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Simvastatin Reduces Burn Injury-induced Splenic Apoptosis via Down-regulation of the TNF- α / NF- κ B Pathway

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Abstract

Objective—Recent studies have suggested that epidermal burn injuries are associated with inflammation and immune dysfunction. Simvastatin has been shown to possess potent anti-inflammatory properties. Thus we hypothesized that simvastatin protects against burn-induced apoptosis in spleen via its anti-inflammatory activity.

Methods—Wild-type, tumor necrosis factor alpha knockout (TNF- α KO) and NF- κ B KO mice were subjected to full-thickness burn injury or sham treatment. The mice then were treated with or without simvastatin and the spleen were harvested to measure the extent of apoptosis. Expression levels of TNF- α and NF- κ B were also determined in spleen tissue and serum.

Results—Burn injury induced significant splenic apoptosis and systemic cytokine production. Simvastatin protected the spleen from apoptosis, reduced cytokine production in the serum and increased the survival rate. Simvastatin decreased burn-induced TNF- α and NF- κ B expression in spleen and serum. TNF- α and NF- κ B KO mice demonstrated lower levels of apoptosis in spleen in response to burn. Simvastatin did not further decrease burn-caused apoptosis and mortality in either strain of KO mice.

Conclusions—Simvastatin reduces burn-induced splenic apoptosis via down-regulation of the TNF- α / NF- κ B pathway.

Keywords

burn; simvastatin; spleen; apoptosis; TNF- α ; NF- κ B

Introduction

Burn patients are at high risk for infection.¹ Burn injury causes significant immune dysfunction, which might be attributable to burn-induced apoptosis in immune organs.^{2,3} As

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Type of study: This is an original study to elucidate how simvastatin reduces burn-induced splenic apoptosis. We used simvastatin to treat burned mice to investigate its effects on splenic apoptosis. We found simvastatin protected mouse spleen from apoptosis by decreasing TNF- α and NF- κ B expression.

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

the thymus degenerates with age in humans, the spleen plays a pivotal role in human immune defenses. This splenic dysfunction is recognized as an important factor in burn-associated infections.⁴ However, it is not clear whether severe burn trauma causes sufficient apoptosis in spleen that could result in its dysfunction.⁵

Simvastatin is a cholesterol-lowering agent that reduces local and systemic inflammatory responses through unknown mechanisms⁶ Previous data has suggested that simvastatin blocks the activities of many important proinflammatory cytokines,^{7,8} such as TNF- α and NF- κ B. These cytokines are found to be actively involved in apoptosis.⁹ However, it is unknown whether simvastatin decreases apoptosis in the spleen.

Tumor necrosis factor- α (TNF- α) is a potent inflammatory cytokine and is produced by many cell types, such as macrophages and CD4⁺ lymphocytes.¹⁰ Upon interaction with its ligand, TNF- α activates NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway that is involved in apoptosis.¹¹ The primary role of TNF- α is in the regulation of immune cells, and is able to induce apoptosis.¹²

NF- κ B is an important down stream component of TNF- α and controls DNA transcription.¹³ NF- κ B plays a key role in regulating the immune response to infection. Upon activation of either T or B-cell receptors, NF- κ B is able to enter the nucleus to upregulate genes involved in T/B-cell maturation, proliferation and apoptosis.¹⁴ NF- κ B is found in almost all cell types and can be activated by apoptotic stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and oxidized LDL.¹⁵ Additionally, NF- κ B controls the expressions of many genes involved in inflammation.¹⁶ We hypothesize that burn injury induces the expression of TNF- α and NF- κ B, which may cause apoptosis in the spleen, and simvastatin inhibits burn-induced apoptosis by suppressing the expression of the TNF- α /NF- κ B pathway.

MATERIALS AND METHODS

Materials

Ketamine and Xylazine were obtained from Henry Schein (Melville, NY). In Situ Apoptosis Detection kit was purchased from Millipore (Temecula, CA). Anti-TNF- α antibody, anti-NF- κ B antibody, anti-GAPDH antibody and NF- κ B ELISA kit were obtained from cell signaling (Danvers, MA). TNF- α , IL-1 α , MCP-1 (Monocyte Chemoattractant Protein-1) and CRP (C-reactive protein) ELISA Kits were from Abcam (Cambridge, MA).

Animals

Wild type C57BL/6 mice, TNF- α KO and NF- κ B KO mice were obtained from Jackson Laboratory (Bar Harbor, Maine). The knockout mice were fertile, viable and displayed no physical or behavioral abnormalities.

All procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Massachusetts General Hospital, and complied with the Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011).

Burn injury in mice

General anesthesia was induced (Ketamine 40mg/kg and Xylazine 5 mg/kg body weight, I.P) prior to the burn injury. Thirty percent total body surface area (TBSA) on the back was shaved and exposed to a 90°C water bath for 9 seconds along a template. The mice received 1.5 mL of saline for resuscitation by immediate intraperitoneal injection after burn injury. Sham control mice were treated in the same manner except being exposed to a room-temperature water bath. Immediately following burn injury, the animals in the statin-treated group were injected with 150µg/kg of simvastatin intraperitoneally, and two additional doses were administered at 8 and 16 hours later. The burned animals and sham burn animals received the same volumes of saline at the same dosing times.

Survival Analysis

In our study, the mortality of the mice subjected to 30% TBSA burn was only 3–5%. Thus, to assess the protective activity of simvastatin on burned mice, we increased the severity of injury by burning 30% TBSA at 90 °C for 9 seconds on the dorsum plus and additional 20% TBSA burn to the ventral skin(the resuscitation fluid prevented visceral organ injury). This combined burn (50%TBSA) produced very high mortality on the day of injury that increased with time after injury. The wild-type and knockout mice were injured with or without simvastatin treatment at 150µg/kg intraperitoneally, three times a day, for six days after the burn. Kaplan-Meier survival curves were drawn and the differences between survival rates were compared by log-rank tests. There were 10 mice in each group.

TUNEL staining

The spleens were fixed for routine histological analysis and 4 µm sections were deparaffinized in xylene and dehydrated in graded ethanol. Apoptotic cells were identified using the Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) technique. The TUNEL-positive cells within the spleen were scored by randomly selecting 10 fields of white pulp per animal. The selected fields were traced by the magnetic lasso tool in Adobe Photoshop CS4 Extended Version (Adobe Systems, San Jose, CA) for analysis. NIH Image J software (ver.1.43, NIH, Bethesda, MD) was used for counting cells.

The data was expressed as the percentage of TUNEL-positive cells/100 splenic lymphocytes in the white pulp. Digitized images were analyzed by two blinded investigators.

Time course of apoptosis in spleen

The burn-injured animals were sacrificed and the spleens were harvested at various time points after injury (8hr, 16hr, 24hr, 2d and 3d after the burn injury, 6 mice for each time point). We performed TUNEL staining to estimate the apoptotic index at each time point. The time point of its peak value was chosen for the following studies.

Western blot analysis

Total spleen protein was determined and identical amounts of protein from each animal were run on 10% SDS-PAGE and transferred onto equilibrated polyvinylidene difluoride membranes by electroblotting. The membranes were incubated at room temperature for one

hour with the primary antibodies of TNF- α or NF- κ B. The levels of protein expressions were normalized to that of GAPDH in the same sample. Bands were quantified by scanning densitometry using the digital Kodak Gel Logic 200 system (Carestream Molecular Imaging, USA).

ELISA Analysis on TNF- α and NF- κ B in the serum

The antigen was added to the wells for the first incubation. After washing, a biotinylated monoclonal antibody specific to TNF- α , NF- κ B, IL-1 α , IL-6, MCP-1 or CRP was added and incubated for one hour. Then streptavidin-proxylase was added followed by incubation for one hour. After washing, the substrate solution was added to produce a colored reaction product.

Serum was collected at 0, 8, 16, 24, 48 and 72 hours after burn injury, to evaluate the time course of TNF- α and NF- κ B expression in the serum.

Statistical Analysis

All results were presented as MEAN $\pm$ SEM. and evaluated by one- and two-way ANOVA. Individual group means were compared with unpaired Student's *t* tests. Survival analysis was performed by the Kaplan-Meier method. The differences in the survival rates were tested for significance by the use of a log-rank test. Differences with *P*-value of less than 0.05 were considered to be significant.

RESULTS

1. Burn induced significant apoptosis in mouse spleen

TUNEL staining of the sections of spleens from the sham mice demonstrated diffusely scattered apoptotic cells at a ratio of 4.2 ± 1.1 % (Fig. 1. A). By contrast, burn induced markedly greater apoptosis with apoptotic index as high as 24.6 ± 4.1 % (Fig. 1. B). The majority of the apoptotic cells were present in the white pulp, suggesting they might be lymphocytes.

The time course study demonstrated that burn-induced apoptosis occurred as early as 8 hours after injury, after which the apoptotic index gradually increased to a maximum level at 24 hours followed by gradual decrease in the next two days (Fig. 1C). As a result, we chose 24 hours after burn as the time point for the rest of our study. Burn induced significant IL-1 α , IL-6, MCP-1 and CRP production in the serum 24 hours after burn (Fig. 1. D).

2. Burn induced TNF- α and NF- κ B expression in spleen and serum

Expressions of TNF- α and NF- κ B in the spleen were mildly detectable in sham-treated mice. Thermal injured mice presented remarkably high TNF- α and NF- κ B expressions in the spleen. A time course study on this expression exhibited that the serum and spleen presented the highest expression of both TNF- α and NF- κ B at 24 hours after the thermal injury (Fig. 2. A, B, C), which coincided with the peak level of apoptosis index. In parallel to the findings in splenic tissues, thermal injury also caused significant elevation of plasma

TNF- α and NF- κ B 24 hours after burn in comparison with normal controls (Fig. 2. D, and E).

3. Simvastatin decreased the splenic apoptosis and increased the survival rate in burned mice

Burned mice treated with simvastatin displayed much fewer apoptotic cells in the spleen ($8.1 \pm 2.1\%$) 24 hours after burn. In contrast, the saline-treated burned mice demonstrated significant apoptosis, which suggests a protective effect of simvastatin on the spleen. However, simvastatin treatment did not alter the distribution pattern of apoptotic cells, most of which were also present in the white pulp of the spleen (Fig. 3. A, B, C). Kaplan-Meier survival curves showed the burned mice treated with simvastatin had a survival advantage over the saline-treated mice ($P < 0.005$), further suggesting a strong protective effect of simvastatin after burn injury (Fig. 3. D). Simvastatin significantly reduced IL-1 α , IL-6, MCP-1 and CRP productions in the serum (Fig. 3. E)

4. Simvastatin reduced TNF- α and NF- κ B expression in spleen tissue and serum

As shown in Figure 2, burn-injured mice showed significant increases in TNF- α and NF- κ B expressions in the spleen and plasma. Treatment with saline did not change the expressions of TNF- α and NF- κ B in serum or spleen of the burned mice. However, when treated with simvastatin, the expressions of TNF- α and NF- κ B were greatly decreased in both spleen and serum of burned mice. These findings suggest that simvastatin reduced the burn-induced TNF- α and NF- κ B expressions (Fig. 4.).

5. TNF- α and NF- κ B KO mice showed less apoptosis in spleen and higher survival rate after severe burn injury

Thermal injury did not induce as much apoptosis in the spleen of KO vs. wild type mice. Also, simvastatin administration did not cause additional reduction of the apoptotic index in spleen of TNF- α and NF- κ B KO mice (Fig. 5. A, C). Kaplan-Meier survival curves showed the TNF- α and NF- κ B KO mice had survival advantage over the burned WT mice, however, there was no significant survival difference between burned KO mice and KO mice treated with simvastatin (Fig. 5. B, D). These findings further suggest that TNF- α and NF- κ B play a central role in burn induced spleen apoptosis and simvastatin might decrease this burn-induced apoptosis through the TNF- α /NF- κ B pathway in mice

DISCUSSION

Infection is one of the most common complications in burn patients. Post-burn immune dysfunction might play an essential role in the susceptibility of infections.¹⁷ Recent data have demonstrated that burn might induce significant apoptosis in immune organs.¹⁸ We postulate that spleen dysfunction might be an important contributing factor to the post-burn infection in humans. Treatments that prevent apoptosis in immune cells may have clinical value in burn patients. In the present study, we evaluated burn-induced splenic apoptosis, and whether simvastatin has any protective effects on the spleen. Also, we investigated the underlying mechanisms for this protection.

In the present study, we found that burn injury induced as much as $24.6 \pm 4.1\%$ apoptosis in spleen white pulp at 24 hours post burn (Fig. 1. A, B and C). Interestingly, we found much fewer cells undergoing apoptosis in the spleen red pulp, suggesting that burn-induced apoptotic cells in the spleen are largely lymphocytes. We postulate that the burn-induced apoptosis is sufficient to affect immune function of spleen. With age, the thymus degenerates and the spleen is recognized to play a central role in human immune function. Therefore, our findings demonstrated that significant lymphocytic apoptosis in the spleen might contribute to vulnerability to infection after burn. Our studies revealed that simvastatin reduced apoptosis in the spleen, thus the protection of immune function may contribute to the increased surviving rate (Fig. 5), although necropsies of the non-surviving animal did not demonstrate any obvious organ damage.

Current data has also showed that a severe skin burn induces significant secretion of proapoptotic cytokines.^{19,20} Among these factors, TNF- α and NF- κ B are recognized as central to the regulation of apoptosis.²¹ In the present study, we found burn caused significant IL-1 alpha, IL-6, MCP-1 and CRP production in the serum which indicated a systemic inflammatory reaction in the burned mice (Fig. 1.D). Particularly, both TNF- α and NF- κ B are significantly increased in mouse serum and spleen after burn injury, with peak levels appearing at 24 hours post burn in both tissues (Fig. 2), which interestingly coincided with the timing of the maximal apoptosis in the spleen. These findings might point toward to TNF- α and NF- κ B as potentiating burn-induced apoptosis in the spleen.

Much data have suggested that simvastatin possesses significant anti-inflammatory properties that we believe may reduce apoptosis through these activities. We found burned mice treated with simvastatin presented much lower IL-1 alpha, IL-6, MCP-1 and CRP expressions in the serum (Fig. 3.E), which suggested the anti-inflammatory property of simvastatin. The present study also demonstrated that burned mice treated with simvastatin presented with much fewer apoptotic cells in the spleen (Fig. 3. A, B and C), which suggested a protective effect. Additionally, we found that simvastatin remarkably reduced the burn-induced expression of both TNF- α and NF- κ B in mouse serum and spleen (Fig. 4), indicating that the TNF- α /NF- κ B pathway may be involved in the induction of apoptosis and simvastatin-related reduction of apoptosis in spleen.

A clear protective property of simvastatin was further demonstrated by a significantly reduced mortality of burned mice (Fig. 3.D). Moreover, burn injured TNF- α /NF- κ B KO mice did not show as many apoptotic cells in their spleen compared to wild type mice. Simvastatin administration to these KO mice did not show an additive reduction in splenic apoptosis (Fig. 5. A and C). Burned TNF- α KO and NF- κ B KO mice had much lower mortality rates than burned WT mice. Simvastatin did not present additive survival benefit in burned KO mice compared with untreated burned KO mice (Fig. 5. B and D). These findings are consistent, and clearly point to simvastatin acting by down-regulation of TNF- α /NF- κ B expression to reduce burn-induced splenic apoptosis in the mouse.

There are many controversies regarding the roles of TNF- α /NF- κ B pathway in apoptosis. Data suggests that TNF- α promotes apoptosis in most cases, but NF- κ B, as a potential downstream factor of TNF- α , was found to possess either a pro- or anti-apoptotic role. On

one hand, NF- κ B enhances the transcription of inhibitory proteins C-FLIP, Bcl-2, and cIAP1 / cIAP2 that interfere with apoptosis signaling.²² On the other hand, NF- κ B activates caspases and cleaves several components of signal proteins including RIP, IKK, and the subunits of NF- κ B itself, which promote apoptosis.²³ Extensive interactions between pathways are mediated by the myriad and often-conflicting effects of apoptotic factors. Moreover, factors, such as cell type, concurrent stimulation of other cytokines, and the nature of the apoptotic stimulus may shift the pathway balance towards a particular direction [.²⁴

Collectively, our data suggests that burn injury induces significant apoptosis and increases TNF- α /NF- κ B expression in serum and spleen. Although we used high dose of simvastatin in the mice, further studies will be necessary to determine the adequate dose for human which will have similar effects as those seen in mice. Simvastatin might play a role, which has not been recognized before, in preventing burn induced spleen apoptosis via down-regulation of the TNF- α /NF- κ B pathway. However, many aspects of this protective role of simvastatin need to be further explored. It remains unclear whether simvastatin exerts its effects directly or indirectly in the down regulation of TNF- α /NF- κ B expression; what type(s) of lymphocytes in the white pulp of the spleen undergo apoptosis after burn injury. The roles of other genes and/or other factors that might be involved in simvastatin treatment also need to be investigated. Finally, it needs to be explored if the results in animal studies can be extrapolated to humans. Moreover, Ahn²⁵ found that different statins behave differently from one another, only the natural statins (simvastatin, mevastatin, lovastatin and pravastatin), not synthetic statins (fluvastatin and atorvastatin) inhibited TNF-induced NF- κ B activation. However, results of the present study strongly suggest a potentially important therapeutic application for simvastatin for preventing splenic dysfunction and related infections in burn patients.

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Figure 1.

Burn induced splenic apoptosis. A. Occasional apoptotic cells were detected in the spleens of sham-treated mice. B. Significantly increased numbers of TUNEL-positive cells were found in the splenic white pulp of the burned mice. C. Burn injury-induced apoptosis was found in the spleen as early as 8 hours after burn, and peaked at 24 hours (n=6 animals per group; sham vs. *: $P<0.05$, sham vs. **: $P<0.001$; within burn group: $P>0.05$ by one-way ANOVA test). D. Burn injury caused significant IL-1 α , IL-6, MCP-1 and CRP production in the serum (* $P<0.01$).

Figure 2.

Time course of TNF- α and NF- κ B expression in the spleen and serum of burned mice. A. Western blot detection of TNF- α and NF- κ B expressions (n=6 in each group). B. Levels of TNF- α expression in splenic tissues normalized to the expression levels of GAPDH (no difference exists among ** groups: $P>0.05$; * vs. **: $P<0.01$; * vs. ***: $P<0.05$). C. Levels of NF- κ B expression in splenic tissues normalized to the expression levels of GAPDH (no difference exists ** groups: $P>0.05$; * vs. **: $P>0.05$; ** vs. ***: $P<0.01$). D. Time course study on the expressions of TNF- α in the serum (ELISA; no difference exists among ** groups: $P>0.05$; * vs. **: $P<0.05$; * vs. ***: $P<0.01$; ** vs. ***: $P<0.05$). E. Time course study on the expressions of NF- κ B in the serum (ELISA; no difference exists among ** groups or *** groups: $P>0.05$; * vs. **: $P<0.05$; * vs. ***: $P<0.01$; ** vs. ***: $P<0.05$). The statistics above are based on one-way ANOVA tests.

Figure 3.

Effects of simvastatin on burned mice. Representative histological images of TUNEL stained spleen sections: A. burned mice; B. Burned mice treated with simvastatin. C. Apoptotic index in each group: burn injury induced significant apoptosis in the spleen, and simvastatin reduced the apoptotic index (n=6 animals per group. * vs. **: $P < 0.001$; no difference exists among * groups or ** groups: $P > 0.05$, two-way ANOVA tests). D. Kaplan-Meier survival curves demonstrated simvastatin-treated mice had a survival advantage over untreated and saline-treated mice (burn vs. burn/saline: $P > 0.05$; sham vs. burn or burn/saline vs. burn/statin: $P < 0.01$). Simvastatin reduced IL-1α, IL-6, MCP-1 and CRP production in the serum of burned mice (* $P > 0.05$; * vs. **: $P < 0.01$).

Figure 4.

Effects of simvastatin treatment on TNF-α and NF-κB expressions in burned mice. A. Western blots analysis of the effects of simvastatin on TNF-α expression in mouse spleen (no difference exists within * groups or ** groups, but significant difference was demonstrated between * and ** groups: $P < 0.05$). B. Western blots analysis of the effects of simvastatin on NF-κB expression in mouse spleen (no difference exists within * or ** groups, but significant difference was found between * and ** groups, $P < 0.05$). C. ELISA test of TNF-α in serum. Burn increased remarkably the expression of TNF-α in serum (No difference exists among * or ** groups: $P > 0.05$; * vs. **: $P < 0.005$ (n=6 animals per group)). D. ELISA test of NF-κB in serum. Burn induced remarkably increased expression of NF-κB in serum (n=6 animals per group. No difference exists among * or ** groups: $P > 0.05$; * vs. **: $P < 0.005$).

Figure 5.

Effects of TNF- α KO and NF- κ B KO on simvastatin treatment and survival rate. A. simvastatin exhibited no additive decrease in spleen apoptosis in TNF- α KO mice after burn injury (no difference within * or *** groups: $P > 0.05$; * vs. ** vs. ***: $P < 0.01$; by two-way ANOVA test). B. simvastatin treatment produced no additive survival benefit in burned TNF- α KO mice (Burn/ TNF- α KO vs. burn/ TNF- α KO/statin: $P > 0.05$; sham vs. burn/ TNF- α KO or burn/ TNF- α KO/statin vs. burn/WT: $P < 0.01$). C. simvastatin exhibited no additive reduction in spleen apoptosis in burned NF- κ B KO mice (no difference among * or *** groups: $P > 0.05$; * vs. ** vs. ***: $P < 0.01$ by two way ANOVA test). D. simvastatin treatment produced no additive survival benefit to burned NF- κ B KO mice (Burn/ NF- κ B KO vs. burn/ NF- κ B KO/statin: $p > 0.05$; sham vs. burn/NF- κ B KO or burn/ NF- κ B KO/statin vs. burn/WT: $P < 0.01$).