REVIEW ARTICLE

Effects of Statins on Angiogenesis and Vasculogenesis

Joan Llevadot and Takayuki Asahara[a]

Servicio de Cardiología. Centro Cardiovascular Sant Jordi. Barcelona. [a]Department of Medicine. Cardiovascular Research. St. Elizabeth's Medical Center. Boston, Massachusetts. EE.UU.

Statins promote the proliferation, migration, and survival of endothelial cells and bone marrow-derived endothelial progenitor cells (angioblasts) by stimulating the serine/threonine protein kinase Akt (also known as protein kinase B) pathway. Like vascular endothelial growth factor (VEGF), the statins promote angiogenesis and vasculogenesis. Therefore, Akt activation may explain some of the beneficial effects of the statins, including postnatal neovascularization.

Key words: *Statins. Angiogenesis. Vasculogenesis.*

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Efecto de las estatinas en la inducción de angiogénesis y vasculogénesis

Las estatinas promueven la proliferación, migración y supervivencia celular de las células endoteliales y las células endoteliales progenitoras (angioblastos) procedentes de la médula ósea a través de mecanismos relacionados con la activación de la serina/treonina proteína cinasa Akt (o proteína cinasa B). De forma similar al factor de crecimiento endotelial vascular (VEGF), las estatinas promueven la angiogénesis y la vasculogénesis. Así pues, la activación de la Akt puede ser responsable de parte de los efectos beneficiosos de las estatinas, incluyendo la neovascularización posnatal.

Palabras clave: *Estatinas. Angiogénesis. Vasculogénesis.*

INTRODUCTION

Statins inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes mevalonate synthesis, the limiting step in cholesterol biosynthesis.[1] The resulting reduction in intracellular cholesterol leads to a compensatory increase in cholesterol uptake by low-density lipoprotein (LDL) receptors and a decrease in plasma cholesterol. The discovery of the statins and their application in subjects with high cholesterol concentrations has made it possible to greatly improve the primary and secondary prevention of coronary artery disease.[2,3] Recently, the effectiveness of the statins in the primary and secondary prevention of coronary artery disease has also been observed in subjects with lower cholesterol levels.[4-6] Aside from reducing LDL cholesterol (C-LDL), the statins have a series of pleiotropic effects on several components of atherosclerosis, including endothelial function, cell migration, inflammation, and the thrombotic tendency of the plaque.[7-12] In normocholesterolemic animals it has been demonstrated that statins have a protective effect against ischemia-reperfusion lesions of the cardiac muscle, probably through mechanisms related to nitric oxide production (NO) by endothelium.[13]

The serine/threonine protein kinase Akt or protein kinase B (PKB) is a multifunctional intracellular regulator of cellular survival, growth and metabolism [14] (Figure 1). In relation to its cardiovascular functions, Akt/PKB acts on the intracellular pathway stimulated by vascular endothelial growth factor (VEGF)[14,15] and angiopoietin,[16-18] promoting cell survival and ensuring adequate vascular development.[19] Constitutive activation of Akt signaling protects cardiomyocytes against apoptosis in ischemia-reperfusion lesions.[20] In addition to its cytoprotective effect, Akt acts as an activator of NO production by the endothelium in response to VEGF and shear stress through its capacity to phosphorylate the endothelial nitric oxide synthase (eNOS) in serines 1179 or 1177,[21,22] thus controlling vasomotor tone.[23] On the other hand, Akt is essential in the migration of endothelial cells to the VEGF-producing focus.[24] Therefore, the capacity of Akt to mediate cell survival, NO production, and VEGF-induced migration suggests that protein kinase Akt can mediate endothelial response to angiogenic stimuli.

Correspondence: Dr. J. Llevadot.
Servicio de Cardiología.
Centro Cardiovascular Sant Jordi.
Via Augusta, 269-273. 08017 Barcelona. España.
E-mail: 27558jlg@comb.es

ABBREVIATIONS

VEGF: vascular endothelial growth factor.
LDL: low-density lipoprotein.
NO: nitric oxide.
PKB: protein kinase B.
eNOS: endothelial nitric oxide synthase.
EPC: endothelial progenitor cells.
FACS: fluorescence-activated cell sorting, a flow cytometry technique.

It has been demonstrated recently that the statins also stimulate the intracellular signaling pathway of protein kinase Akt/PKB[25-27] in endothelial cells[25] and the endothelial progenitor cells (EPC) of bone marrow,[26,27] thus inducing both angiogenesis[25] and vasculogenesis.[26] The effects of the statins on the kinetics of EPC have also been demonstrated in humans by Vasa et al.[28] This article reviews the effect of the statins on the induction of angiogenesis[25] and vasculogenesis[26] through mechanisms related with Akt activation.[25-27]

ANGIOGENESIS AND VASCULOGENESIS

Angiogenesis and vasculogenesis are responsible for the development of the vascular system in the embryo.[29-32] Vasculogenesis is the process of blood vessel formation from endothelial progenitor cells (angioblasts) that migrate and fuse with other endothelial progenitor cells and differentiate into endothelial cells while forming new blood vessels. In contrast, angiogenesis is the process of the extension of the blood vessels that have formed by budding new capillaries through the migration and proliferation of previously differentiated endothelial cells (Figure 2).

It was initially thought that the vasculogenic process was restricted to embryonal development, whereas angiogenesis (which also occurs in the embryo) was the only process involved in neovascularization in adults. However, the paradigm of postnatal neovascularization was reviewed recently and it was discovered that

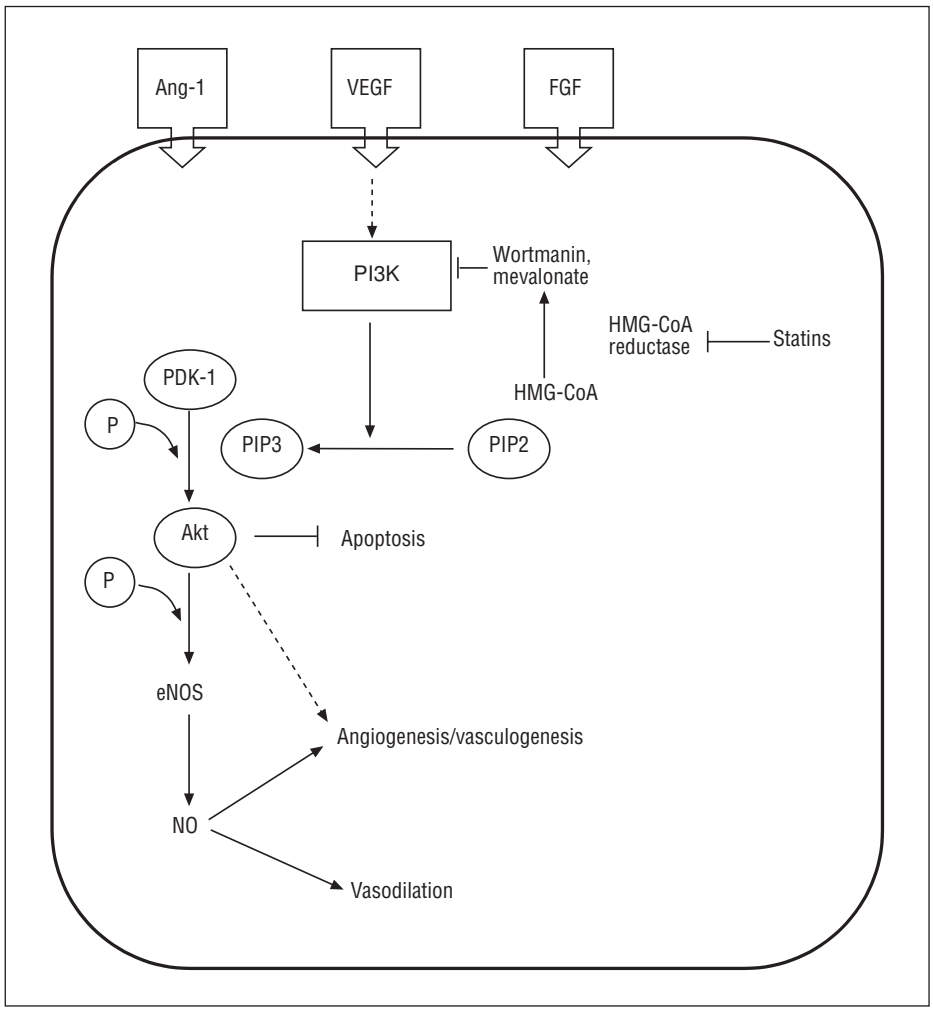

Fig. 1. Statins, Akt signaling and angiogenesis/vasculogenesis. Angiopoietin 1 (Ang-1), VEGF and fibroblast growth factor (FGF), when bound to their membrane receptors, induce the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) by phosphatidylinositol 3-kinase (PI3K). PIP3 formation is necessary for the phosphorylation of Akt protein kinase by PDK-1 kinase. Statin treatment increases the phosphorylation of Akt, while wortmanin (a PI3K inhibitor) prevents it. Mevalonate, the product of HMG-CoA reductase, also inhibits PI3K and the subsequent phosphorylation of Akt. Therefore, the statins, by inhibiting HMG-CoA reductase and the production of mevalonate, increase Akt phosphorylation and, at the same time, the phosphorylation and activation of endothelial nitric oxide synthase (eNOS), nitric oxide synthesis (NO), and a variety of physiological effects induced in angiogenesis and vasculogenesis. Akt also prevents endothelial cell apoptosis.

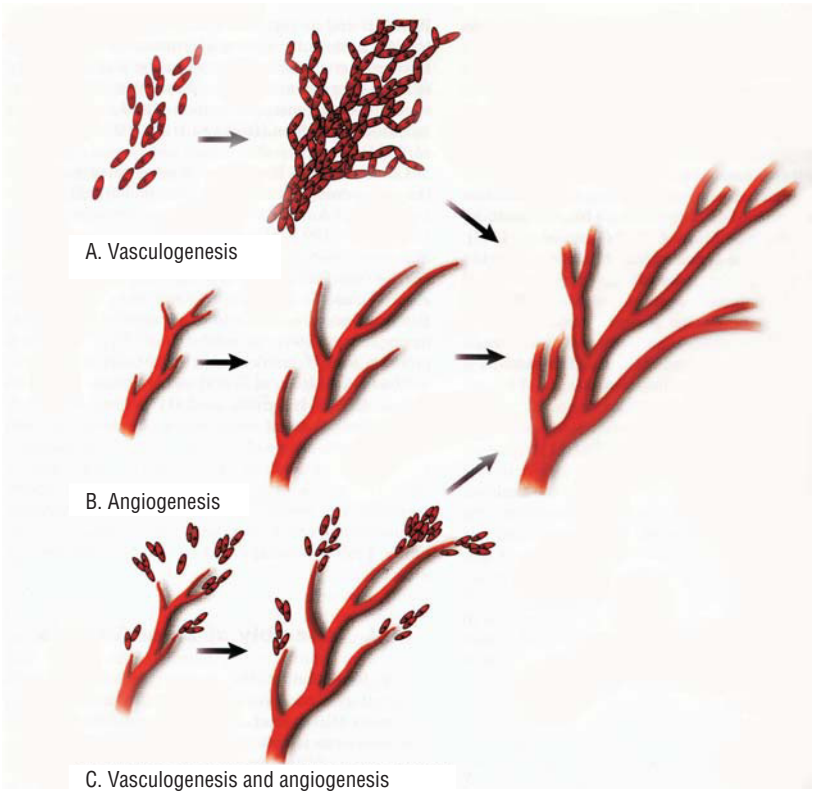

Fig. 2. Schematic representation of angiogenesis and vasculogenesis. (A) Vasculogenesis is the aggregation of angioblasts or endothelial progenitor cells to form blood vessels. Angioblasts coalesce in situ or migrate to form blood vessels in distant sites. (B) Angiogenesis is the formation of new blood vessels from pre-existing vessels by the proliferation and migration of differentiated endothelial cells. (C) Angiogenesis and vasculogenesis also can occur simultaneously. (Taken from Cleaver et al.[29])

endothelial progenitor cells circulating in peripheral blood,[33] are incorporated by neovascularization foci in adult animals,[34] they increase in number in response to tissue ischemia,[35] and they promote the development of collateral blood vessels after their expansion *in vitro* and later transplantation.[36] These studies have established that both angiogenesis and vasculogenesis are responsible for neovascularization in adults.

A third mechanism that probably contributes to the development of collateral vessels is the increase in the size and caliber of pre-existing arteriolar collateral connections, a process called arteriogenesis.[37] The presence and number of these native collateral vessels vary widely between individuals and species. When a vessel becomes occluded, there is an increase in the velocity of blood flow through pre-existing collateral vessels and an increase in luminal shear stress, factors that contribute to the maturation of the collateral vessels, particularly those of intermediate size.

Methods of study *in vitro*

The development of techniques for the culture of endothelial cells has made it possible to understand the processes involved in angiogenesis.[38] Endothelial cells in culture retain the capacity to respond to factors that stimulate or inhibit angiogenesis as well as the capacity to form endothelial tubes *in vitro*. Assays of cellular proliferation allow the effect of a certain substance on endothelial cell proliferation to be analyzed. The migration of endothelial cells toward a solution containing a certain substance, separated by a permeable membrane, can be examined in a Boyden chamber. The mechanisms of tubular endothelial formation and the effect of a certain substance on tubules can be studied using two-dimensional or three-dimensional assays. With these techniques, the processes of formation of the endothelial lumen and the influence of the extracellular matrix on capillary development are analyzed.[38] Finally, cultures of endothelial cells allow the study of the molecular pathways involved in angiogenesis processes.

Recently, by using cell selection techniques and special culture media, techniques developed to study differentiated endothelial cells have been used to study endothelial progenitor cells.[33-36]

Methods of study *in vivo*

Although techniques *in vitro* enable a preliminary analysis to be made of angiogenesis and vasculogenesis, many factors that can influence or modulate these processes *in vivo*.[38] In order to study the mechanisms of blood vessel formation *in vivo*, different biological

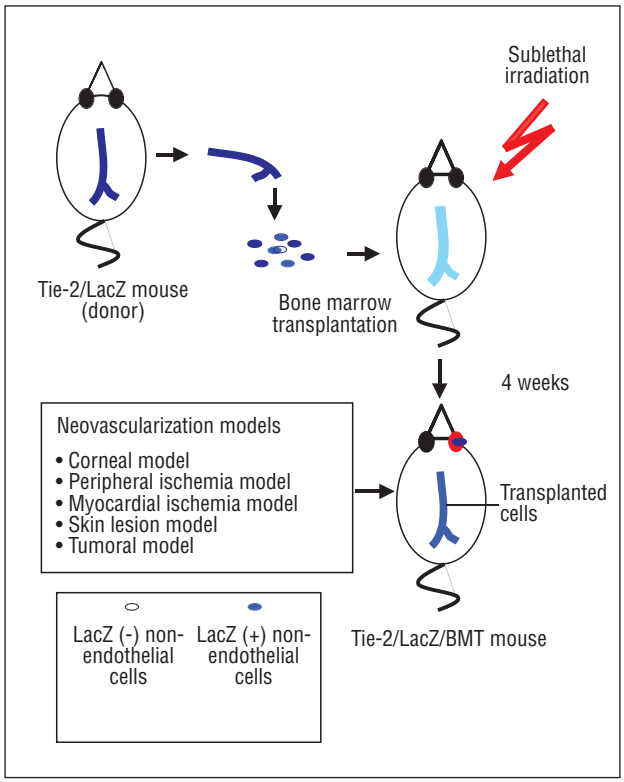

Fig. 3. Murine model of bone marrow transplantation for the study of vasculogenesis. The bone marrow donors used are transgenic mice that constitutively express the gene LacZ (which encodes beta-galactosidase) regulated by a specific endothelial promoter, Tie-2. The bone marrow is extracted and transplanted to a mouse receptor whose bone marrow has been sublethally irradiated. After a period of 4 weeks to achieve the reconstitution of the transplanted bone marrow, one or more interventions are made in the mouse receptor (the case shown is a corneal model) to stimulate neovascularization. After these interventions, the animals are sacrificed and a histological study is made to detect beta-galactosidase expression. With X-GAL stain, cells that express beta-galactosidase acquire a bluish color. The use of a specific promoter for endothelial cells allows the blue cells that have been incorporated into neovascularization foci to be identified as cells of endothelial lineage.

systems have been developed to quantify or demonstrate the effect of a certain substance: mouse cornea models, chicken embryo chorioalantoid membrane, or spongy implants.[38] These systems require the sacrifice of the animal so they only capture the effect in a specific moment. In order to study the temporal evolution of events in a single tissue, intravital microscopy techniques have been developed for the skin on the back or skull of the mouse.[39] Finally, the development of genetic engineering techniques has made it possible to study the effect of the suppression (knock-out) or addition (knock-in) of a gene to the processes of vasculogenesis and angiogenesis.

The study of postnatal vasculogenesis and the effect of certain substances on vasculogenesis processes has been possible thanks to the use of a flow-cytometry techniques, fluorescence-activated cell sorting (FACS), special techniques for culturing endothelial progenitor cells (EPC) from peripheral blood, and murine bone marrow transplantation models.[33-36] FACS is used to detect and quantify EPC in peripheral blood using antibodies against the surface antigens of these cells. The influence of drugs or growth factors on the number of these cells in peripheral blood can be analyzed this way. The special techniques of cell selection and culture developed by our group have also made it possible to detect and quantify EPC. In the murine model of bone marrow transplantation, bone marrow cells from a mouse donor are transplanted to a mouse receptor with a gene that encodes the elaboration of a substance that allows it to be detected later (Figure 3). In our case the gene encoded the elaboration of beta-galactosidase by endothelial cells. Selective expression is achieved because this gene is regulated by a specific endothelial cell promoter, Tie-2, in the mouse donor. Therefore, only endothelial cells from the bone marrow of the animal donor will express the beta-galactosidase that can be detected in the animal receptor. If the biological experiments described above are performed after bone marrow transplantation in the animal receptor, we can analyze the influence of a certain substance on vasculogenesis by quantifying the number of endothelial progenitor cells derived from the bone marrow.

EFFECT OF STATINS ON THE INDUCTION OF ANGIOGENESIS AND VASCULOGENESIS

Investigations made in our laboratory and elsewhere have demonstrated that the statins stimulate the intracellular signaling pathway of the protein kinase Akt/PKB,[25-27] which promotes both angiogenesis[25] and vasculogenesis.[26] In addition, Vasa et al also have been able to demonstrate in humans the effects of statins on the kinetics of EPC.[28]

Effects *in vitro* of statins

The statins rapidly activate protein kinase Akt/PKB in endothelial cells[25] and EPC,[26,27] thus increasing the phosphorylation of eNOS and the subsequent production of NO. Akt activation by statins promotes the proliferation, migration, and cellular survival of endothelial cells and EPC, as well as the formation of the vascular structure. In addition, the inhibition of Akt by the use of adenovirus that encode dominant negative forms of Akt causes inhibition of the effects induced by statins. The potential of statins in tissue regeneration processes was demonstrated earlier in osteoblasts. In these cells, statins increased the proliferation and level of activity, consequently increasing bone formation.[40]

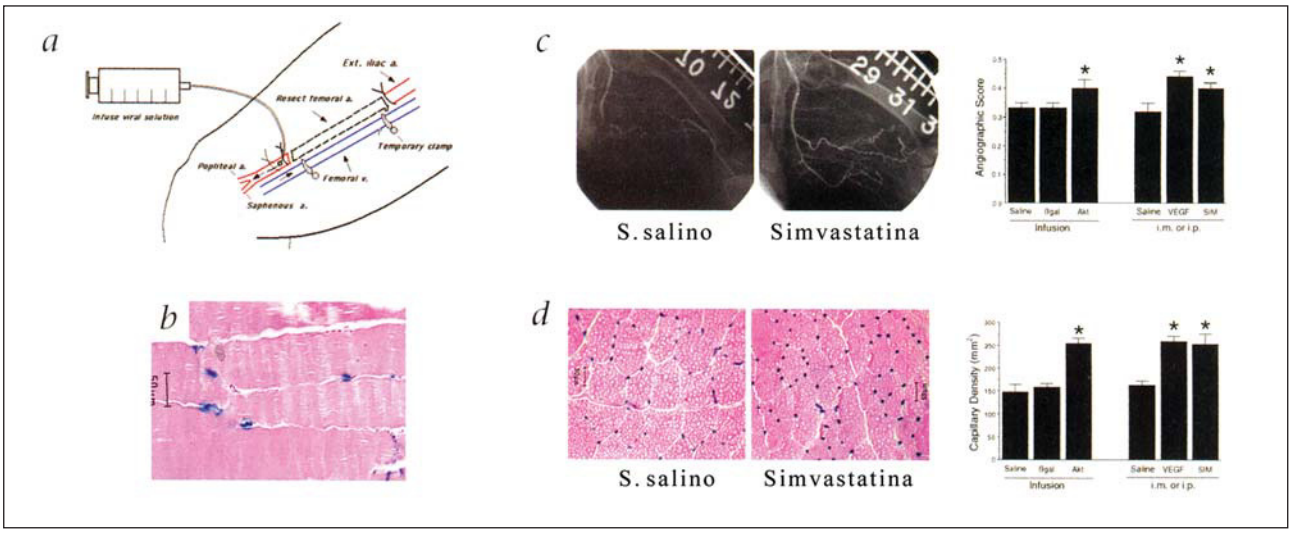

Fig. 4. Statin-induced increase in neovascularization in the leg of a rabbit in response to unilateral resection of the femoral artery. *a*) The femoral artery and its branches are dissected. The genetic transfer to endothelium is made by infusion of adenovirus that encode beta-galactosidase (Ad-βgal) or Akt (Ad-myrAkt) in the distal femoral artery and incubation during 15 min while temporarily clamping the femoral vein. *b*) On the third day, the gastrocnemius muscle is extracted and the X-GAL stain is made to determine the transgenic distribution in histological preparations stained with hematoxylin-eosin. *c*) Angiography through the internal iliac is performed to analyze the formation of collateral vessels in different treatment groups. In angiographies made at 40 days, an increase in the formation of collateral vessels is visible in the animals that received 0.1 mg/kg simvastatin by intraperitoneal injection compared to the animals that were intervened but were only injected saline solution. Quantitative analysis of the collateral vessels was made in the control group, simvastatin-treated group, and the group of animals that received an intramuscular injection of Ad-VEGF. The angiographic score was analyzed in the experimental groups that received an infusion of saline solution, Ad-βgal and Ad-myrAkt 31 days after surgery. *d*) Staining for alkaline phosphatase in the adductor muscle of the ischemic leg revealed a greater capillary density in the group of animals treated with simvastatin with respect to the group control 40 days after surgery. The data from each experiment are presented as mean±SD (n=6 rabbits in each of the treatment groups, **P*<.05 compared to the group of infusion or injection of saline solution, by single-tailed analysis of the variance). (Taken from Kureishi et al.[25])

Although the mechanisms of Akt activation by statins are not accurately known, it is probable that phosphatidylinositol 3-kinase (PI3K) signaling is involved because this process is blocked by wortmanin and LY294002, two inhibitors of the enzyme (Figure 1). In addition, the inhibition of HMG-CoA reductase is necessary, since the activation of Akt by simvastatin was inhibited by the addition of mevalonate to incubation (Figure 1). Mevalonate is necessary, not only for the biosynthesis of cholesterol, but also in the production of ubiquinone, dolichols and isoprenoids, which are essential in several cell processes. Although the statins stabilize the messenger RNA (mRNA) of eNOS by modifying isoprenoid synthesis,[41] we did not observe changes in protein synthase eNOS values. In this sense, it is important to emphasize that the increase in mRNA concentration was later (24 h) than the activation of eNOS phosphorylation by Akt (15 min). This shorter activation time is consistent with the changes induced by statins in the production of NO and in the vasodilation observed in aortic annuli *ex vivo*.[42]

Effects *in vivo* of statins

The statins and activation of intracellular Akt signaling promote angiogenesis in models of peripheral ischemia developed in normocholesterolemic rabbits.[25] In animals that received statins, higher perfusion pressures, a larger number of collateral vessels, and a greater capillary density were observed (Figure 4). On the other hand, the statins increase the number of endothelial progenitor cells in peripheral blood in both mice[26,27] and humans.[28] In addition, the statins increase corneal neovascularization in normocholesterolemic mice, in part due to vasculogenesis from EPC obtained from bone marrow (Figures 3 and 5).[26] Using the murine model of bone marrow transplantation, it was possible to demonstrate a greater number of EPC from bone marrow in the corneas of mice treated with statins. Therefore, statins have an important effect on EPC kinetics, as had been demonstrated previously with VEGF or granulocyte and monocyte-colony stimulating factor (GM-CSF),[35] and statin-induced mobilization of these cells could increase postnatal neovascularization.

CONCLUSIONS

Statins promote the proliferation, migration, and cellular survival of endothelial cells and EPC obtained from bone marrow through mechanisms related to the activation of serine/threonine protein kinase Akt or

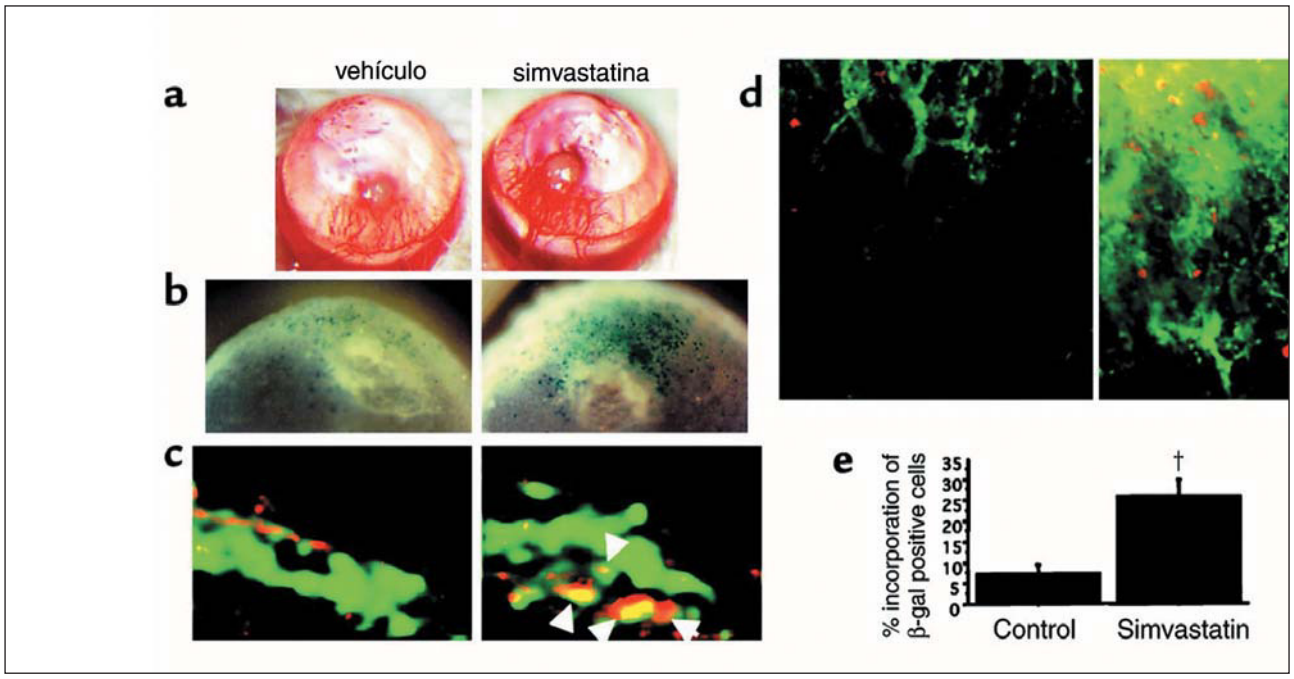

Fig. 5. Increase in corneal neovascularization by statin-induced vasculogenesis. *a*) Representative photographs showing corneal neovascularization (left, vehicle; right, simvastatin). *b*) X-gal staining of whole corneas. The bluish points are cells that express beta-galactosidase (left, vehicle; right, simvastatin). *c*) Representative microphotographs of the histochemical study of fluorescence in paraffin-embedded corneas from Tie2/LacZ/BMT mice (left, vehicle; right, simvastatin). The presence of doubly positive cells is evidence that endothelial progenitor cells (EPC) obtained from bone marrow have been incorporated by the neovascularization foci (vasculogenesis). The red color in this case indicates beta-galactosidase and the green color shows the specific endothelial marker isolectin B4. The doubly positive cells (yellow color) are EPC obtained from bone marrow that have been incorporated by the new vessels. *d*) Representative microphotographs of the histochemical study of fluorescence in whole corneas from Tie2/LacZ/BMT mice (left, vehicle; right, simvastatin). The red color shows beta-galactosidase and the green color shows the specific endothelial marker lectin BS-1. *e*) Quantification of the EPC derived from transplantation incorporated in the neovasculature. Expressed as the ratio between number of EPC and total number of endothelial cells forming the new vessels. **P*<.05. (Taken from Llevadot et al.[26])

PKB. In a way similar to VEGF, statins promote angiogenesis and vasculogenesis. Therefore, Akt activation can be responsible for some of the beneficial effects of statins, including postnatal neovascularization.

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