The Effect of Atorvastatin (Lipitor) on the Duration of Survival of Allogeneic Skin Graft and the Growth of B16F10 Mmelanoma Cells in Mice

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ABSTRACT

Aims: To evaluate the immunomodulatory effect of atorvastatin on skin allograft survival and on tumor growth in mice.

Study design: Experimental Study

Place and Duration of Study: Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine, American University of Beirut; 2011-2012.

Methodology: BALB/c mice were transplanted with skin allografts from C57BL/6 mice and given either atorvastatin alone or in combination with immunosuppressive agents. Average survival days of skin allografts was recorded and serum levels of interleukin-1β and interferon-γ were quantified. BALB/c mice and C57BL/6 mice challenged intraperitoneally with B16F10 melanoma cancer cells (cancer cell line syngeneic to C57BL/6 mice) and were then treated with atorvastatin. They were observed regularly for tumor growth.

Results: The results indicated that in transplant mice atorvastatin given alone or in combination with immunosuppressive agents prolonged allograft survival time in spite of a non-significant change in serum cytokine levels. Furthermore, atorvastatin treatment enhanced tumor growth in C57BL/6 mice and promoted tumor growth in BALB/C mice.

Conclusion: The results obtained are supportive of atorvastatin being an immunosuppressive agent. It is hypothesized that the immunosuppressive effect of atorvastatin could be related to its effect on membrane cholesterol of antigen presenting cells (APC) resulting in hindering the ability of APCs to present antigen to T-lymphocytes.

Keywords: Immunosuppression, Skin Transplantation, Cancer, Statins, Cytokines

1. INTRODUCTION

Statins are widely used clinically for the prevention of primary and secondary cardiovascular diseases owing to their potent plasma cholesterol reduction properties [1]. Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the enzyme that catalyzes the rate limiting step in the biosynthesis of cholesterol. Inhibition of HMG-CoA reductase by statins prevent the production of cholesterol and of other downstream intermediates including geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) that are involved in post-translational modification of
essential proteins including nuclear lamins, Rho, Ras and Rac GTPases [2]. These proteins serve crucial functions in cell migration, differentiation and proliferation [3]. Extensive studies on the non-cholesterol lowering effects of statins have generated convincing evidence that statins possess pleitropic effects and can alter numerous aspects of key biological systems including the immune system [4]. Hot et. al. reported that simvastatin or rosuvastatin inhibited the pro-inflammatory effects of IL-17 and TNF on endothelial cells. [5]. The 2 cytokines have been implicated in the Pathogenesis of some autoimmune diseases including rheumatoid arthritis and psoriasis. Earlier we reported that atorvastatin suppressed γ-interferon, IL-4 and antibody levels, in mice immunized with egg albumin [6]. Moreover, it has been observed that acute rejection episodes were less, and duration of kidney graft survival was longer in recipients that were given statins in addition to conventional immunosuppressive therapy [7]. Statins have been suggested as potential therapeutic agents to treat a wide range of immune-related diseases including autoimmune diseases, cancer, asthma and graft rejection [8,9,10].

2. MATERIAL AND METHODS

2.1 Atorvastatin and immunosuppressive agents

Atorvastatin (Lipitor, Pfizer, New York, NY, USA) tablets were pulverized and suspended in phosphate buffered saline (PBS). Cyclosporine A suspension (Sandimmune, Novartis, Basel, Switzerland) was diluted in olive oil. Prednisone (Cortancyl, Sonafi Aventis, Paris, France) was dissolved in PBS. Mycophenolate mofetil (Cellcept, Roche, Basel, Switzerland) was dissolved in PBS. The amount injected intraperitoneally in a volume of 0.3ml when given alone or 0.1ml when given in combination is given in Table 1.

Table 1. Treatment of BALB/c mice that received a skin transplant obtained from C57BL/6 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td>Oil</td>
</tr>
<tr>
<td>3</td>
<td>Prednisone (P), 20mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>Atorvastatin (AS), 40mg/kg</td>
</tr>
<tr>
<td>5</td>
<td>Mycophenolate mofetil (MMF), 100mg/kg</td>
</tr>
<tr>
<td>6</td>
<td>Cyclosporine A (CSA), 20mg/kg</td>
</tr>
<tr>
<td>7</td>
<td>P, 10mg/kg + MMF, 50mg/kg + CSA, 10mg/kg</td>
</tr>
<tr>
<td>8</td>
<td>AS, 20mg/kg + P, 10mg/kg + MMF, 50mg/kg + CSA, 10mg/kg</td>
</tr>
</tbody>
</table>

*intraperitoneal injections were given to balb/c mice 2 days prior to, and every other day after the c57bl/6 shin was transplanted.
2.2 Animals
BALB/c and C57BL/6 mice aged 6 – 8 weeks old were obtained from the animal care facility at the American University of Beirut Faculty of Medicine. Mice were maintained in an air-conditioned room (21 ± 1 °C) under a 12-h light/dark cycle and fed ad libitum. All animals were acclimatized for at least 1 week before experimentation. Animal welfare ethical policies were respected.

2.3 Effect of atorvastatin on skin allograft survival
In the case of the effect of atorvastatin in comparison with established immunosuppressive agents on the survival of skin transplants, 8 groups of BALB/c mice were used, each group consisting of 9 mice. They were treated as indicated in Table 1. Pharmacological agents were tested alone or in combination. Intraperitoneal injections were given to BALB/c mice 2 days prior to, and every other day after the C57BL/6 skin was transplanted. Blood was collected by cardiac puncture from 3 mice from each group at days 5 and 10 post-transplantation and on rejection day and was used to determine interleukin-1β (IL-1β) and interferon-γ (IFN-γ) levels by Enzyme-Linked Immunosorbant Array (ELISA).

2.4 Transplantation
The skin transplantation procedure described by McFarland and Rosenberg [11] was adopted with some modifications. In brief, a fully mismatched allogeneic skin transplantation procedure was performed two days after the first injection was given. Recipient and donor mice were anesthetized with a mixture 0.025ml of xylazine (20mg/ml, Interchemie, Castenray, Holland) and 0.075ml ketamine (50mg/ml, Rotexmedica Trittau, Germany) for the procedure. Skin allografts of 2 x 1 cm from black C57BL/6 mice were grafted onto the back of BALB/c white mice. An intravenous dressing was used to cover the graft. For best results, an additional gauge tape was wrapped around the mouse. Transplanted mice were then put on a warm-pad until they woke up. Dressing was removed and the allograft was observed daily. Signs of graft rejection included hair loss, scar formation and shrinkage and necrosis of graft. Graft rejection was defined as >80 percent destruction of the allograft.

2.5 Enzyme-Linked Immunosorbant Array (ELISA)
Serum interleukin-1β (IL-1β) and interferon-γ (IFN-γ) levels were determined by ELISA using single analyte ELISArray IL-1β kit and single analyte ELISArray IFN-γ kits (SA Biosciences, Fredrick, MD, USA). The procedure described by the manufacturer was followed.

2.6 B16F10 melanoma cells
B16F10 melanoma cells are syngeneic to C57 BL/6 mice [12, 13]. They were maintained as monolayers in vitro in RPMI-1640 containing 1% L-Glutamine and Hapes complemented with 1% penicillin-streptomycin and 10% heat inactivated fetal bovine serum [14, 15]. A suspension containing 0.25 million cells/0.3ml was used.

2.7 Effect of atorvastatin on growth of B16F10 melanoma cells in C57BL/6 and BALB/c mice
Two groups of BALB/c mice and 2 groups of C57BL/6 mice were used. One group of each strain was given a daily intraperitoneal (i.p.) injection of 0.3ml of PBS and the other group of each strain was given a daily ip injection of AS (40mg/kg). One week after the first injection, each mouse in both groups was challenged with 0.3ml of the tumor cell suspension. Treatment was sustained on daily basis following tumor challenge. Mice were observed and weighed regularly for health assessment.
3. RESULTS

3.1 Graft Survival

It was noted that in all treated groups there was a prolongation of graft survival of 7 to 13.5 days as compared to the controls. There was an average prolongation of graft survival of 7 (±3.5) days in the group that received atorvastatin alone compared to the control group. Figures 1 and 2 are the appearance of the skin grafts in a mouse that was treated with AS and a mouse treated with PBS. Moreover, when atorvastatin was added to triple therapy (P + CSA + MMF) graft survival was prolonged an additional 3.5 (±0.7) days when compared to the triple therapy alone (Figure 3).

Figure 1. Skin graft on day 13. BALB/c mice were transplanted with skin allografts from C57BL/6 mice and treated with atorvastatin. The allograft showed minor signs of necrosis with limited scar formation and shrinkage.
Figure 2. Skin allograft on day 9 post-transplantation. BALB/c mice were transplanted with skin allografts from C57BL/6 mice and treated with i.p injections of PBS. Note the signs of rejection including extensive necrosis, shrinkage and hair loss.
Figure 3. Average number of days of skin allograft survival. Groups were given i.p injections of either the vehicle or the assigned treatment every two days starting two days prior to transplantation.

AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mycophenolate Mofetil.

3.2 Serum IL-1β and IFN-γ Levels

There was no significant difference in serum levels of IL-1β between treated and control groups at any time point. Similarly, expression of IFN-γ did not vary significantly compared to the control groups for all analyzed time points (Figures 4 and 5).

Figure 4. Serum interleukin-1β (IL-1β) levels determined by ELISA in different groups of mice. On specified days (D) mice were sacrificed and serum obtained for IL-1β analysis. Mice in P and MMF group did not survive to rejection day.

RD = rejection day; AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mofetil Mycophenolate.
Figure 5. Serum IFN-γ levels determined by ELISA. On specified days (D) mice were sacrificed and serum obtained for IFN-γ analysis. Mice in P and MMF group did not survive to rejection day.

RD = rejection day; AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mycophenolate.

3.3 Effect of atorvastatin on growth of the B16F10 melanoma

There was an increase in body weight of BALB/c mice treated with AS as well as in the size of their abdomens. Aspirates obtained from one mouse was rich in tumor cells on day 18 post-tumor injection. Dissection revealed an abnormally large mass in the lower abdomen (Figure 6). However, the increase in abdomen size in surviving BALB/c mice subsided after 18 days post-tumor challenge. On the other hand, growth of tumor on day 16 post-tumor challenge in the AS- treated C57BL/6 mice was apparent when compared to the C57BL/6 mice that were not treated with AS. (Figure 7).
Figure 6. BALB/c mice on day 18 post-tumor injection treated with atorvastatin. Note tumor growth in the lower abdomen.
Figure 7. C57BL/6 mice on day 16 post-tumor injection treated with either atorvastatin (left) or PBS (right).

DISCUSSION

The introduction of immunosuppressive agents was a breakthrough in organ transplantation and has increased one year allograft survival rates by more than 80% [16]. However, it has been reported that they disrupt proper functioning of organs and they have been associated with severe side effects such as, increased frequencies of opportunistic infections, nephrotoxicity and cancer [17].

Statins, or HMG-CoA reductase inhibitors, are a family of drugs that are commonly used to treat hypercholesterolemia and to prevent primary and secondary cardiovascular diseases. Evidence is growing that suggests that statins have multifactorial cholesterol-independent immunomodulatory properties that extend to include beneficial effects in conditions such as organ transplantation, rheumatoid arthritis, multiple sclerosis and renal diseases [18, 1, 19]. Statins are well tolerated and easy to administer making them ideal for their use in any pathological condition.

It has been demonstrated in this study that atorvastatin given at a dose of 40mg/kg in a murine model of skin allograft transplantation displayed immunomodulatory effects. It appeared that atorvastatin given as a monotherapy had effectively increased mean survival time of allografts. Allograft recognition can be direct or indirect [20]. In the former case,
Antigen Processing/Presenting Cells (APC) migrate from the graft to secondary lymphoid tissue where they activate T helpers and T cytotoxic cells. The activated T cytotoxic cells then migrate to the graft and destroy it. In the latter case which occurs later, post-transplantation, donor antigens are processed by recipient APC which in turn activate T helper and T cytotoxic cells in the secondary lymphoid tissue. Again, the T cytotoxic cells get access to the graft and destroy it. In both cases, Major Histocompatibility Complex (MHC) molecules expressed on the surface of APC are involved. It is possible that atorvastatin through its action on membrane cholesterol temporarily modifies membrane MHC and delays the activation of T lymphocytes. When atorvastatin was given in combination with immunosuppressive agents, in particular cyclosporine A, a prolongation of skin graft survival was observed. Cyclosporine A inhibits the CYP3A4 complex in the liver which metabolizes atorvastatin [21]. By doing so, cyclosporine A would mediate the potentiation of atorvastatin by increasing its bioavailability.

On the other hand, the increase in mean survival time was not associated with a reduction in serum pro-inflammatory cytokine production. Indeed, when IFN-γ and IL-1β serum levels were quantified, no change was detected between atorvastatin group and control group. Similarly, immunosuppressive monotherapy and combination therapy did not result in any significant change in the expression level of serum IFN-γ and IL-1β albeit a net increase in the mean survival time of skin allografts was observed. There are at least 2 explanations supporting these results: 1- The variation in the expression level of these two cytokines may be significant in the site of transplantation and not in serum. Monocytes mature into macrophages when they reach their target organ and IL-1β is a cytokine that is mostly expressed by activated macrophages and endothelium cells. So it is likely that IL-1β may be expressed only locally and that any change in serum may not be detectable. 2- The selected time points to study the expression level of serum IFN-γ and IL-1β may not correspond with the actual physiological changes. When the immune response is triggered during allograft rejection, some pro-inflammatory cytokines are expressed early after transplantation and are mainly secreted by cells of the innate immune system while others are expressed later during the immunological response and contribute to the development and the maintenance of the adaptive immune response. IL-1β is secreted by macrophages which are involved in the early response and IFN-γ is required for early events including the activation of macrophages and T-cytotoxic cells [20]. Therefore, the analysis of serum IFN-γ and IL-1β expression level at earlier time points using more sensitive techniques might have been more appropriate.

However, these results are not in agreement with previous studies showing that different immunosuppressive treatment [22, 23, 24] in murine models of skin transplant could inhibit IFN-γ secretion over a time course that overlaps the studied period of time. The reasons for the variance in the results might be due to factors such as the treatment protocol and the quantification techniques used.

An association between immunosuppressive therapy and cancer has been reported [25]. Some tumors can be considered as allografts since they possess foreign antigens and are supposedly rejected by an active immune system. The C57BL/6 tumor cell line (B16F10) can grow only in C57BL/6 black mice. This tumor cannot cross histocompatibility barriers. In other words, it cannot grow in other strains of mice. Our preliminary experiments indicated that the C57BL/6 tumor cells grew in BALB/c mice treated with atorvastatin. Atorvastatin-treated BALB/c mice challenged intraperitoneally with tumor cells had extended abdomens, and dissection of one mouse revealed a large mass. In other AS-treated BALB/c mice belonging to the same group tumors started to regress at 18 day post-tumor challenge. Perhaps the effect of atorvastatin was overrun by the active immune system. In agreement...
with the hypothesis that atorvastatin suppresses the immune system, tumor growth rate was enhanced by atorvastatin treatment compared to PBS-treatment in C57BL/6 mice.

4. CONCLUSION

In conclusion, it appears that atorvastatin may increase the number of survival days of skin allografts and promote tumor growth in murine models. The results of the serum pro-inflammatory cytokine analysis of transplant mice does not provide conclusive evidence about the mechanisms behind the immunomodulatory effects of statins. Nonetheless, the prevention of acute rejection suggests that statins therapy may at least down-regulate Th1-mediated immune response, thus allowing better survival of the skin allografts. In addition, the observed findings that atorvastatin induced tumor growth across histocompatibility barriers tend to support this assumption. It could be hypothesized that the immunosuppressive effect of atorvastatin is related to its effect on membrane cholesterol of antigen presenting cells (APC) resulting in hindering the ability of APCs to present antigen to T-lymphocytes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS’ CONTRIBUTIONS

Alexander M. Abdelnoor; designed the research proposal, overlooked the bench work and revised and edited the draft of the manuscript.

Nabil Zeidan; did the bench work and wrote the draft of the manuscript.

Fadi El-Rami; with Nabil Zeidan did the bench work.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

REFERENCES


