Effect of candesartan on the expression of sclera-choroidal intercellular adhesion molecule-1 in hypercholesterolemic models

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OBJECTIVE: To evaluate the effect of blocking the angiotensin II AT-1 receptor by the systemic administration of candesartan on the expression of intercellular adhesion molecule-1 in the sclera and choroid of hypercholesterolemic rabbits.

METHODS: New Zealand rabbits were divided into 3 groups, as follows: GI, which was fed a rabbit standard diet; GII, which was fed a hypercholesterolemic diet; and GIII, which received hypercholesterolemic diet plus candesartan. Samples of the rabbits’ sclera and choroid were then studied by hematoxylin-eosin staining and histomorphometric and immunohistochemical analyses for intercellular adhesion molecule-1 expression.

RESULTS: Histological analysis of hematoxylin- and eosin-stained sclera and choroid revealed that macrophages were rarely present in GI, and GII had significantly increased macrophage numbers compared to GIII. Moreover, in GII, the sclera and choroid showed a significant increase in thickness in comparison to GI and GIII. GIII presented a significant increase in thickness in relation to GI. Sclera and choroid immunohistochemical analysis for intercellular adhesion molecule-1 expression revealed a significant increase in immunoreactivity in GII in relation to GI and GIII. GIII showed a significant increase in immunoreactivity in relation to GI.

CONCLUSION: Candesartan reduced the expression of intercellular adhesion molecule-1 and consequently macrophage accumulation in the sclera and choroid of hypercholesterolemic rabbits.

KEYWORDS: Renin-Angiotensin System; Cholesterol; Cell Adhesion Molecules; Macrophages; Choroid; Sclera; Macular Degeneration.

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In atherosclerosis, macrophages decrease atherosclerotic plaque formation (7). In ARMD, experimental studies have shown that the inhibition of macrophage functions improves the neovascular condition (8). Thus, drugs that inhibit the mobilization and/or adhesion of monocytes to the inflamed tissue may be effective not only for treating atherosclerosis, but also for preventing the development of ARMD. Additionally, previous studies have suggested that the activation of the renin-angiotensin system is related to the physiopathogenesis of ARMD and that the blockade of this system is related to its regression (9,10).

The objective of this study was to determine the effects of candesartan-mediated blockage of the angiotensin II AT-1 receptor on ICAM-1 expression and the accumulation of macrophages in the sclera and choroid of hypercholesterolemic rabbits.

**MATERIALS AND METHODS**

**Experimentation environment**

The procedures described in this study were performed at the Surgical Technique Laboratory at Pontificia Universidade Católica do Paraná, Graduation, Curitiba/PR (PUC-PR) and the Study Center of the Angelina Caron Hospital. The animals were kept in an animal facility (macroenvironment) on 12/12-hour light cycles, with air changes and room temperature controlled between 19 and 23°C. During the experimental period, the animals were fed water and standard Nuvital® rabbit chow (Nuvital, Colombo, Brazil) ad libitum. Animals used and experiment design

In total, 33 white adult male rabbits (New Zealand) with a mean age of 3.6 months were selected for this study. The animals were divided into three groups as follows: Group I (GI), normal diet group, 8 rabbits; Group II (GII), hypercholesterolemic diet group, 13 rabbits; and Group III, candesartan-treated hypercholesterolemic group, 12 rabbits. During the 56-day study, the animals in GII and GIII were fed a specific diet, including 5% cholesterol (Sigma-Aldrich®). The daily amount of food per animal was 600 grams (11). The diet, Nuvital® Lab (Nuvital, Colombo, Brazil) plus 1% cholesterol (Sigma-Aldrich®). The daily amount of food per animal was 600 grams (11). The diet, Nuvital® Lab (Nuvital, Colombo, Brazil), does not alter lipid metabolism in the animals. For the GIII group, 2.62 mg/kg/day of candesartan cilexetil (AstraZeneca, London, United Kingdom) was administered by oral gavage from day 1 through day 56. On day 56, the animals from GI and GIII were euthanized. Animals from GI were fed only Nuvital® Lab chow and were euthanized on day 28. Serum levels of total cholesterol, triglycerides and glucose were determined in fasting rabbits at the beginning of the experiment and the time of euthanasia. Euthanasia was performed by intravenous anesthesia with 5 ml pentobarbital, and the rabbit eyes were immediately placed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate, pH 7.4, for 4 hours. The eyes were then used for the morphometrical and immunohistochemical analyses of the choroid-sclera complex.

**Tissue preparation**

After fixation, the samples were evaluated macroscopically. A coronal section was performed at the level of the optic nerve, and the ocular globe was divided into two equal halves. The lower half was stored for future study, and the upper half was dehydrated, diaphanized and embedded in paraffin using a Leica® TP 1020 – Automatic Tissue Processor (Leica, Wetzlar, Germany). A Leica® EG1160 paraffin-embedding device was used to produce the paraffin blocks. A Leica® RM2145 Microtome was used to obtain 5-µm-thick sections for histology. The sections were placed on glass slides smeared with albumin, stained with hematoxylin-eosin and mounted with 24 × 900-mm coverslips using Entellan Mounting Media (Merck, Darmstadt, Germany).

**Scleral and choroidal histomorphometry**

For quantitative analysis, the evaluated sections were stained with hematoxylin-eosin. With the aid of a 4x objective lens and a blue overhead projector marker, the semi-sectioned ocular globe was divided manually into 10 equal segments from the pars plana to the contralateral pars plana. Segment images were obtained (10 images of each eye) using an Olympus BX50 microscope (Olympus, Tokyo, Japan) connected to a Sony BX50 camera (Sony Corporation, Tokyo, Japan). In each captured image, four linear morphometric measurements were taken using the Image ProPlus® software supplied with the BX50 (Media Cybernetics Inc., Silver Spring, MD). Accordingly, 40 measurements were taken from each eye to evaluate choroidal and scleral thicknesses. Finally, the mean of the four measurements of each of the 10 segments was obtained. The results are expressed in micrometers.

**Tissue preparation and immunohistochemical analysis**

The histological slices were first blocked against endogenous peroxidase. They were then washed in deionized water and incubated in a moist chamber at 95°C for 20 minutes for antigen retrieval, after which the endogenous peroxidase was blocked again. Primary mouse anti-ICAM-1 monoclonal antibody (1:100, Novocastra, Newcastle upon Tyne, UK) was then added to the slides. Next, the slices were incubated with a secondary antibody, Envision® System labeled polymer-HRP anti-mouse (DakoCytomation, Carpinteria, CA, USA), at room temperature for 30 min. The slices were then stained by incubation with freshly prepared DAB substrate (DakoCytomation, Carpinteria, CA, USA) for 3 to 5 minutes. Finally, the slides were counterstained with Mayer hematoxylin and mounted.

Positive and negative controls were used in all evaluations, and a masked observer initially analyzed the slides. In this analysis, positive and negative results for the marker detected by anti-ICAM-1 were recorded. The positive areas, which were a brownish color, were studied utilizing color morphometry. This procedure was performed by capturing images of three consecutive fields that were close to the optic nerve head with a BX50 Olympus microscope (Olympus, Tokyo, Japan) coupled to a Sony camera (Sony Corporation, Tokyo, Japan), Model DXC-107A, using a 40x objective. The computer program, Image Pro Plus, enabled the observer to select and color the positive areas and automatically determined the immunoreactive area in square micrometers. The data obtained were compiled in a Microsoft Excel spreadsheet (Redmond, WA) for statistical analysis. The immunoreactive area represents the sum of all positive areas in each of the three fields studied. This color morphometry method was previously used in other studies (12).
RESULTS

The differences in thickness of the sclera-choroidal complexes among the groups are shown in Figure 1. Table 1 shows the thicknesses of the sclera and choroid of the three groups in micrometers.

Sclera and choroid morphometric analysis

A significant increase in the thickness of the sclera and choroid was observed in GII compared to GI and GIII (p<0.001). The thickness in GIII was significantly higher compared to GI (p=0.008).

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Statistical analysis

To compare the defined groups for treatment in relation to the quantitative variables, one-way analysis of variances (ANOVA) was performed. Multiple comparisons were made using the LSD (least significant difference) test. The baseline and euthanasia time evaluations within each group were analyzed using Student’s t-test for paired samples. Normality was assessed with the Shapiro-Wilk test. Logarithmic transformation was performed for variables showing skewed data. Values of p<0.05 indicated statistical significance. Statistica 8.0 (StatSoft. Inc. 2300 East 14th Street Tulsa, Oklahoma, USA) was used for data processing.

Ethics

Animals were handled in compliance with the principles established by the Association for Research in Vision and Ophthalmology (ARVO), and the protocol was approved by the Animal Research Committee of the Pontificia Catolica Universidade do Parana.

DISCUSSION

In this study, rabbits were fed a cholesterol-enriched diet, and the effects of candesartan on ICAM-1 expression and macrophage accumulation in the sclera and choroid were assessed. As has been demonstrated in the cardiovascular system (3,13), dyslipidemia caused significant increases in ICAM-1 expression in the sclera and choroid in GI and GII animals (Table 2). The role of immunoglobulins in the formation of atheromatous plaques in the great vessels is well defined. ICAM-1 induces the adhesion of the circulating monocytes to the vascular endothelium. Once in the arterial wall intima, these cells are transformed into macrophages, which contribute to the formation of atherosclerotic plaque (3,13). In the normal eye, ICAM-1 immunoglobulin is expressed at low levels in the choroid and retina vascular endothelium, retinal pigment epithelium (EPR), Bruch’s membrane and external limiting membrane (14-16). However, in pathological conditions, such as in exudative ARMD, a significant increase in ICAM-1 expression has been observed in the EPR cells and choroidal complexes among the groups are shown in Figure 1. Table 1 shows the thicknesses of the sclera and choroid of the three groups in micrometers.

Table 1 - Sclera and choroid thicknesses in micrometers.

| Variable                      | Group | n  | Mean | Median | Min  | Max  | Standard Deviation | p-value* |
|-------------------------------|-------|----|------|-------|------|------|--------------------|----------|
| Choroid and Sclera Morphometry| GI    | 8  | 232.9| 221.4 | 192.9| 307.7| 35.8               |          |
|                               | GII   | 13 | 382.5| 387.9 | 254.0| 475.0| 60.5               |          |
|                               | GIII  | 12 | 284.8| 278.5 | 240.0| 390.5| 43.4               | <0.001   |

*ANOVA, p<0.05.

GI vs. GII: p=0.001; GI vs. GIII: p=0.008; GI vs. GIII: p<0.001.

GI - Normal diet group; GII - Cholesterol-enriched diet group; GIII - Cholesterol-enriched diet plus candesartan diet group.

number of histiocytes were observed in the sclera-choroidal complex from GII animals (Figure 1.2). The sclera-choroidal complexes from GIII animals (Figure 1.3) had fewer histiocytes than GII, but more than GI.

Immunoreactivity to ICAM-1

The hypercholesterolemic group (GII) exhibited a significant increase in immunoreactivity to ICAM-1 compared to GI and GIII (p<0.001). Immunoreactivity was also significantly increased in GIII in relation to GI (p=0.041). Table 2 shows the values of the areas immunoreactive to ICAM-1 antibody in square micrometers, as determined by the color morphometry technique.

In GI animals, the choroid-sclera complex was predominantly stained blue, revealing a low level of ICAM-1 immunoreactivity (Figure 2.1). Conversely, in GII animals, the choroid-sclera complex was predominantly orange, revealing a high level of immunoreactivity (Figure 2.2). Figure 2.3, which represents the GII choroid-sclera complex, shows that the ICAM-1 immunoreactivity level in GII was higher than in GI (Figure 2.1) and lower than in GIII (Figure 2.2).

Lab variables

At baseline, there were no significant differences in the laboratory variables of the animals, both within the same group and between groups. Additionally, the rabbits did not show significant alterations in their glycemic values at the time of euthanasia. Conversely, at the time of euthanasia, total cholesterol, which was approximately 42 mg/dl at baseline, reached 2200.00 mg/dl in GII and 923.6 mg/dl in GIII. These increases were significant compared to GI. Triglyceride was approximately 53 mg/dl at baseline; at the end of the experiment, it increased to 168 mg/dl in GII and 171 mg/dl in GIII, which was a significant increase compared to GI.

Sclera and choroid analysis with hematoxylin-eosin

The sclera and choroids obtained from animals in GI had very few histiocytes (Figure 1.1). Conversely, a large number of histiocytes were observed in the sclera-choroidal complex from GII animals (Figure 1.2). The sclera-choroidal complexes from GIII animals (Figure 1.3) had fewer histiocytes than GII, but more than GI.

Figure 1 - C: Choroid; S: Sclera; H: Histiocytes. Figure 1 shows the sclera and choroid from the GI group. They were of normal thickness, with very few histiocytes. Figure 2 shows the sclera and choroid from the GII group. They were of increased thickness, which was mainly due to the high number of histiocytes. Figure 3 shows the sclera and choroid from the GII group. There were fewer histiocytes in GIII than in GII, and the thickness of the sclera and choroid were higher than in GI and lower than in GII.

Figure 2.1, which represents the GI sclera choroid complex, reveals a high level of immunoreactivity (Figure 2.2). Conversely, in GII animals, the sclera choroid complex from GII animals (Figure 2.1). The sclera-choroidal complexes among the groups are shown in Figure 1. Table 1 shows the thicknesses of the sclera and choroid of the three groups in micrometers.
vessels (9,17-18). Due to the role of ICAM-1 in regulating leukocyte adherence (5), the increased expression of this immunoglobulin may account for the increase in macrophages and, consequently, the increased thickness of the sclera-chorioid complex (Table 1), which was observed here and previously in other studies (12,19).

In this study, by inducing hypercholesterolemia, we attempted to create a physiopathogenic condition that is similar to ARMD, in which the accumulation of oxidized lipids attracts macrophages, which can clear the oxidized lipids (4,20). Besides producing vascular endothelial growth factor (VEGF), macrophages are also sources of pro-inflammatory and pro-angiogenic cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF), which mediate inflammatory responses and contribute to the formation of the sub-retinal neovascular membrane (21-25). It is important to note that oxidized low-density lipoprotein (LDL) is a powerful stimulus that increases the expression of chemotactic and adhesion molecules, which then attracts macrophages to the inflammatory tissue. This process occurs in both atherosclerosis and ARMD (4,20). It is also known that not only the oxidized but also the native LDL induces the increase in the ICAM-1 expression (26). Hence, LDL, which is deposited on the Bruch’s membrane, formed by the degradation of photoreceptor outer segments (20-27), can increase the expression of these adhesion molecules, a determinant aspect in the increase of macrophages and development of AMRD.

As AMRD and atherosclerosis present similar physiopathogenic mechanisms (1,2,3,13), it is expected that the drugs used to treat these diseases will have equivalent characteristics and performances. Candesartan blocks the angiotensin II AT-1 receptor, thereby preventing angiotensin-mediated effects. It is important to note that angiotensin II induces the increased expression of cytokines (IL-6, IL-1, TNF-α), chemokines (monocyte chemotactic protein-1 – MCP-1) and leucocyte adhesion molecules (selectins P, E and L; integrins α1 and b2; VCAM; ICAM) (28,29). Moreover, among all of the renin-angiotensin system (RAS) blockers, candesartan is considered to have the strongest affinity to the AT-1 receptor. This class of drug is widely used to treat arterial hypertension and has been shown to reduce the levels of soluble ICAM-1 in hypercholesterolemic patients (30). Experiments have demonstrated that the blockade of AT-1 receptors of the RAS reduces the numbers of macrophages, monocytes and T lymphocytes in atheromatous plaques (7). In the present study, the use of candesartan to block the angiotensin II AT-1 receptor (GIII) significantly reduced the expression of ICAM-1 in the sclera and choroid compared to GII (p<0.001) (Table 2), which may have reduced the thickness of the GIII sclera-choroid complex in relation to GII (p<0.001) (Table 1).

In a recent study, our group showed that olmesartan reduced the expression of MCP-1 and the accumulation of macrophages in the sclera and choroid of hypercholesterolemic rabbits (31). Besides blocking the binding of angiotensin II to the ATI receptors, olmesartan exerts a mild modulatory activity on PPAR-gamma receptors (32). Thus, the olmesartan-mediated decrease of macrophages in the sclera-choroid complex could also be accounted for by the drug’s agonistic effect on the PPAR-gamma receptors (12). In the present study, the RAS blockade was induced by candesartan, which does not exert this agonistic effect on PPAR-gamma receptors. This finding shows that RAS blockade alone can decrease the accumulation of macrophages in the sclera-choroid complex, as occurs in the great vessels (33).

It has been demonstrated that the blockade of the angiotensin II AT-1 receptor reduces the presence of macrophages and the expression of inflammatory markers in the RPE and choroid, thus significantly affecting the formation of the sub-retinal neovascular membrane (9,10,34). Hence, by playing an important role in the pathological processes of angiogenesis and inflammation (35), the angiotensin II AT-1 receptor may represent a new target for the treatment of ARMD.

In this study, we used immunohistochemistry to analyze the sclera and choroid of hypercholesterolemic rabbits. Immunohistochemistry, when used on paraffin-embedded material, enables the researcher to locate and identify proteins in the analyzed tissues. The authors acknowledge that while the Western blot technique offers high sensitivity for detection and would improve the analysis of the studied protein, it usually requires fresh or frozen tissues. As the ocular globes were fixed in paraformaldehyde and then embedded in paraffin, it was not possible to complement the study with the Western blot technique.

### Table 2 - Area showing immunoreactivity to ICAM-1 antibody, in square micrometers, obtained from the color morphometry analysis.

| Variable                                | Group | n  | Mean       | Median    | Min | Max      | Standard Deviation | p-value* |
|-----------------------------------------|-------|----|------------|-----------|-----|----------|--------------------|----------|
| Area immunoreactive to ICAM-1          | GI    | 8  | 607.0      | 218.5     | 50.2| 2200.4   | 858.7              |          |
|                                         | GII   | 13 | 3600.2     | 3288.7    | 1867.7| 6368.8   | 1377.3             |          |
|                                         | GIII  | 12 | 1231.3     | 1267.6    | 119.9| 3048.4   | 951.3              | <0.001   |

*Nonparametric Kruskal-Wallis test, p<0.05.
GI - Normal diet group; GII - Cholesterol-enriched diet group; GIII - Cholesterol-enriched diet plus candesartan diet group.

Figure 2 - C: Choroid; S: Sclera. 1) GI sclera and choroid. The prevalence of the bluish color demonstrates the low levels of immunoreactivity to ICAM-1. 2) GII sclera and choroid. The prevalence of the orange color demonstrates the high levels of immunoreactivity to ICAM-1. 3) GIII sclera and choroid. The orange areas indicate immunoreactivity to ICAM-1. Specifically, this reactivity was higher than in GI and lower than in GII.
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AUTHOR CONTRIBUTIONS

Torres RJ participated in the study conception and design, analysis and interpretation of data, manuscript writing, final approval for manuscript submission and general supervision of the research group. Luchini A participated in data collection and manuscript writing. Torres RR participated in data collection, study planning and final manuscript revision for submission. Oliveira LR participated in the critical review of the manuscript, data analysis and interpretation and final approval for manuscript submission. Torres CL and Torres RA participated in data collection, data analysis and interpretation and final approval for manuscript submission. Olandoski M participated in statistical analysis and final approval for manuscript submission. Nagashima S provided material and technical and administrative support and participated in statistical analysis and final approval for manuscript submission. Noronha I participated in critical review of the manuscript, general supervision of the study group and final approval for manuscript submission. Percoma DB participated in final approval for manuscript submission and was responsible for the research fundraising and general supervision of the research group.

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