nm. MPO activity of a known amount of MPO units (Sigma-Aldrich) was used as reference.

Quantitative real-time reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from pulverized tissues with TRizol® reagent (Invitrogen, Paisley, UK). cDNA was synthesized from total RNA with SuperScript Reverse Transcriptase kit (Invitrogen). Quantitative real-time RT-PCR reaction was performed with iQ5 Real-Time PCR Detection System (Biorad, Hercules, CA, USA) using rat primers for TNF-α, macrophage inflammatory protein (MIP)-1α, MIP-2, cytokine-induced neutrophil chemoattractant (CINC)-1, E-selectin, inter-cellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1. Data were normalized for expression of internal controls, β-actin and GAPDH.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). All parameters were evaluated by one-way ANOVA with Bonferroni’s post hoc test.
Results

Stability of the model
SAH was induced at Day 0 in male Wistar rats and treated for four consecutive days with IFN-β. At Day 7, placeto-
treated rats showed a case fatality rate of 50%, whereas
IFN-β-treated animals showed a case fatality rate of 63%,
although this difference was not statistically significant. All
animals that did not survive the protocol died within the
first three days after the induction of SAH.

IFN-β inhibits the influx of neutrophils into the lung
As a parameter for the number of infiltrating neutro-
phils we determined myeloperoxidase (MPO)-activity in
total lung homogenates at Day 7 post-SAH. SAH-an-
imals had a significant increase in MPO-activity com-
pared to sham-animals (P < 0.01), which could be
efficiently blocked by IFN-β (P < 0.01; Figure 1a).

IFN-β inhibits the expression of endothelial activation
markers and pulmonary chemokines
Because neutrophil influx is dependent on the activation
of endothelial cells, we studied the effect of SAH on
endothelial adhesion molecules in lung tissue. Compared
to sham-animals, SAH increased pulmonary expression
of E-selectin (P < 0.01), inter-cellular adhesion molecule
(ICAM)-1 (P < 0.001), and vascular cell adhesion mole-
cule (VCAM)-1 (P < 0.01). IFN-β normalized the
expression of E-selectin (P < 0.01), ICAM-1 (P < 0.01)
and VCAM-1 (P < 0.01) compared to saline-treated
SAH-rats (Figure 1b-d).

To establish whether the attraction of neutrophils is
correlated with the expression of chemokines, we next
questioned whether SAH induced the expression of pul-
monary macrophage inflammatory protein (MIP)-1α,
MIP-2, and cytokine-induced neutrophil chemoattract-
tant (CINC)-1. All chemokines were significantly
increased in lungs from SAH-rats (P < 0.05). Treatment
with IFN-β completely abolished the SAH-induced
upregulation of the chemokines MIP-1α (P < 0.01),
MIP-2 (P < 0.01), and CINC-1 (P < 0.05) (Figure 1e-g).

IFN-β reduces pulmonary TNF-α expression
To determine whether SAH induced pro-inflammatory
cytokine expression in the lung, we measured TNF-α
expression. SAH resulted in a significant three-fold
increase in pulmonary TNF-α expression compared to
 sham-animals (P < 0.01). IFN-β treatment significantly
decreased the SAH-induced TNF-α expression to the
levels observed in IFN-β treated sham-animals
(P < 0.05; Figure 1h).

Discussion

We report here that SAH induces the influx of neutro-
phils into the lung and expression of pulmonary adhesion
molecules, chemokines, and TNF-α. More importantly,
we are the first to show that IFN-β effectively abolishes
the SAH-induced expression of all pro-inflammatory
mediators in the lung.

SAH rats showed a case fatality rate of 50% after seven
days. The mortality observed by us is common in
experimental SAH-models and resembles other findings
[15-17]. Treatment with IFN-β resulted in a case fatality
rate of 63%, although this increase did not statistically
differ from the placebo group.

Neutrophils play an essential role in the development
of lung inflammation. We observed a four-fold increase
in MPO, indicating that neutrophils are recruited to the
lung following SAH. The influx of neutrophils involves a
complex cascade of events. The early response cytokine,
TNF-β, most probably initiates the inflammatory
response by activating the endothelial cells resulting in
increased chemokine expression and upregulation of
adhesion molecules [18]. In support of this concept we
showed increased expression of pulmonary TNF-α
following SAH. In addition, SAH induced enhanced pul-
monary expression of the endothelial adhesion mole-
cules E-selectin, ICAM-1, and VCAM-1, which play a
pivotal role in the rolling across the endothelium and
firm adhesion of neutrophils to the endothelium, respec-
tively [18]. Furthermore, we observed marked SAH-
induced upregulation of the chemokines MIP-1α,
MIP-2, and CINC-1, which are responsible for the
cytotactic activity of neutrophils. Therefore, we conclude
that SAH induces a pro-inflammatory environment in
the lung which may represent an important risk factor
for the development of NPE.

Although several groups reported the occurrence of
lung injury after brain injury, the underlying mechan-
isms are largely unknown. It has been suggested that
increased intracranial production of pro-inflammatory
cytokines results in the release of systemic pro-inflam-
matory mediators, thereby promoting secondary organ
injury [8]. Although this is a tempting hypothesis, we
show here de novo synthesis of pro-inflammatory me-
diators, suggesting that spill-over of cytokines is not the
primary cause of lung inflammation following SAH. A
second proposed mechanism underlying secondary
organ injury is increased capillary permeability elicited
by catecholamines due to sympathetic nerve stimulation
after brain injury [7]. Catecholamines can activate the
SAH was induced in male Wistar rats. Rats were treated with IFN-β for four consecutive days starting at two hours after the induction of SAH. Seven days post-SAH total lung homogenates were prepared and analyzed for inflammatory markers. (a) MPO activity corrected for the amount of protein. (b-d) Expression of endothelial activation markers E-selectin, ICAM-1, and VCAM-1. (e-g) Expression of chemokines MIP-1α, MIP-2, and CINC-1. (h) Expression of TNF-α. (b-h) Data are normalized for the expression of β-actin and GAPDH. Sham: N = 4; Sham/IFN-β: N = 4; SAH: N = 7; SAH/IFN-β: N = 6. *** P < 0.001, ** P < 0.01, * P < 0.05; SAH vs sham or SAH/IFN-β vs SAH. MPO, myeloperoxidase; MIP, macrophage-inflammatory protein; CINC, cytokine-induced neutrophil chemoattractant.
transcription factor NFκB in macrophages thereby promoting the production of TNF-α chemokines, and adhesion molecules [19,20]. Therefore, we propose that sympathetic activation of the lung could have led to the local release of cytokines and chemokines in our model.

The major finding of our study was that IFN-β treatment strongly attenuates the SAH mediated pulmonary inflammation. The decreased influx of neutrophils in response to IFN-β administration was accompanied by decreased expression of TNF-α, chemokines, and adhesion molecules in the lung. Although this is an interesting finding, one should keep in mind that IFN-β therapy may also worsen bacterial pneumonia [21], although until now only one case-report has been published. However, the increase in incidence of bacterial pneumonia was only described for a situation in which long-term treatment with IFN-β was applied [22]. IFN-β is a potent immunomodulator with diverse effects. Several studies have shown that IFN-β reduces the migration of inflammatory cells across the blood-brain barrier [12]. This is likely accomplished by reducing the expression of endothelial adhesion molecules, ICAM-1 and VCAM-1, and by downregulating the production of chemokines [23,24]. Although the immunomodulatory effects of IFN-β have been described in brain-related diseases including multiple sclerosis and focal cerebral ischemia, we are the first to describe the modulatory effects of IFN-β in pulmonary inflammation. The exact mechanisms by which IFN-β attenuates SAH-induced lung inflammation need to be further clarified. In our study, IFN-β was administered systemically; therefore it may be possible that IFN-β had a direct effect on pulmonary cells. For example, Kiss et al. showed that IFN-β treatment ameliorated vascular leakage in ALI via upregulation of 5’-nucleotidase (CD73) on pulmonary endothelial cells [25]. Although we did not look at CD73, it could be a possible explanation for our findings. IFN-β could also have an indirect effect by inhibiting either the activation of sympathetic nervous system or reducing the systemic pro-inflammatory environment and subsequently preventing the upregulation of pro-inflammatory mediators in the lung. Although we did not measure catecholamines, we could not see an effect of IFN-β treatment on TNF-α levels in the blood (data not shown). Finally, IFN-β could have a direct effect on cerebral inflammatory responses after SAH, thereby indirectly regulating the lung inflammation. Our preliminary data do not confirm this hypothesis since IFN-β did not have any effect on the SAH-induced cerebral inflammation (manuscript in preparation, Tiebosch et al.)

In conclusion, SAH induces a pro-inflammatory environment in the lung, which can be efficiently blocked by IFN-β. Therefore, our data strongly suggest that IFN-β may be an attractive clinical candidate to prevent SAH mediated lung inflammation.

Conclusions
We show here that subarachnoid hemorrhage (SAH) results in the upregulation of pro-inflammatory mediators in the lung as well as recruitment of neutrophils into the lung. In addition, we report that treatment with IFN-β completely abolishes the SAH-induced pulmonary inflammation. Our data imply that SAH is associated with pulmonary inflammation and that IFN-β may be an attractive therapeutic candidate to prevent SAH-mediated lung inflammation.

Key messages
- Subarachnoid hemorrhage in rats is associated with increased pulmonary inflammatory mediators.
- Subarachnoid hemorrhage results in increased neutrophil influx into the lung.
- IFN-β treatment completely abolishes the subarachnoid hemorrhage-induced pulmonary inflammation.

Abbreviations

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Authors’ contributions
PC performed the experimental work, interpreted the results and drafted the manuscript. IT and RZ performed the experimental work and were responsible for critical review of the manuscript. PM participated in study design and was responsible for critical review of the manuscript. RD, CH, JK and WB supervised the study, were involved in interpreting the results and correcting the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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