3-Deazaadenosine Prevents Adhesion Molecule Expression and Atherosclerotic Lesion Formation in the Aortas of C57BL/6J Mice

Gerhard Walker, Alexander C. Langheinrich, Elisabeth Dennhauser, Rainer M. Bohle, Thomas Dreyer, Jörg Kreuzer, Harald Tillmanns, Ruediger C. Braun-Dullaeus, Werner Haberbosch

Abstract—Adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) play an important role during the development of atherosclerosis. 3-Deazaadenosine (c\textsuperscript{2}Ado), an adenosine analogue, inhibits endothelial-leukocyte adhesion and ICAM-1-expression in vitro. We hypothesized that c\textsuperscript{2}Ado is able to prevent the expression of adhesion molecules and atherosclerotic lesion formation in female C57BL/6J mice. The animals were placed on an atherogenic diet with or without c\textsuperscript{2}Ado for 9 weeks. Frozen cross sections of the proximal ascending aorta just beyond the aortic sinus were stained with oil red O, hematoxylin, and elastic van Gieson’s stains and were analyzed by computer-aided planimetry for fatty plaque formation and neointimal proliferation. Monoclonal antibodies against CD11b (macrophages), VCAM-1, and ICAM-1 were used for immunohistochemistry. Mice on the atherogenic diet demonstrated multiple (5.4±1.6 per animal) lesions covering 3.4±2.8% of the endothelium and a marked neointima when compared with control mice (4501±775 versus 160±38 µm\textsuperscript{2}, \textit{P}<0.001). Mice on the cholesterol-rich diet without c\textsuperscript{2}Ado showed strong endothelial coexpression of ICAM-1 and VCAM-1. Moreover, there was a 10-fold increase in monocyte accumulation on the endothelial surface (33.3±4.9 versus 3.8±1.2, \textit{P}<0.004). In contrast, in mice treated with c\textsuperscript{2}Ado, expression of ICAM-1 and VCAM-1 as well as monocyte adhesion and infiltration were almost completely inhibited. Furthermore, these mice did not show any fatty streak formation or neointima formation (125±32 µm\textsuperscript{2}). Our results demonstrate that c\textsuperscript{2}Ado can inhibit diet-induced fatty streak formation and the expression of endothelial ICAM-1 and VCAM-1 in C57BL/6J mice. This may provide a novel pharmacological approach in the prevention and treatment of atherosclerosis. (\textit{Arterioscler Thromb Vasc Biol. 1999;19:2673-2679.})

Key Words: adenosine analogues • atherosclerosis • cell adhesion molecules • hypercholesterolemia • immunohistochemistry

Adhesion of leukocytes to the endothelial cell layer and their subsequent migration into the vascular wall are believed to play a key role during the development of atherosclerotic lesions. Monocytes/macrophages and lymphocytes, for example, are ubiquitous at all stages of atherosclerotic plaque development and propagate the local inflammatory process. Furthermore, lipid-laden macrophages accumulate within the plaque, thereby leading to instability with consequent rupture, thrombosis, and acute vessel closure.\textsuperscript{1–3}

It has widely been appreciated that the inhibition of leukocyte adhesion and migration may have protective effects on plaque development.\textsuperscript{1–6} Cellular adhesion and migration are mediated by various molecules of the selectin, integrin, and immunoglobulin superfamily, such as the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Previous studies have demonstrated increased expression of VCAM-1 and ICAM-1 on the surface of endothelial and vascular smooth muscle cells of human plaques and in experimental models of atherosclerosis.\textsuperscript{7–13} A recent study of C57BL/6 mice with homozygous mutations for ICAM-1 suggests a direct relationship between the amount of this molecule expressed in the vessel wall and the formation of atherosclerotic lesions.\textsuperscript{14}

3-Deazaadenosine (c\textsuperscript{2}Ado), a structural analogue of adenosine, is an anti-inflammatory drug that has been shown to inhibit monocyte chemotaxis and phagocytosis.\textsuperscript{15–18} The mechanisms underlying c\textsuperscript{2}Ado’s actions are generally thought to be mediated through the inhibition of cellular methylation reactions.\textsuperscript{15,19} However, it has also been suggested that some of its biological actions are independent of these mechanisms.\textsuperscript{17,20–22} For example, c\textsuperscript{2}Ado reduces tumor necrosis factor-α–induced macrophage adhesion to endothelial cells in vitro via the selective inhibition of ICAM-1 synthesis.\textsuperscript{23} The underlying molecular mechanisms have not yet been fully clarified.
Because of its anti-inflammatory properties, c3Ado has been studied in a clinical trial in patients with rheumatoid arthritis, and the drug has been tested for its antiviral (HIV-1) activity. Despite its intriguing properties with respect to cell adhesion, cell migration, and phagocytosis, the drug has never been tested in animal models of vascular proliferative disease.

We hypothesized that c3Ado inhibits in vivo leukocyte adhesion and concomitant formation of atherosclerotic lesions through inhibition of the expression of endothelial cell adhesion molecules, such as VCAM-1 and ICAM-1. The atherosclerotic model of female C57BL/6 mice was chosen for this study. These animals are susceptible to fatty lesion formation that closely resembles early atherosclerotic plaques detected in humans.

**Methods**

**Animals, Atherogenic Diet, and Experimental Design**

Six- to 8-week-old female C57BL/J6 mice (Charles Rivers Wiga, Sulzfeld, Germany) with an average body weight of 20 g were randomly divided into 3 groups:

**Group 1**

Group 1 included control animals (n=9) maintained on a normal mouse diet (Altromin, Standard Diet).

**Group 2**

Group 2 also included 9 animals receiving an atherogenic diet based on normal mouse chow but differing in total fat content (10% versus 5%), protein content (15.4% versus 22%), and cholesterol content (1% versus 0%). The total energy was 3790 versus 3000 kcal/kg.

**Group 3**

Group 3 included 9 animals on an atherogenic diet as described above, and c3Ado (Southern Research) was mixed into their food at a final concentration of 0.04 mg/g, according to a daily oral dose of 10 mg/kg body weight of c3Ado per animal.

**Experimental Design**

The average intake of food for each animal was 5.2 g/d. Food and water were replenished every 2 days, and the volumes consumed for each cage were recorded. The mice were kept in accordance with standard animal care requirements and were maintained on a 12-hour light/dark cycle with autoclaved water in a temperature-controlled environment. All animals remained healthy during the experimental period. After 9 weeks, mice were humanely killed by inhalation of trichlormethane. The heart and ascending aorta were removed and rinsed with Dulbecco’s PBS (Life Technologies). The lower half of the heart was transected along a line between the tips of the atria to obtain a base for the emerging ascending aorta. This procedure allowed exact vertical embedding of the aorta in Tissue-Tek (Sakura) for optimal plane cross sections. The sections were frozen in LN2 and stored at −80°C until further study.

**Quantification of Atherosclerotic Lesions and Neointimal Proliferation**

A modification of the method described by Paigen et al was used to evaluate aortic lesion formation. The frozen tissue blocks were placed on a cryotome, and 8-μm serial sections of the ascending aorta were collected on coated glass slides until we were able to locate the most cranial portion of the aortic sinus by examining unstained sections. Once this section (No. 1) was identified, the cranial 35 sections, covering 280 μm of the ascending aorta, were used for further evaluation. Every fifth section of the first 280 μm of the ascending aorta was stained with oil red O (Riedel de Haen) and counterstained with hemalum (Merck) and elastic van Gieson’s (Chroma-Gesellschaft, Schmid GmbH) stains. Lesion area as well as intimal and medial area were blindly determined using a video-computer-aided microscopy planimetry system (Zeiss; video camera 3 CCD, Sony; ×40 lens magnification; IBAS-2 with IBAS version 2.0 Standard, Kontron).

The number of lesions was counted by reviewing every fifth section. This procedure resulted in 40 μm between each evaluated section and has been shown to give a valid estimation of the degree of atherosclerosis. Lesions were defined as oil red O–positive areas in the aortic wall. Size of the lesions was determined as the length of lesion along the luminal aortic perimeter, which was related to the total luminal aortic perimeter in the section.

The extent of neointimal proliferation was quantified by measuring the area (in microns squared) of the neointima and the media in each ascending aorta from 3 sections (No. 10, 20, and 30). In each section, 4 sectors (at 0°, 90°, 180°, and 270°) of the vessel wall were analyzed in a defined window of 63×63 μm to measure neointimal area enclosed by the endothelial layer and internal elastic lamina. Medial area was determined by measuring the area enclosed by the internal elastic lamina and external elastic lamina. The ratio of neointimal to medial areas (NI/MI) was averaged for each animal.

**Immunohistochemical Analysis**

Serial cryostat sections (8 μm) from the following 3 parts of the ascending aorta were chosen: 8 to 32 μm, 120 to 144 μm, and 240 to 264 μm distal to the aortic sinus. The frozen sections were fixed in ice-cold acetone and dried for 10 minutes. Sections were then incubated for 10 minutes in a 1:1000 dilution of mouse serum (Sigma Chemical Co). After being rinsed with RPMI 1640 (Life Technologies), the sections were incubated for 40 minutes at room temperature with a 1:100 dilution of monoclonal rat anti-mouse antibody against ICAM-1 (CD54) or VCAM-1 (CD106, Dianova GmbH). Detection of monocyes/macrophages was performed using monoclonal rat anti-mouse antibody CD11b (MAC-1, Serotec Ltd), and a polyclonal anti–von Willebrand factor antibody (rabbit anti-human vWF, Dako) was used to demonstrate endothelial integrity.

After additional washing steps with Tris buffer (USB) and incubation with a secondary antibody (AffiniPureMouse anti-rat IgG, 1:400, Dianova; and for the polyclonal anti-human vWF antibody, AffiniPureMouse anti-rabbit, Dianova, 1:600) for 10
minutes, followed by incubation with a linking antibody (Dualsystem-Brückenantikörper, Dianova, 1:600) for 10 minutes, the sections were incubated with an alkaline phosphatase–anti–alkaline phosphatase complex (APAAP, 1:50, Dianova) for 30 minutes. Development of the sections was performed in new fuchsin developing solution. The sections were finally counterstained with hemalum (Merck) for 10 seconds. Control sections were treated with linking secondary antibody and APAAP complex only.

Quantification of Immunohistochemical Staining
The intensity of staining with ICAM-1 and VCAM-1 was scored from 1 to 4 as follows: score 1=no staining, score 2=weak staining, score 3=moderate staining, and score 4=strong staining of the vascular cells. The number of CD11b-positive cells was counted in 3 section per animal, and the localization of the cells was classified as adherent to the wall or localized in the intima or media. Quantification was performed by 2 experienced operators (R.M.B. and A.C.L.) who were blinded to the study protocol.

Determination of Total Plasma Cholesterol Levels
Blood was collected via the tail vein from all mice after overnight fasting before initiation of the experiment, after 35 days’ feeding of the different diets, and at the time of death. Total plasma cholesterol levels were determined using the CHOD-PAP-method (Boehringer Mannheim) as described previously.27

Statistical Analysis
Results were analyzed by 2-way ANOVA with repeated measures and 1-way ANOVA with pairwise contrasts by the Scheffé test. Differences in expression of ICAM-1, VCAM-1, and CD11b-positive monocytes were analyzed with the nonparametric Kruskal-Wallis 1-way ANOVA. Values of neointimal area and NI/M ratio were averaged for all groups, and differences were analyzed with the Kruskal-Wallis 1-way ANOVA as well. Data shown are mean±SEM. All tests were performed with SPSS for Windows version 6.1.3.

Results
Characteristics of the Groups
Cholesterol concentrations of study groups 2 and 3, measured on days 35 and 65, were not statistically different. The animals of both groups consumed similar amounts of food. As well, weight development was the same. For quantification, refer to Table 1.

Atherosclerotic Plaque Development
Fatty streak development in the ascending aorta was examined and quantified on day 65. Control mice did not show any atherosclerotic changes, whereas the animals of study group 2 showed multiple lipid-containing lesions covering the vessel wall of the ascending aorta, as demonstrated by oil red O staining (Figure 1). The fatty streaks were mainly located around the aortic cusps but also in more distal parts of the ascending aorta, and the average number of lesions per animal in this group was 5.4±1.6. The percentage of the aortic lining covered by plaque was 3.4±2.8% (see Table 2). In contrast, animals of group 3, which received c3Ado in addition to the atherogenic diet, had no detectable lesions within the aortic root.

Furthermore, neointimal development was completely prevented in animals treated with c3Ado despite the atherogenic diet, closely resembling the control situation. As expected, mice in group 2 demonstrated a strongly developed neointima (Figure 2). Planimetric analysis of the intimal area of the proximal ascending aorta demonstrated a marked increase of the intima (4501±775 μm²) when compared with the control

| TABLE 2. Comparison of Neointimal Area, Number of Fatty Streak Lesions, and Percentage of Surface Covered by Plaque |
|---------------------------------|-------------|-----------------|------------------|
|                                 | Neointimal  | No. of          | % Surface Covered |
|                                 | Area, μm²   | Lesions         | by Lesions       |
| Group 1, control                | 160±38      | 0±0             | 0±0              |
| n=9                            |             |                 |                  |
| Group 2, atherogenic            | 4501±775*   | 5.4±1.6*        | 3.4±2.8*         |
| diet (n=9)                      |             |                 |                  |
| Group 3, atherogenic            | 125±32      | 0±0             | 0±0              |
| diet + c3Ado (n=9)              |             |                 |                  |

Values are expressed as mean±SEM of 7 sections per animal. Comparison between group 2 (atherogenic diet) and group 3 (atherogenic diet plus c3Ado) demonstrates significant differences in all tested parameters. Beginning from the most cranial portion of the aortic sinus, 35 sections covering 280 μm of the ascending aorta were used for quantification.

* P<0.001, analyzed by mean-rank sums obtained with the Kruskal-Wallis test.

Walker et al 3-Deazaadenosine and Atherosclerosis 2675
mice value of 160±38 \mu m^2, P<0.001). c′Ado treatment of mice fed the atherogenic diet resulted in complete inhibition of neointimal proliferation (125±32 \mu m^2, P<0.001). Neointimal thickness as measured by the NI/M ratio was reduced by 94% in these mice when compared with mice on an atherogenic diet alone (0.002±0.0004 versus 0.033±0.005, P<0.001). The NI/M ratio in the c′Ado group did not differ significantly from that of control mice, which was found to be 0.003±0.0006.

Monocyte/Macrophage Accumulation and Expression of Adhesion Molecules

Animals fed the atherogenic diet showed CD11b-positive monocytic cells adhering to the endothelium (Figure 3) or located within the neointima of the ascending aorta. The mean number of these cells was 10-fold higher in group 2 than in animals of group 3, which had been treated with c′Ado. In sections from control animals on the standard diet, no monocytes/macrophages could be detected at all. Table 3 summarizes the results after quantification of monocytes/macrophages of 3 sections per animal within the different groups.

We furthermore examined the expression of the adhesion molecules VCAM-1 and ICAM-1, which have both been demonstrated to be directly involved in monocyte/macrophage adhesion and may therefore play an important role in atherosclerotic lesion formation.\textsuperscript{7,8} Immunohistological staining demonstrated intense endothelial expression of VCAM-1 and ICAM-1 in all analyzed frozen sections of mice on the atherogenic diet (Figure 4). Both adhesion molecules were abundantly expressed in the intima of the ascending aorta, from the aortic cusps to 280 \mu m distal from this point. In striking contrast, the expression of VCAM-1 and ICAM-1 was completely absent in mice treated with c′Ado as it was in control animals (Figure 4). Quantification of adhesion molecule expression is given in Table 3. Control staining with anti–von Willebrand factor demonstrated the integrity of the endothelium in all sections investigated (data not shown).
TABLE 3. Comparison of Endothelial VCAM-1 and ICAM-1 Expression and Quantification of Monocytes/Macrophages

<table>
<thead>
<tr>
<th></th>
<th>VCAM-1 Expression Score</th>
<th>ICAM-1 Expression Score</th>
<th>Number of Monocytes/Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, control</td>
<td>1.1±0.1</td>
<td>1.0±0.1</td>
<td>0±0.1</td>
</tr>
<tr>
<td>(n=9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2, atherogenic diet (n=9)</td>
<td>3.3±0.2*</td>
<td>2.9±0.3*</td>
<td>33.3±4.9†</td>
</tr>
<tr>
<td>Group 3, atherogenic diet+c3Ado (n=9)</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>3.8±1.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM of 3 sections per animal. Comparison between group 2 (atherogenic diet) and group 3 (atherogenic diet plus c3Ado) demonstrates significant differences in all tested parameters. Beginning from the most cranial portion of the aortic sinus, 35 sections covering 280 μm of the ascending aorta were used for quantification.

*P<0.001 and †P=0.004, analyzed by mean-rank sums obtained with the Kruskal-Wallis test.

Discussion

In this study, we could demonstrate that the adenosine analogue c3Ado inhibits early plaque formation and neointimal formation in the ascending aorta in C57BL/6J mice on an atherogenic diet. This antiatherogenic effect was accompanied by a marked suppression of endothelial VCAM-1 and ICAM-1 expression and the prevention of monocyte adhesion to the vascular wall.

Our study is the first to investigate c3Ado’s effect on the development of vascular proliferative disease in vivo. Because we were interested in blocking the early events during atherosclerosis development, we used the hyperlipidemic model of plaque formation in female C57BL/6J mice. This model is well established, and the lesions were found to be similar to fatty streak formation detected in the early phase of atherosclerosis in humans.26,28,29 In agreement with the published literature, we were able to reproducibly induce fatty lesion formation in untreated mice covering 3.4±2.8% of the examined surface area of the ascending aorta after 9 weeks on a fat-rich diet.

Importantly, c3Ado did not have any influence on blood cholesterol levels. Cholesterol levels were high in both groups and were slightly higher than reported in other investigations.26,30 Thus, c3Ado prevents lesion formation independently of the cholesterol level. Furthermore, body weight development was the same in both groups on the atherogenic diet. They also consumed the same amount of atherogenic food (5.2±0.5 g versus 5.1±0.6 g), thereby excluding a negative effect of the drug on the appetite.

Various studies in animal models have shown that 1 of the earliest events detectable after starting a cholesterol-rich, atherogenic diet is the adherence of blood monocytes and lymphocytes to the vascular endothelium. They subsequently migrate into and accumulate within the intima, take up oxidatively modified LDLs, and finally transform into foam cells.1,31,32 Endothelial recruitment of monocytes depends on the expression of cellular adhesion molecules such as VCAM-1 and ICAM-1.23 Treatment of apo E–deficient mice with monoclonal antibodies directed against ICAM-1, for example, attenuated macrophage homing to atherosclerotic plaques.34 Furthermore, the increased expression of adhesion molecules during the early stages of atherosclerosis has been demonstrated in several animal models6,10 and in human atherosclerotic plaques.7,12,35 Although these studies did not prove a causal relationship between adhesion molecules and plaque formation,7–11 their importance for atherosclerosis development has widely been appreciated.1,3–6 New clinical data have shown a significant association between increasing plasma concentrations of soluble ICAM-1 and risk for future myocardial infarction.36

This study focused on VCAM-1 and ICAM-1, the adhesion molecules important for monocyte adhesion. Our data suggest that cAdo inhibits plaque formation through prevention of VCAM-1 and ICAM-1 expression and the concomitant inhibition of monocyte recruitment on the endothelial surface. However, this study does not prove causality. In endothelial cells in vitro, c3Ado has been shown to prevent monocyte adhesion and inhibition of ICAM-1 expression.23 c3Ado additionally affects the expression of other molecule critical for the development of atherosclerosis. For example, the thrombin-stimulated production of platelet-derived growth

Figure 4. c3Ado prevents expression of adhesion molecules VCAM-1 and ICAM-1. Typical cross sections of the aortic root of an animal on the atherogenic diet (A and B), an animal on the atherogenic diet plus c3Ado (C and E), and a control mouse (D and F). Immunohistochemical staining for ICAM-1 (A, C, and D) and VCAM-1 (B, E, and F) with monoclonal rat anti-mouse antibodies with the APAAP method and counterstaining with hemalum. A through F, original magnification ×100; bar=5 μm. For semiquantitative analysis refer to Table 3.
factor and the expression of endothelial leukocyte adhesion molecule-1 could have been prevented by c3Ado in human endothelial cells.37 The drug not only prevents monocyte adhesion, but it also inhibits monocyte chemotaxis and phagocytosis.15,18,38 Furthermore, it prevents tumor necrosis factor-α production and has also been shown to promote apoptosis in monocytic cell lines.39,40 Unfortunately, we had not enough blood to determine the amount of white cells to rule out the possibility that c3Ado causes leukopenia. In the study reported by Smith et al24 in humans, the serum concentration of 600 pg/mL c3Ado obviously did not alter cell count, and the mice treated with c3Ado in the present study did well and had no signs of infection.

Nevertheless, our results are consistent with the hypothesis of a direct relationship between expression of adhesion molecules and lesion formation. Furthermore, our findings suggest that the inhibition of endothelial ICAM-1 and VCAM-1 expression prevents diet-induced plaque formation. The mechanisms by which c3Ado exerts its effect are the focus of ongoing work. However, other mechanisms may also play an important role. For example, c3Ado is a potent inhibitor of essential transmethylation reactions, which are required for 3 chemoattractant-mediated functions in monocytes/macrophages: (1) chemotaxis, (2) the stimulated release of arachidonic acid from membrane phospholipids, and (3) superoxide production.19 Furthermore, c3Ado is known to reduce transcriptional activation of platelet-derived growth factor, endothelial-leukocyte adhesion molecule-1,37 and ICAM-1 mRNA selectively. These absorbing properties of c3Ado may contribute to the drug’s protective activity as an anti-inflammatory agent.

On the basis of these results, we conclude that c3Ado, which has already been tested in patients with rheumatoid arthritis,24 is a potent drug in preventing the expression of inflammatory cellular adhesion molecules, monocyte/macrophage recruitment, and the development of atherosclerotic lesions. This drug may therefore represent a new pharmacological approach for the prevention and treatment of atherosclerosis.

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References


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