Resident Peritoneal Inflammatory Cells are Pivotal in the Development of Experimental Atherosclerosis

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Aim: Based on evidence that ionizing radiation can ameliorate chronic and autoimmune diseases in patients and experimental animals, we investigated the effects of radiation on the induction and development of experimental atherogenesis.

Methods: Male New Zealand rabbits were divided into 5 groups and given an atherogenic diet for 90 days. Peritoneal and thoracic areas (9 Gy) were irradiated on the 1st and 45th days for groups 1 and 2, the 45th day for groups 3 and 4, and not at all for group 5. Prior to irradiation, the peritoneal cavity of animals from groups 1 and 3 was washed with buffered saline. Cells collected by peritoneal washing were reinfused into the peritoneal cavity of the same animal after irradiation. Animals from groups 2 and 4 were intraperitoneally injected with saline as a control.

Results: Despite similar lipid profiles among the experimental groups, the percentage of aortas covered by plaques was remarkably reduced (p<0.001) among animals submitted to irradiation (groups 2 and 4). These differences were completely abolished in irradiated animals reconstituted with their own peritoneal cells.

Conclusions: These findings point to an important role of resident inflammatory peritoneal cells in experimental atherogenesis.


Key words: Atherogenesis, Rabbits, Peritoneal cells, Ionizing radiation

Introduction

Atherosclerosis is a chronic inflammatory disease of multiple origins characterized by an accumulation of lipoproteins and mononuclear cells in the subendothelial space of blood vessels. The initial stages of the disease involve the recruitment of blood leukocytes, while later stages involve many inflammatory mediators, regulated by cells of both the innate and adaptive immune systems. Recently, the role of different leukocyte subpopulations has been proposed in the pathophysiology of this disease. In this context, the contribution of these cells to early atherogenesis has emerged, and details regarding the origin and type of the inflammatory cells involved in this process provide insights for new approaches to therapy.

Several reports have demonstrated that periodic irradiation known as “total lymphoid irradiation” can improve the health and survival of mice in certain autoimmunity models (reviewed by Loor et al.) as well as humans.

Recently, Brito et al. investigated the possible role of B-1 cells in the evolution and fate of systemic lupus erythematosus (SLE) in female lupus-prone New Zealand Black/New Zealand White (NZB/NZW) F1 mice. The investigation was based on the fact that B1-b cells reside within the peritoneal cavity...
in an extra medullar reservoir, being capable of migrating to distant lesions and spontaneously proliferating in stationary cultures of adherent peritoneal cells. Interestingly, these cells have a hybrid monocyte/macrophage pattern. An increased rate of differentiation into adherent phagocytic cells was triggered by components of the extracellular matrix, inducing the B-1 cells to produce high levels of several cytokines.

In addition, having been completely destroyed by ionizing radiation, the cells repopulated the peritoneal cavity in 45 days in mice. Further, B-1 cells participate in the genesis of inflammatory giant cells, are endowed with tolerogenic properties, and facilitate the growth and metastatic potential of experimental melanoma. e Brito et al. demonstrated that, in this particular model of SLE (NZB/NZW) F1 mice, irradiation significantly halted the development of SLE, even though B-1 cells seemed not to be affected by radiation in (NZB/NZW) F1 mice. Interestingly, a dramatic decrease in mortality in irradiated animals was observed since no control mice survived over 10 months, whereas 80% of irradiated specimens were alive at 16 months of age without symptoms of lupus disease.

Here we investigated the possible role of resident inflammatory peritoneal cells in the induction and development of atherogenesis by diet in rabbits. The results suggested that selective irradiation abrogated atherogenesis in the model and induced significant remission of established lesions. Still, these results seem to be dependent on the presence of resident inflammatory peritoneal cells.

### Methods

#### Animals

Male New Zealand white rabbits were kept in individual cages on a 12-hour dark/light cycle and fed a 0.5% cholesterol-enriched diet for 90 days plus water ad libitum. The protocol was in accordance with the institution’s guidelines for the care and use of laboratory animals and was approved by the local ethics committee. As shown in Table 1, animals were randomly assigned to one of five groups: groups 1 and 2 received irradiation of the peritoneal and pleural cavities on the 1st and 45th days, groups 3 and 4 were similarly irradiated but only on the 45th day; and group 5 was not irradiated. Prior to irradiation, the peritoneal cavity of the animals from groups 1 and 3 was washed with a 0.9% saline solution in order to collect resident cells. The cell suspension was maintained at 4°C. Following irradiation, the cell suspension obtained from each rabbit was re-inoculated into their peritoneal cavity.

#### Irradiation of Peritoneal and Pleural Cavities

Animals were anesthetized with ketamine hydrochloride (Ketalaf®, Pfizer) and xylazine (Rompum®, Bayer). Shortly after, they were fixed in a Styrofoam bed, in dorsal decubitus, for simulation (Acuity Simulator Varian) and transportation to the cobalt unit (60 Co source, CGR, ALCION II, MEV, Varian). Each animal received a radiation dose of 9 Gy, collimated to the thorax and abdomen, at a dose rate of 1.3 Gy min⁻¹ with parallel and opposed fields.

#### Peritoneal Wash

Prior to the irradiation procedure, the peritoneal cavity was washed with saline (0.9%) and the sample collected was re-infused in the animals of groups 1 and 3 immediately after irradiation. Only saline replaced the wash solution collected from animals of groups 2 and 4. In some animals, histology (Giemsa) and flow cytometry (using RAM-11, and IgM) were performed to identify the presence of inflammatory cells.

#### Flow Cytometry, and Histomorphometrical Analysis

Animals were sacrificed on the 90th day and aortas were carefully removed from their origin in the heart to the iliac bifurcation. The aortas were cut longitudinally, fixed in 10% formalin, and stained with the Sudam Red dye to identify lipid-enriched areas. A histomorphometrical analysis was conducted using the Image Tool® software to estimate the percentage of the aorta covered by plaques. To determine plaque composition, immunohistochemical analyses were done using RAM-11 (DAKO) for macrophages and 1A4 (DAKO) for smooth muscle cells. Collagen content was examined by Picrosirius staining plus polarization microscopy. Flow cytometry was used to measure the number of peritoneal cells before and after irradiation.

### Table 1. Experimental protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days of treatment</th>
<th>n</th>
<th>Time of irradiation</th>
<th>Peritoneal cell reinfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90</td>
<td>8</td>
<td>1st and 45th days</td>
<td>yes</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>8</td>
<td>1st and 45th days</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>8</td>
<td>45th day</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>8</td>
<td>45th day</td>
<td>no</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>10</td>
<td>Not irradiated</td>
<td>no</td>
</tr>
</tbody>
</table>
Laboratory Measurements

The lipid profile of all experimental animals was processed in the Central Laboratory of UNIFESP. Samples maintained at \(-80^\circ\text{C}\), were defrosted at room temperature for two hours and the lipid profile was obtained by enzymatic methods. The SERA-PAK reagent was used for determining total cholesterol and triglyceride (Bayer) levels and HDL-C IMMUNO FS (DiaSys) was used for determining HDL-C levels, in ADVIA 1650 (Bayer, Germany). The effects of irradiation on blood cells were examined at baseline and weekly up to four weeks after the procedure (Cell Dyn 3700, Abbott, USA). Concentrations of enzymes in liver were determined using an AU 640 (Olympus, USA).

Statistical Analysis

Data are expressed as the mean and SEM. Results were analyzed using a one-way ANOVA. Differences were considered statistically significant at a value of \(p<0.05\).

Results

Animals

At baseline, no differences were observed in the weight or lipid profile of the animals from the different experimental groups (data not shown). After treatment, as shown in Table 2, the weight and lipid profile were similar between the five groups of rabbits.

Characterization of Peritoneal Cells and the Effects of Irradiation

Numbers of peritoneal cells with the monocyte/macrophage morphology (Fig. 1) decreased markedly after irradiation (Fig. 2). A decrease of 78.4% at the gate characterizing lymphocytes was observed. The possible B-1 phenotype of these lymphocytes could not be determined with certainty when commercial monoclonal anti-rabbit IgM and anti-rabbit macrophage antibodies were used.

Evolution of Atherosclerosis and Effects of Radiation

As shown in Table 3 and Fig. 3 and 4, animals subjected to irradiation exhibited a remarkable reduction in the area of the aorta covered by plaques. Conversely, irradiated animals reinfused with peritoneal cells had the same percentage of the aorta covered as control animals.

Animals irradiated on day 45 had smaller areas of plaques when compared with non-irradiated controls suggesting some controlling effect of irradiation on established lesions. This interpretation is based on results showing that reinfused animals irradiated on day 45 had more plaques than non-reconstituted ani-
mals. Immunohistochemical analyses to determine the composition of plaques showed high macrophage and low smooth muscle cell content (as shown in Fig. 5 A, B, C, D), as well as high macrophage and high smooth muscle cell and collagen content (Fig. 5 E, F, G, H), in all groups.

**Effects of Irradiation on Hematologic Parameters**

As shown in Table 4, there was no apparent effect of irradiation on red blood cells. However, there was a progressive decrease in leukocyte numbers from baseline to four weeks, mainly neutrophils (~54.01%) and lymphocytes (~73.64%). Platelets were less affected by irradiation.

**Table 3. Areas of aortas, areas of plaques and percentage of aortas occupied by plaques according to group**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 (n=8)</th>
<th>Group 2 (n=8)</th>
<th>Group 3 (n=8)</th>
<th>Group 4 (n=8)</th>
<th>Group 5 (n=10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of aorta*</td>
<td>1,303 (92)</td>
<td>1,133 (52)</td>
<td>1,105 (50)</td>
<td>968 (25)</td>
<td>1,351 (92)</td>
<td>0.003</td>
</tr>
<tr>
<td>Area of plaque †</td>
<td>842 (162)</td>
<td>287 (101)</td>
<td>497 (123)</td>
<td>81 (11)</td>
<td>924 (156)</td>
<td>0.001</td>
</tr>
<tr>
<td>Aorta covered by plaques, % ‡</td>
<td>70.7 (7.3)</td>
<td>23.5 (7.5)</td>
<td>43.8 (10.5)</td>
<td>8.4 (1.3)</td>
<td>64.3 (8.8)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Aorta and plaque areas are in mm²; aorta covered by plaques is the percentage of aortas positively stained by Sudam Red dye (%). Data are the mean (SEM). *Group 4 < group 1 (p=0.019); group 4 < group 5 (p=0.003). †Group 1 > group 2 (p=0.036); group 1 > group 4 (p=0.002); group 2 < group 5 (p=0.007); group 4 < group 5 (p<0.001). ‡Group 1 > group 2 (p=0.002); group 1 > group 4 (p<0.001); group 2 < group 5 (p=0.005); group 3 > group 4 (p=0.029); group 4 < group 5 (p<0.001). Comparisons were made with ANOVA followed by the Tukey test.

**Discussion**

Our findings point towards a surprising new aspect of the atherogenetic process, that an extra medullar reservoir of inflammatory cells in the peritoneal cavity is extensively involved in atherogenesis. This assumption is supported by the observation that irradiation of the thoracic-abdominal region of rabbits maintained on an atherogenic diet almost completely abolished plaque formation. This effect might be due to depletion of lymphoid reactive cells from lymph nodes and other organs. In fact, after irradiation, a progressive decrease in leukocyte numbers was noted. Nevertheless, this interpretation was ruled out based
on experiments in which resident peritoneal cells were washed out from the peritoneal cavity before irradiation with reinfusion of these cells after irradiation. Under these experimental conditions, the intensity of plaque formation was similar to that observed in control animals, showing that the reduction in the number of blood leukocytes did not prevent plaques for developing in irradiated animals, highlighting the contribution of inflammatory cells from other sources, such as the resident peritoneal cells. Interestingly, the irradiation exerted some anti-atherogenic effect even in animals on the atherogenic diet for 45 days, in which plaques had already developed. In other words, irradiation might not only block the formation of plaques but arrest/regress the development of lesions that have already formed. The mechanisms by which irradiation exerts its anti-atherogenic effects are not entirely clear. Irradiation is known to kill intestinal endothelial cells and induce chronic diarrhea, so the absorption of cholesterol-enriched food would be decreased in irradiated animals. However, the reconstitution of irradiated animals with peritoneal cells reestablished the atherogenesis. In addition, irradiation neither changed the levels of liver enzymes in serum (Table 4), nor affected the microscopic analysis of the liver or spleen (data not shown).

Irradiation depletes approximately 78.4% of lymphoid-like cells in the peritoneal cavity of normal rabbits and in the mouse, the existence of a population of lympho/myeloid cells denominated B-1 cells is

Fig. 3. Representative aortas from five experimental groups.

Group 5 was used as a control (non-irradiated animals) receiving the atherogenic diet. In groups 3 and 4, the animals were irradiated on day 45, but only those from group 3 received their own peritoneal cells after irradiation. In groups 1 and 2, the animals were irradiated twice (on the 1st and 45th days) but only those from group 1 were reconstituted with their own cells after irradiation. Note that irradiation attenuated the development of atherosomas while reinfusion of the peritoneal cavity with resident peritoneal cells allowed plaque formation similar to control animals.

Fig. 4. Figure shows box-plots of the distribution of atheromas in the animals.

Fig. 4a indicates the total plaque area among groups. Groups 1 and 5 showed a greater area when compared with groups 2 and 4; ANOVA, p ≤ 0.001. Fig. 4b indicates the total area of the aorta covered by plaques among the groups. Groups 1, 3 and 5 showed a greater percentage of area occupied by plaques than did group 4. Group 2 showed a smaller percentage when compared with groups 1 and 5; ANOVA, p ≤ 0.001.
well established. It has also been demonstrated that these cells facilitate parasite infectivity\(^{26}\), promote tumor growth\(^{19, 20}\), have tolerogenic properties\(^{18}\), are radiosensitive\(^{13}\), migrate from the peritoneal cavity to distant inflammatory lesions and differentiate into a mononuclear phagocyte\(^{14, 16}\). Nevertheless, there are no reports in the literature considering the possible existence of B-1 cells in the peritoneal and pleural cavities of rabbits. Using commercial monoclonal antibodies and a marker for macrophages we did not succeed in characterizing with certainty the B-1 cell subtype in the peritoneal cavity of the rabbit. Therefore, the role of these cells in atherogenesis remains speculative.

The involvement of B-1 cells in the pathogenesis of atherosclerosis has been reported by Silverman \textit{et al.}\(^{27}\). The authors postulated that modified LDL particles could be highly stimulatory for certain B-1 clones. In their opinion, during the evolution of the adaptive immune system, the neo-self antigenic milieu may have been exploited for the natural selection of primordial clonal specificity. We strongly agree with this important concept, and the extra-medular reservoir of B1-b cells in the peritoneal cavity might represent a new paradigm in the field of atherosclerosis, with this clone of inflammatory cells recognized as

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Table 4. Effects of irradiation on hematologic and liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>1-wk after irradiation</th>
<th>2-wk after irradiation</th>
<th>3-wk after irradiation</th>
<th>4-wk after irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>12.7 (0.4)</td>
<td>15.0 (0.9)</td>
<td>14.4 (0.6)</td>
<td>13.7 (0.1)</td>
<td>13.1 (1.0)</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>38.2 (1.2)</td>
<td>43.0 (1.4)</td>
<td>43.0 (1.5)</td>
<td>39.8 (1.3)</td>
<td>39.2 (2.6)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>3,923 (1,136)</td>
<td>3,910 (370)</td>
<td>3,510 (1,135)</td>
<td>2,990 (310)</td>
<td>1,467 (230)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1,533 (559)</td>
<td>2,614 (951)</td>
<td>2,289 (995)</td>
<td>1,583 (21)</td>
<td>705 (111)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>9 (9)</td>
<td>2 (2)</td>
<td>83 (41)</td>
<td>135 (113)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Basophils</td>
<td>64 (33)</td>
<td>73 (73)</td>
<td>26 (21)</td>
<td>57 (22)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,324 (330)</td>
<td>891 (223)</td>
<td>817 (181)</td>
<td>787 (117)</td>
<td>349 (76)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>222 (93)</td>
<td>331 (285)</td>
<td>296 (157)</td>
<td>183 (59)</td>
<td>15 (6)</td>
</tr>
<tr>
<td>Platelets</td>
<td>305,667 (32,539)</td>
<td>234,500 (35,500)</td>
<td>189,333 (75,393)</td>
<td>400,000 (51,000)</td>
<td>243,333 (49,640)</td>
</tr>
<tr>
<td>AST</td>
<td>29.2 (2.6)</td>
<td>28.0 (3.0)</td>
<td>33.3 (8.4)</td>
<td>27.5 (8.5)</td>
<td>52.0 (10.6)</td>
</tr>
<tr>
<td>ALT</td>
<td>39.0 (3.4)</td>
<td>27.5 (3.5)</td>
<td>29.0 (4.3)</td>
<td>35.5 (17.5)</td>
<td>45.7 (9.9)</td>
</tr>
</tbody>
</table>

Hematologic parameters observed in rabbits at baseline and after irradiation (\(n = 5\)). Values are expressed as the mean (SEM). Hemoglobin (g/dL); Hematocrit (%); Leukocytes (number/\(\mu\)L); AST = aspartate aminotransferase; ALT = alanine aminotransferase.

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Fig. 5. Histological and immunohistochemical analyses in segments of aorta (×100).

A and B show Hematoxylin and Eosin staining; B and F show RAM-11-positive staining (brown) for macrophages; C and G show 1A4-positive staining for smooth muscle cells; D and H show Picrosirius staining for collagen.
part of our evolution, initially as an important defense mechanism for maintenance of cellular homeostasis, but now as an important pathway for atherosclerotic plaque development.

How could the oxidized lipoprotein particles trigger immune responses involving the peritoneal inflammatory cells? In fact, it was reported that oxidized epitopes from low-density lipoproteins were recognized by a natural phosphorylcholine-specific germ-line-encoded B-1 cell antibody, T15. These findings support the notion of interaction between atherosclerosis and these inflammatory cells.

Summarizing, our study adds to the study of atherosclerosis in at least two ways. First, we developed a new promising experimental model to examine the role of peritoneal inflammatory cells in the atherogenic process, and second, we added the novelty of radiation to the conventional diet-induced atherosclerosis in the rabbit model. Furthermore, through the reinfusion of peritoneal inflammatory cells after irradiation, we established the relevance of these cells to atherogenesis while concomitantly demonstrating an outstanding attenuation of plaque formation when these cells are not reinfused.

The characterization of these inflammatory cells and the pathways for both differentiation and migration to the vessel wall will constitute the next step in the study of this complex disease.

Acknowledgments

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