Tunicamycin Inhibits Diabetes

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Key Words

Tunicamycin • Endoplasmic reticulum stress • Oxidative stress • Lymphocytic choriomeningitis virus • Diabetes

incidence of virus-induced diabetes and improved survival rates. Conclusion: Tunicamycin may offer therapeutic opportunities for T cell-mediated autoimmune diseases such as diabetes.

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Abstract

Background: Autoimmune diseases are characterized by a breakdown of immunologic tolerance, and this breakdown can lead to life-threatening or lifelong disorders. Moreover; drugs that are used to treat these diseases are few in number and are associated with many serious adverse effects. **Methods:** We used the rat insulin promoter-glycoprotein mouse model to analyze the role of tunicamycin in the process of autoimmune diabetes; the P14 mouse model to analyze the effect of tunicamycin on CD8⁺ T cells; *chop* knockout mice to analyze the role of tunicamycin on an endoplasmic reticulum stress model; and fluorescence-activated cell sorting, quantitative real-time polymerase chain reaction, and histologic methods. **Results:** We found that a single dose of tunicamycin reduced the activation and pancreatic infiltration of CD8⁺ T cells. This activity delayed the

Introduction

Diabetes mellitus is an insulin homeostasis disorder characterized by metabolic abnormalities in carbohydrate and lipid metabolism. Type 1 diabetes is primarily immune-mediated. The loss of beta cells is due to a T cell-mediated autoimmune attack, which results in the failure of the pancreas to produce insulin [1-5].

Tunicamycin is produced by the bacterium *Streptomyces lysosuperificus* and is a mixture of homologous nucleoside antibiotics that inhibit the enzyme GlcNAc phosphotransferase (GPT). GPT catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate, which blocks the synthesis of asparagine-linked glycoproteins. Tunicamycin inhibits the synthesis of all N-linked glycoproteins (N-glycans) and causes cell cycle arrest in

the G1 phase. In addition, it is used in biology as an experimental tool that induces an unfolded protein response [6-11].

In this study we analyzed the effects of tunicamycin in suppressing the immune response. We found that a single dose of tunicamycin significantly reduces T-cell proliferation and delays the incidence of diabetes in a model of virus-induced type 1 diabetes.

Materials and Methods

Mice, treatment, viruses

Lymphocytic choriomeningitis virus (LCMV) strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Virus titers were measured with a focus-forming assay as described previously [12]. All mice used in this study were maintained on the C57BL/6 genetic background. Rat insulin promoter-glycoprotein (RIP-GP) mice, which express the LCMV glycoprotein as a transgene under the rat insulin promoter, were used for analysis of autoimmune diabetes [13]. P14/CD45.1 mice expressing the LCMV GP33 peptide-specific T-cell receptor (TCR) as a transgene were used for T-cell studies [14]. KL25 knockin mice express a LCMV specific B cell receptor [15]. Mice lacking the *chop* gene were maintained on the C57BL/6 background [16]. Animals were kept in single ventilated cages. Animal experiments were carried out with the authorization of the Veterinäramt of Nordrhein Westfalen, Germany, and in accordance with the German laws for animal protection, the institutional guidelines of the Ontario Cancer Institute, or both. Mice exhibiting symptoms of sickness or showing substantial weight loss during LCMV infection were considered dead for purposes of statistical analysis and were euthanized. Tunicamycin was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in dimethyl sulfoxide (DMSO) for intravenous treatment of the animals.

Diabetes measurement

Blood glucose concentrations were measured with an Elite Meter (Bayer, Tarrytown, NY). Mice were considered to be diabetic if the glucose concentration was higher than 14 mM for 2 consecutive days.

Histology

In virus infection experiments, histologic analyses were performed on snap-frozen tissue [12]. Sections of pancreas were stained with the rat monoclonal antibodies anti-CD8 (BD Pharmingen, San Diego, CA), with anti-major histocompatibility complex I (MHC-I) (eBioscience, San Diego, CA), and with guinea pig anti-insulin (Dako, Carpinteria, CA).

Fluorescence-activated cell sorting analysis

Tetramer staining, surface staining, and intracellular fluorescence-activated cell sorting (FACS) staining were performed as described previously [12]. Briefly, splenocytes or

peripheral blood lymphocytes were stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramers (GP33/H-2Db) for 15 minutes at 37°C. They were then stained with anti-CD8 peridinin chlorophyll protein complex (PerCP; BD Biosciences, Franklin Lakes, NJ) for 30 minutes at 4°C. Tetramers were provided by the National Institutes of Health (NIH) Tetramer Facility.

Lymphocyte transfer

Splenocytes from P14/CD45.1 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and injected intravenously into 4 groups of mice. On the next day, 2 groups were infected with 2×10^4 plaque-forming units (PFU) LCMV-WE. After 12 hours, one group of the uninfected and infected mice was given 20 nmol of tunicamycin intravenously; the other mice served as a control group and were left uninfected.

In vitro T-cell proliferation

The proliferation of CD8⁺ T cells *in vitro* without antigen was performed as described previously [17]. Briefly, T cells were sorted with the MACS Pan T Cell Isolation Kit (130-090-861, Miltenyi Biotec, Bergisch Gladbach) and then labeled with CFSE. T-cell activation was achieved in a 24-well plate coated with anti-CD3 (5 µg/mL; 14-0031-85, eBioscience) with or without soluble anti-CD28 (2 µg/mL; 553294, BD Pharmingen).

Reverse-transcription polymerase chain reaction

Total RNA was extracted from splenocytes with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Peqlab Biotechnologie GmbH, Erlangen, Germany). The RNA was reverse-transcribed into cDNA by using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). Gene expression analysis was performed with assays from Applied Biosystems (Carlsbad, CA; glyceraldehyde-3-phosphate dehydrogenase [GAPDH] [4352339E-0806018], *Chop* [Mm00492097_m1], interferon-gamma [IFN-γ] [Mm99999071_m1]), and interleukin-2 [IL-2] [Mm99999222-m1]). Gene expression values were then calculated with the ddCt method; the mean of the control group was used as a calibrator to which all other samples were compared. Relative quantities (RQs) were determined with the equation $RQ = 2^{-ddCt}$.

In vivo killer assay

C57BL/6 mice were infected with 200 PFU of LCMV-WE. On day 7, CD45.1⁺ splenocytes were labeled either with GP33 peptide or with CFSE and were transferred to the infected mice in a ratio of 1:1. After 30 minutes, the number of GP33-labeled splenocytes was compared to the number of CFSE-labeled peptide-negative splenocytes.

Statistical analysis

Data are expressed as means ± S.E.M. Differences between two groups were analyzed for statistical significance with Student's *t*-test. Statistical significance was set at the level of $P < 0.05$.

Fig. 1. Tunicamycin inhibits lymphocytic choriomeningitis virus–induced diabetes. A: Rat insulin promoter-glycoprotein (RIP-GP) mice, expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein under the rat insulin promoter, were infected with 200 plaque-forming units (PFU) LCMV-WE. One group of mice was treated with 20 nmol tunicamycin on day 4 after infection. Induction of diabetes was monitored (n = 6). B: Survival of mice was analyzed (n = 6).

Fig. 2. Tunicamycin reduces the infiltration of CD8⁺ T cells to the pancreas. Rat insulin promoter-glycoprotein (RIP-GP) mice were infected with 200 PFU LCMV-WE. One group of mice was treated with 20 nmol tunicamycin on day 4 after infection. On day 6, mice were killed, and beta-islet cells were stained with immune fluorescence antibodies (n = 3). Scale bar, 100 μ m.

Results

Tunicamycin inhibits LCMV-induced diabetes

To analyze the role of tunicamycin in diabetes we used RIP-GP mice, which express a viral antigen (LCMV glycoprotein) under the rat insulin promoter. Priming of CD8⁺ T cells with LCMV-WE induces the proliferation of β -islet epitope–specific CD8⁺ T cells; these cells subsequently destroy pancreatic β -islet cells, and this destruction leads to diabetes [13]. To test the effect of

tunicamycin in diabetes, we infected RIP-GP mice with 200 PFU LCMV-WE (day 0) and then treated one group of mice additionally with 20 nmol tunicamycin on day 4. Treatment with tunicamycin substantially delayed the onset of diabetes; mice treated with tunicamycin did not show any obvious sign of disease until day 15. In contrast, diabetic mice that were not given tunicamycin exhibited high glucose concentrations beginning on day 10 and died within 40 days (Fig. 1 A, B).

Tunicamycin reduces the infiltration of CD8⁺ T cells to the pancreas

To investigate the effect of tunicamycin on the infiltration of CD8⁺ T cells to the pancreas, we used 2 groups of RIP-GP mice that had been infected with LCMV-WE. One group was treated intravenously with 20 nmol tunicamycin on day 4, and the other group was left untreated. After 6 days of infection, mice were killed. Pancreas slices were stained with fluorescence antibodies against β -islet cells, CD8⁺ T cells, and MHC-I. Treatment with tunicamycin reduced the infiltration of T cells and the expression of MHC-I (Fig. 2).

Tunicamycin induces rapid apoptosis in proliferating T cells

To investigate whether tunicamycin specifically reduces the number of CD8⁺ T cells and whether its suppressive activity also affects other immune cells, we infected mice with 200 PFU LCMV and administered 20 nmol tunicamycin on day 6. On day 8, mice were killed and immune cells were counted. We found that the administration of tunicamycin primarily reduced the number of CD8⁺ T cells (Fig. 3 A). The main reduction was in NP396-specific and GP33-specific T cells (Fig. 3 B). Moreover, we analyzed the effect of tunicamycin on the proliferation of specific B cells. We used B cells from the spleens of KL25 mice, which are specific for LCMV-GP. The B cells were labeled with CFSE and incubated with LCMV with or without 10 μ M tunicamycin. We did not find obvious effect of tunicamycin on the proliferation of these specific B cells (Fig. 3 C).

Tunicamycin acts directly on T cells

To determine whether the reduction in the number of CD8⁺ T cells is direct, we transferred CFSE-labeled splenocytes from P14/CD45.1 mice into 4 groups of C57BL/6 mice. One group was infected with 2×10^4 PFU of LCMV-WE; the second group was also infected (2×10^4 PFU) and was additionally treated with 20 nmol tunicamycin on day 1; the third group was not infected but was only treated with 20 nmol tunicamycin; and the fourth group served as a control group. The mice were killed on day 3, and the proliferation of the labeled splenocytes was analysed in FACS. The addition of tunicamycin in the absence of T-cell activation had only a mild influence on the survival of CD8⁺ T cells (Fig. 4A, B). T-cell activation in the absence of tunicamycin led to rapid CFSE dilution and expansion of CD8⁺ T cells. In the presence of tunicamycin, the number of CD8⁺ T cells decreased rapidly to a level even lower than the original

Fig. 3. Tunicamycin primarily kills CD8⁺ T cells. A-B: C57BL/6 mice were infected with 200 PFU LCMV-WE. On day 6 after infection, one group of mice was treated with 20 nmol tunicamycin. On day 8, mice were killed, and numbers of subsets of cells in the spleen (A) ($n = 3$) and of LCMV-specific CD8⁺ T cells in the blood (B) were counted ($n = 3$). One of two similar experiments is shown. C: Two million KL25 splenocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE) and activated with LCMV-WE in the presence or absence of 10 μ M tunicamycin. After 48 h, B-cell proliferation was measured by fluorescence-activated cell sorting (FACS) ($n = 4$).

Fig. 4. Tunicamycin acts directly on T cells. A-B: P14/CD45.1 splenocytes were labeled with CFSE and transferred to C57BL/6 mice. Mice were then infected with LCMV-WE or left uninfected in the presence or absence of 20 μ M tunicamycin. The proliferation capability (A) and the number (B) of T cells were determined by FACS (n = 3). C-D: MACS-sorted T cells were labeled with CFSE and stimulated with anti-CD3 and with or without anti-CD28, in the presence or absence of 10 μ M tunicamycin. After 3 days, the CFSE content (C) and the absolute number of CD8⁺ T cells (D) determined by FACS (n = 6). E: C57BL/6 mice were treated with 20 μ M tunicamycin or left untreated. P14/CD45.1 splenocytes were labeled with CFSE and transferred to the mice with or without LCMV-WE infection. The proliferation of T cells was determined by FACS (n = 4).

cell count (Fig. 4 A, B). This finding suggests that activated T cells are highly sensitive to treatment with tunicamycin but that non-activated T cells are only slightly sensitive to this treatment.

To eliminate the effect of tunicamycin on the expression of MHC-I, we cultured CFSE-labeled CD8⁺ T cells in anti-CD3-coated 24-well plates and stimulated them with or without CD28. One group each of the stimulated and unstimulated cells was treated with tunicamycin on day 0. After 3 days, the absolute number of CD8⁺ T cells and the CFSE content were measured with FACS. Tunicamycin directly inhibited the Proliferation of the CD8⁺ T cells (Fig. 4 C, D), and this finding excluded the possibility that tunicamycin affects the antigen-presenting pathway.

To restrict the effect of tunicamycin on MHC-I, we treated C57BL/6 mice with tunicamycin. Twelve hours later we injected mice with CFSE-labeled splenocytes from P14/CD45.1 mice. After 48 hours, the proliferation of the transferred CD8⁺ T cells was measured with FACS and was found to be at a normal level (Fig. 4 E).

Tunicamycin increases the expression of the chop gene in T cells

Tunicamycin has a strong effect on endoplasmic reticulum (ER) stress and specifically on the *chop* gene; under circumstances of stress this effect can lead to apoptosis [18-20]. Therefore, we next measured the expression of *chop* mRNA in T cells. We stimulated splenocytes from P14 mice with GP33 and incubated the cells with 10 μ M tunicamycin for 48 hours. Tunicamycin increased the expression of *chop* (Fig. 5 A). To determine whether this enhanced *chop* expression was the reason for the reduced proliferation of T cells, we injected *chop*^{-/-} mice

Fig. 5. Tunicamycin induces the expression of *chop* in T cells. A: P14 splenocytes were activated with GP33 peptide in the presence or absence of 10 μ M tunicamycin. After 48 hours, *chop* gene expression was measured (n = 6). B: *Chop* knockout mice and C57BL/6 mice were infected with 2×10^6 PFU LCMV-WE. On day 4, mice were treated with 20 nmol of tunicamycin. CD8⁺ T cells in the blood were counted on day 8 (n = 3).

Fig. 6. Tunicamycin reduces the expression of interleukin 2 and interferon γ . A-B: After being cultured in a 24-well plate, 4×10^6 P14 splenocytes were activated with GP33 peptide in the presence or absence of 10 μ M tunicamycin. After 24 hours, RNA was isolated, and the expression of interleukin-2 (IL-2, A) and interferon gamma (IFN- γ , B) mRNA was measured by real-time polymerase chain reaction (RT-PCR) (n = 6). C: C57BL/6 mice were infected with 200 PFU of LCMV-WE strain. On day four, mice were treated with 20 nmol of tunicamycin intravenously. On day 8 after infection, the cytotoxic capability of CD8⁺ T cells was determined with an *in vivo* killer assay (n = 4).

Fig. 7. The comparison of tunicamycin to dexamethasone. A: C57BL/6 mice were infected with 2×10^6 PFU LCMV-WE. On day 4, mice were treated once with 20 nmol of tunicamycin intravenously, or daily with 20 μ g of dexamethasone intraperitoneally. Viral titers were analyzed on day 11 after infection (n = 4-6). B: C57BL/6 mice were injected with 10, 20, or 40 nmol tunicamycin or with 10, 20, or 40 μ g of dexamethasone. Survival was monitored.

with 2×10^6 PFU LCMV-WE, with or without 20 nmol of tunicamycin. We found that *chop*^{-/-} mice were also sensitive to tunicamycin, a finding suggesting that factors other than *chop* contributed to the rapid death of CD8⁺ T cells (Fig. 5 B).

Tunicamycin inhibits the production of cytokines by proliferated T cells

T cells begin to produce cytokines after activation. This cytokine production enhances proliferation (IL-2) and is beneficial for controlling the virus (IFN- γ) [21, 22]. We

used real-time polymerase chain reaction (RT-PCR) to measure the mRNA expression of IL-2 and IFN- γ by T cells. Splenocytes from P14 mice were stimulated with GP33 peptide and were either treated with 10 μ M tunicamycin for 48 hours or left untreated. Tunicamycin reduced gene expression (Fig. 6 A, B), a finding suggesting that cytokine production is also affected by tunicamycin.

Next we measured the cytotoxic activity of CD8⁺ T cells after tunicamycin treatment. The cytolytic activity of CD8⁺ T cells was significantly reduced when mice were treated with tunicamycin (Fig. 6 C).

Next we compared the immunosuppressive effects of tunicamycin to those of a standard immunosuppressive therapy (dexamethasone). First, we analyzed the ability of both regimens to control the virus and found that both treatments led to the persistence of LCMV (Fig. 7 A). Toxicity studies showed that once the dose was increased, the administration of tunicamycin led to death (Fig. 7 B). This finding suggests that the therapeutic window of tunicamycin is narrow.

Discussion

In this study we found that tunicamycin can prevent type 1 diabetes by reducing the proliferation of T cells, primarily active antigen-specific CD8⁺ T cells. This strong effect of tunicamycin could also be important for treating other types of diseases that are related to autoimmune responses. However, because of the narrow application window of tunicamycin, specific cell toxicity will probably be required. The results of this study may also suggest a new approach to the treatment of diabetes in humans, once the therapeutic dose, the proper route of administra-

tion, and the accompanying adverse effects have been determined.

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K.S.L. studied and wrote the manuscript; N.S. performed most of the experiments and prepared the initial manuscript; N.H. performed many experiments; B.G. and N.G. performed experiments; P.A.L., P.P., and D.H. analyzed the data; and T.M. helped with experiments regarding *chop* knockout mice.

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