Evaluation of the cholesterol influence in type II collagen-induced arthritis in DBA/1J mice: an autoradiographic study

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Abstract

In order to verify the cholesterol influence in RA severity in DBA/1J mice, we quantified the cholesterol present in the knee joints of normal (N) and with collagen II induced arthritis (CIA). Forty male DBA/1J mice, were divided in normal (n=20) and CIA group (n=20). Mice in CIA group were injected with 100 µg of collagen II emulsified in Freund's complete adjuvant. Sixteen DBA/1J (8 N and 8 CIA) received an injection of 2.96×10^6 Bq of ^3H -cholesterol and were anesthetized and sacrificed. Semi-fine sections were covered with LM-1 emulsion, exposed for six weeks and developed. Collagen induced edema, erythema and dysfunction of knee joints in CIA group. Radioactive cholesterol was located more on the synovial membrane, where we found the greatest density of silver grains, significantly ($P < 0.0001$) higher in group CIA vs. controls (61 ± 2.3 X 18 ± 0.7). We conclude that the cholesterol deposits on the synovial membrane is related to CIA severity.

Keywords: autoradiography • cholesterol • DBA/1J mice • rheumatoid arthritis • type II collagen

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology. Immunogenetic studies demonstrate that the RA carriers have

molecules coded by alleles of HLA class II DR₄ and DR₁ genes responsible not only for the development, but probably also for the severity of the disease [1–3].

Progressive accumulation of lipids in the cells and matrix of superficial layer of articular cartilage may be of pathogenetic significance because it is in this area that the earliest lesions of arthritis appear [4].

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The inflammatory reaction is characterized by the release of humoral and cellular inflammatory mediators resulting in alterations of the micro-vascular tonus and permeability [5]. The inflammation in RA can cause systemic and metabolic alterations affecting plasmatic as well as synovial fluid lipid profile [6, 7]. The cholesterol accumulation in the capillaries, caused by the elevation of lipid blood levels, triggers the formation of intra-vascular lesions of the femur and humerus condyles [8-10].

The animal model DBA/1J mouse is genetically susceptible to acquire the arthritis induced by type II collagen (CIA) when injected emulsified in Freund's complete adjuvant. This arthritic type is outset by a cellular immune response, against the heat shock protein (HSP65) of the mycobacteria present in the adjuvant [11-14]. After this stimulus, the animal's T cells react against its own synovial membrane by a mechanism of cross-reaction [15, 16].

The autoradiographic qualitative or quantitative evaluation is of great contribution in the evaluation in normal or pathological systems [17, 18].

In our study we used as radioactive isotope ^3H (tritium). This is used more in technical autoradiography due to its low energy emission. This isotope emits electrons with short trajectories (1 μm in water). To obtain a good resolution, besides the ideal radioactive emission, the tissue section and the emulsion thickness should be thin enough to assure that all the particles with perpendicular trajectory reach the emulsion [19-22].

Although strong evidence shows that low levels of serum lipids act in the prevention of several diseases including RA, little is known about the influence of cholesterol on its evolution and severity.

The aim of this work was to verify the cholesterol influence in CIA severity using autoradiographic technique. In this study the cholesterol in the knee joints of DBA/1J mice (N and CIA) was quantified as compared to the histopathological aspect of the CIA development. We also investigated the lipid profile and its relationship to the inflammatory condition in CIA.

Materials and methods

Animals

Forty male DBA/1J mice were provided by Brazil's National Cancer Institute (Basic Research Center-INCa), aged 21 days, they were housed 5 in a cage, under appropriate conditions, with a controlled temperature and light cycle. Twenty were considered the control group (N) and twenty the CIA group. All mice in CIA group received type II collagen as inductor of autoimmune arthritis. In the same day, eight mice in each group received tritiated cholesterol then sacrificed. Mice without cholesterol injection were used as autoradiography controls. The university standing committee on animal research approved the protocols. The investigation conforms to the "Guide for the Care Use of Laboratory Animals" published by US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Diet

The DBA/1J mice (N and CIA), were given a diet elaborated according to that set down by (AIN-93) [22] for rodents in growth period (g/100g): corn starch 52.95%; casein 20.09%; sucrose 10.0%; soy bean oil 7.0%; cellulose 5.0%; minerals mixture 3.5%; vitamins mixture 1.0%; L-cystine 0.3%; choline bitartrate 0.2%. A rigorous weekly control of the diet consumption (g/day/diet) and the weight gain of the animal (g/day/animal) [24] was kept.

Lipid profile

In the 5th month of diet, the DBA/1J mice (N) after 12 hours fasting were anesthetized with ether and bled by the orbital plexus. The samples were immediately centrifuged (2000rpm/10 min.), and serum separated for lipid profile analysis: triglycerides (TG), total cholesterol (TC), and high density lipoprotein (C-HDL), were checked by enzymatic method [25]. The same procedure was carried out with the DBA/1J mice after CIA developed.

Induction of CIA

In the 6th month of diet, the CIA was induced in DBA/1J mice with bovine type II collagen (Sigma) emulsified in an equal volume of Freund's complete adjuvant. We injected 100 μg of collagen intradermally at the base of the tail of the DBA/1J mice [26-29]. On the day 21 the animals were injected intraperitoneally with 50 μg of

Fig. 1 Reticular grid used for counting the silver grains superimposed in the tissue.

type II collagen. The arthritic index was evaluated by the severity degree for each paw from 0 to 2 (edema, erythema and deformity) from the 28th day of the first immunization as previously described [12, 26].

Administration of the ³H-Cholesterol

On the same day, the ³H-cholesterol was injected into sixteen DBA/1J mice (8 N and 8 CIA) intraperitoneally, with a dose of 2.96×10^6 Bq. After three hours (time necessary for a systemic distribution) [30], the mice were submitted to deep anesthesia with ether, and bled until death by the orbital plexus with pre-washed Pasteur pipettes with heparin (Liquemine). The knee joints were dissected and processed histologically. Blood samples were analyzed for lipid profile (CIA). A normal animal without the ³H-cholesterol injection was used as background for the autoradiography.

Histological processing

The knee joints fragments were dissected, fixed and processed by the standard method of the electron

microscopy (glutaraldehyde-osmium tetroxide). The 2 μm semi-fine sections, were obtained by a Ultramicrotome–Leica (Ultracut UCT). We used an optical microscope for counting the silver grains in the synovial membrane. The semi-fine sections were deposited on the slide surface (2.0 x 10.0 cm) in a drop of water, and dried at 50° C in a thermal foil (Fisatom – Brazilian Industry). Staining with panoptic methylene blue was used for the histopathological evaluation of the articular structures. The slides for autoradiography were stained with Regaud's hematoxylin before applying the emulsion [31, 32].

Autoradiography

To cover the sections, we used the liquid emulsion recommended by Kopriwa and Leblond [33] LM-1, for optical microscopy (Amersham/England), that is constituted of silver-bromide crystals embedded in gelatin. After being dried the slides were kept in light-proof plastic boxes at 4°C. The exposure time was of 6 weeks in order to increase the probability of the silver-bromide crystals to be reached by the β⁻ emission, sensitizing and reducing them to metallic silver (silver grains). We used the D19b to develop the slides.

Quantification of silver grains

An Olympus BH-2 microscope with a 100 x objective under immersion was used for counting the silver grains [34]. The silver grains were quantified according to the scheme shown in the Fig. 1, taking 10 squares at random and counting the grains present in each square. The autoradiogram photomicrographs were made on Olympus BH-2 microscope, with 100x objective under immersion. The histopathological findings of the knee joints were analysed on sections with a 40x objective.

Statistics

The Student's "t" test was applied for autoradiographic and lipid profile comparison. Eight slides were used for each animal.

Results

Our goal was to verify the cholesterol influence in the severity of CIA, without the interference of drugs. For this, we established a comparative study, in which the clinical evaluation, histopathology and autoradiography are taken into consideration in the interpretation of the results.

At the end of six months of administration of diets, the daily calory consumption of both groups was similar.

The clinical signs (edema, erythema and deformity) of CIA group, showed cumulative arthritic index varying from 2.0 to 4.0 for DBA/1J mice.

Histopathological evaluation

We observed in CIA group, infiltration of the mononuclear cells in the inflamed synovia, and

Table 1 Evaluation of ³H-cholesterol between groups N (control) and CIA (with collagen II induced arthritis) of DBA/1J mice.

Regions quantified	Group (N)	Group (CIA)
Articular cartilage	24 ± 1.3	35 ± 1.4
Synovial membrane	18 ± 0.7	61 ± 2.3
Articular space	18 ± 0.7	27 ± 2.2

The values represent the mean ± standard error (SE). Student's "t" test for evaluating the number of silver grains showed high significance ($P < 0.0001$).

erosion in the articular cartilage with the presence of osteoclasts. Fig. 2A shows the normal knee components of DBA/1J mice (N). The knee joints of CIA group showed typical CIA with accentuated erosion of the articular cartilage. Osteoclasts are observed in the destroyed cartilage Fig. 2B.

Autoradiography

We evaluated the ³H-cholesterol of the N and CIA groups. The CIA group had a greater density of silver grains, mainly in the synovial membrane area. Quantification of silver grains is outlined in Table 1. The corresponding photomicrographs can be seen in Fig. 3.

Table 2 Lipid profile of DBA/1J mice (n=8) after five months of diet. Comparison between the groups N (control) and CIA (with collagen II induced arthritis), (mg/dL).

Groups	TC	HDL-C*	LDL-C*	VLDL-C	TG*
Control	80.8 ± 3.44	53.2 ± 2.67	16.0 ± 2.26	13.0 ± 1.95	63.8 ± 3.49
With CIA	84.6 ± 1.86	45.5 ± 1.18	23.4 ± 2.07	16.0 ± 0.50	80.1 ± 2.50

The values represent the mean ± standard error (SE). * $p < 0.05$.

Fig. 2 Knee joints of DBA/1J. In **A**, the normal aspect with the articular structures preserved. Note the condyle of the femur with an integral articular cartilage and slight synovial membrane. In **B**, note the erosion in the condyle of the femur due to inflammatory reaction caused by CIA (Methylene blue, magnification 100 x).

Lipid profile

Lipid profile of DBA/1J mice after five months of diet were established (Table 2). The mean and standard error (SE) was calculated for total cholesterol (CT) and its fractions (C-HDL, C-LDL and C-VLDL), and triglycerides (TG). Comparing the two groups (N and CIA), there were alterations of the lipid profile in CIA group, with increased values of C-LDL and TG and decreased C-HDL.

Discussion

In this autoradiographic study, it was evident that the CIA group had an increase of the cholesterol in the knee areas when compared to controls. The metabolic alterations of CIA include rupturing of the plasmatic membranes of articular chondrocytes, due to action of the proteolytic enzymes released by the inflammatory cells present in the synovia [35-37]. After the release of intracellular cholesterol it is deposited in the extracellular cartilage matrix, in the articular space and the synovial membrane. According to James *et al.*, 1998 [6], an excess of C-LDL was found in the synovial fluid of patients with RA. In rodent studies an incorporation of C-LDL by peritoneal macrophages was observed. In our study, the greatest cholesterol increase found in the synovial membrane is probably due to: (i) a

wider injury in this area, owing to inflammatory cells accumulation caused by CIA; (ii) the fact of being a rich vascularized tissue, favoring a higher migration of the defense cells arising from the blood; and (iii) the phagocytosis taking place by type A synoviocytes [38].

In the morphological study, we observed in the CIA group a thickened and inflamed synovial membrane, superficial erosion of the articular cartilage of the femur and tibia condyles, and the presence of osteoclasts. In our experimental RA work, at the severe peak of the disease we did not find any damaged bone, only cartilage was altered. We also studied the exit of intracellular cholesterol and of the plasmatic membranes of the chondrocytes, which were destroyed by the proteases of the inflammatory cells released by the main cartilaginous extracellular matrix, synovial space and membrane. This interpretation is similar to those described by Varani *et al.*, 1994 [37] and Curtis *et al.*, 1999 [10].

Studies of patients with active RA show alterations in the lipid plasma profile, with decrease in the values of C-HDL, and increase in C-LDL values [39]. We observed that the CIA group had lipid profile values altered when compared to controls. The CIA group presented increased levels of C-LDL, decreased levels of C-HDL ($p < 0.05$), and the TG values were also high ($p < 0.05$). These results agree with those of Cabana *et al.*, 1996 [40], in their studies with 3 species of animals, where it

Fig. 3 The cholesterol presence was assessed by visualizing silver grains as black marks superimposed on the tissue. Cholesterol was quantified by the grain density in groups N (control) and CIA (with collagen II induced arthritis). **A, C, E:** hyaline cartilage, synovial space and synovial membrane of group N; **B, D, F:** the same joint regions of CIA group (450 x).

was observed that the TG increase was related to C-HDL decrease during the severe phase of the disease. High levels of low density lipoprotein (LDL) and low levels of high density lipoprotein (HDL) are well known risk factors for atherosclerosis and important in the development of cardiovascular disease. Cardiovascular and atherosclerotic events are the most common causes of death in patients with RA [41]. We verified an increase in C-LDL and in TG, and a decrease of C-HDL. The increase of oxydized C-LDL provides multiple pro-inflammatory biological effects according to James *et al.*, 1998 [6].

We conclude that the inflammation caused by CIA brought changes in the lipid profile, mainly TG, C-HDL and C-LDL, all statistically significant ($p < 0.05$), contributing to a worse inflammatory reaction, which leads to cartilage erosion and extracellular matrix reabsorption by osteoclasts. The cartilage erosion is due to the presence of collagenases and specific proteases present in chondrocytes and in inflammatory cells of the synovial tissue with CIA, which could just have favored the exit of the intracellular cholesterol to the extracellular medium just as much as its incorporation in the compromised tissue, in attempt to maintain cellular viability. Consequently the blood viscosity increased causing progressive ischemia, leading to cellular death and contributing to the agravation of the inflammatory reaction. Our results suggest that the cholesterol is implied in the severity of CIA in DBA/1J mice.

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