Effect of 1α-25-dihydroxyvitamin D_3 on intimal hyperplasia developing in vascular anastomoses: a rabbit model

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Abstract

Introduction: A common problem encountered in routine daily practice of cardiovascular surgery is migration of smooth muscle cells leading to intimal hyperplasia developing at vascular anastomosis sites which then causes luminal narrowing. The aim of this study was to investigate the antiproliferative effect of 1,25 $(OH)_2D_3$ on intimal hyperplasia.

Material and methods: Twenty-one male white New Zealand rabbits weighing 2-3 kg were selected. There were 3 groups of animals each consisting of 7 rabbits. Group 1 was the control group. Group 2 was the sham group and group 3 consisted of rabbits receiving 1,25 $(OH)_2D_3$. The right carotid arteries of the subjects in groups 2 and 3 were transected and re-anastomosed. A daily dose of 25 ng 1,25 $(OH)_2D_3$ per 100 g body weight was administered for 14 days to rabbits in group 3. Rabbits in group 2 were not subject to any pharmaceutical agent. All the subjects were sacrificed at the end of the 28th postoperative day. Their right carotid arteries were resected and then investigated histopathologically.

Results: Intimal thickness and intimal area were measured as significantly lower in group 1 when compared with the other groups (p = 0.004). In group 3, the ratios of thickness of tunica intima/thickness of tunica media and area of tunica intima/area of tunica media were significantly lower than those of group 2 (p = 0.015, p = 0.003).

Conclusions: 1,25 $(OH)_2D_3$, the active metabolite of vitamin D, reduces the intimal hyperplasia developing after vascular anastomoses.

Key words: carotid artery, vitamin D, intimal hyperplasia.

Introduction

Reconstructive surgery is a commonly used method as a treatment modality in obstructive arterial disease. Recently, the success of these interventions has been worse then expected due to spontaneous thrombus formation or development of stenosis. Contrary to acute occlusion immediately after vascular reconstructive interventions, where acute thrombus formation takes place, neointimal hyperplasia – developing after migra-

tion of smooth muscle cells and accumulation of collagen – plays an important role in pathogenesis of constriction or restenosis of the late period [1, 2]. Vitamin D is a significant regulator of calcium metabolism. It has an antiproliferative effect on some cell systems as well as on smooth muscle cells [3, 4]. Accordingly, in this study, we investigated the effect of 1,25 $(OH)_2D_3$ – the active form of vitamin D – on intimal hyperplasia.

Material and methods

This study was conducted according to the "Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care)" and we obtained approval from the Local Experimental Animal Ethics Committee of Dokuz Eylul University.

During the course of the study, all rabbits were routinely fed with rabbit chow and tap water ad libitum. The rabbits were maintained on a 12 : 12-h light : dark cycle in an environmentally monitored room with a ventilation system at a temperature of 20 ±2°C. Twenty-one male white New Zealand rabbits weighing 2-3 kg were selected.

There were 3 groups of animals each consisting of 7 rabbits. Group 1 was the control group. Group 2 was the sham group and group 3 consisted of rabbits receiving 1,25 $(OH)_2D_3$. Animals in group 1 were not subject to any intervention and were sacrificed at the end of the 28th day using 150 mg of thiopental sodium per kg body weight (Pental, Ibrahim Ethem Ulagay, Istanbul, Turkey). Their right carotid arteries were resected. Preoperatively, auricular marginal veins of the rabbits were cannulated in group 2 and 3. Intramuscular injection of the mixture of 50 mg/kg body weight ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul, Turkey) and 5 mg/kg body weight xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) was performed. Intravenous cefazolin sodium (Sefazol, Mustafa Nevzat, Istanbul, Turkey) was administered at a dose of 50 mg/kg body weight for antibiotic prophylaxis. Providing aseptic conditions, a vertical right jugular incision was performed and a 3-cm segment of carotid artery was explored. Then, heparin sulfate (Liquemine, Roche, Turkey) was administered intravenously at a dose of 100 IU/kg body weight and the carotid artery was clamped proximally and distally with bulldog clamps. The same artery was then transected and re-anastomosed using 8/0 polypropylene suture. Subcutaneous and cutaneous tissues were closed. No additional drug was administered to the animals in group 2 and they were then sacrificed at the end of the 28th postoperative day using 150 mg of thiopental sodium per kg body weight. Their right carotid arteries were then resected. During the postoperative period, animals of group 3 received subcutaneous injections of 1,25 $(OH)_2D_3$ (Calcijex, Abbott, Turkey) at a daily dose of 25 ng/100 g body weight for 14 days. These animals were also sacrificed at the end of the 28th postoperative day as described above.

Histopathological examination

For light microscopic examination, segments of the carotid anastomotic site obtained from the sacrificed rabbits were immediately placed in 10% formalin in phosphate buffer overnight, processed by routine histological methods and embedded in paraffin blocks. Paraffin blocks were placed in a Leica RM2125 rotary microtome (Germany) and sections of 5 µm thickness were obtained. The sections were stained with both hematoxylin and eosin and Masson's trichrome stain in a standard manner. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan) equipped with a high-resolution video camera (Olympus DP71, Japan). Anastomotic intimal hyperplasia was calculated at the site of maximum thickness by quantitative morphometric analysis with digital image analysis software (UTSCSA; Image tool version 3,0). The thickness of media, which was near to the anastomotic site and maintained normal structure, was also calculated. The areas of tunica intima and tunica media, diameters of vessels and their luminal areas were also calculated. All histomorphometric procedures were performed blindly.

Statistical analysis

Results are presented as medians (minimum – maximum values). All data were analyzed by Mann-Whitney test. Values of p below 0.05 was considered statistically significant.

Results

In groups 2 and 3, where vascular injury was formed by anastomosis, median intimal thickness was significantly higher than that of group 1, where the native arteries were kept without surgical intervention ($p = 0.04$) (Table I). The median intimal thickness was measured as < 0.01 mm in group 1, whereas this was measured as 0.105 (0.089-0.169) mm and 0.083 (0.080-0.120) mm, in group 2 and 3, respectively. The median intimal area was measured as 0.0307 (0.0187-0.0545) mm^2 in group 1, whereas this was measured as 0.1364 (0.0595-0.2001) mm^2 and 0.1497 (0.0470-0.2793) mm^2, in group 2 and 3, respectively. This difference was also statistically significant ($p = 0.004$, $p = 0.009$). During histological examination of the sections, this increase in intimal thickness and area was observed to originate from increased collagen accumulation and proliferation of smooth muscle cells due to vascular injury (Figures 1 A-C).

Although the median intimal thickness of the subjects in group 3 (anastomosis + 1,25 $(OH)_2D_3$)

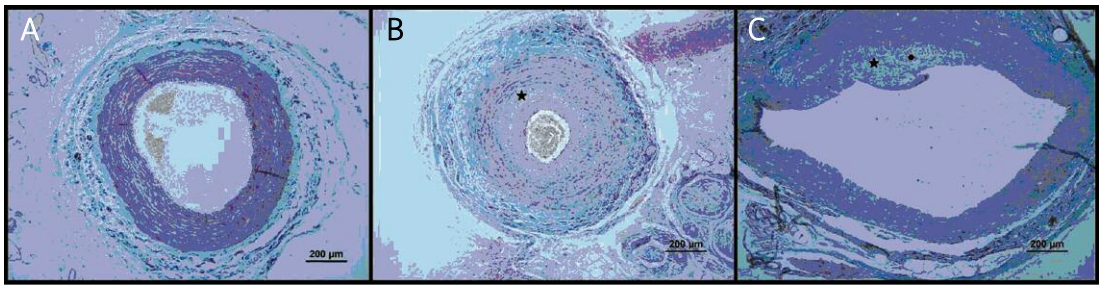
Table I. Histopathological measurements related to tunica intima and media

Variable	Group 1 (Control) (n = 7)	Group 2 (Sham) (n = 7)	Group 3 (Vitamin D) (n = 7)	Value of p		
				Group 1-group 2	Group 1-group 3	Group 2-group 3
Intima thickness [µm]	7.56 (6.48-8.18)	105.1 (89.70-169.36)	83.285 (80.30-120.52)	0.004	0.004	0.180
Media thickness [µm]	176.36 (161.06-179.94)	144.23 (105.14-190.74)	176.535 (123.77-237.38)	0.177	0.931	0.180
Intima/media ratio	0.0435 (0.04-0.05)	0.82 (0.64-0.89)	0.53 (0.38-0.70)	0.004	0.004	0.015
Intima area [mm^2]	0.0307 (0.0187-0.0545)	0.1364 (0.0595-0.2001)	0.1497 (0.0470-0.2793)	0.004	0.009	0.818
Media area [mm^2]	0.5327 (0.4447-0.5494)	0.4253 (0.2786-0.5994)	0.6069 (0.3032-1.4859)	0.537	0.177	0.132
Intima area/media area	0.06 (0.42-0.99)	0.34 (0.09-0.52)	0.20 (0.13-0.25)	0.04	0.04	0.003

was measured as lower than that of subjects in group 2 (anastomosis only), this difference was statistically insignificant (p = 0.180) (Table I). In addition, median thickness of tunica media of the subjects in group 3 was higher than that of subjects in group 2, but with no statistical significance (p = 0.180) (Table I). Nevertheless, in group 3, the ratio of thickness of tunica intima/thickness of tunica media was significantly lower than that of group 2 (p = 0.015) (Table I). This ratio was calculated as 0.53 (0.38-0.70) in group 3, whereas it was calculated as 0.82 (0.64-0.89) in group 2. Although the median intimal area in group 3 was calculated as higher than that in group 2, this difference was statistically insignificant (p = 0.818) (Table I). Comparing these two groups in terms of median area of tunica media, it was calculated as 0.6069 (0.3032-1.4859) mm^2 and 0.4253 (0.2786-0.5994) mm^2 in group 3 and group 2, respectively. This difference was statistically insignificant. But, the primarily considered parameter is the ratio of area of tunica intima/area of tunica media when someone talks about intimal hyperplasia. This ratio was calculated as 0.20 (0.13-0.25) and 0.34 (0.09-0.52) in groups 3 and 2, respectively. This difference between ratios was statistically significant (p = 0.003) (Table I). In histological sections it was observed that 1,25 $(OH)_2D_3$ inhibited smooth muscle cell proliferation and collagen accumulation significantly, thus reducing the ratio of intima thickness/media thickness (Figure 2).

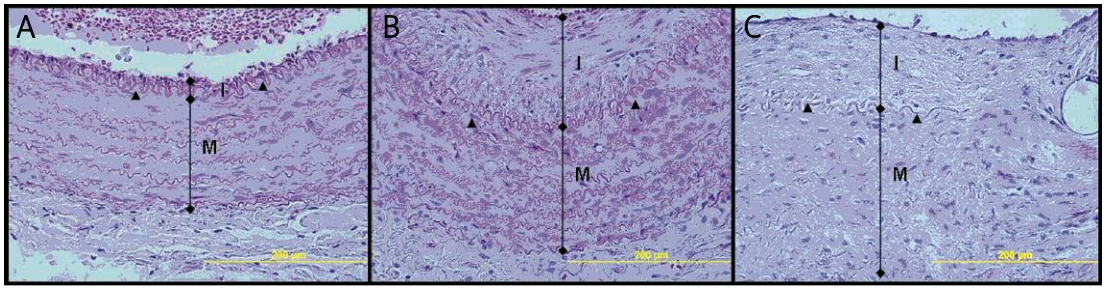
Discussion

The two most common methods used to create vascular injury in experimental settings regarding intimal hyperplasia are balloon catheterization and anastomosis models [1, 5-7]. Balloon catheterization creates intimal injury mostly whereas the anastomosis model injures all layers of the vessel wall. During balloon catheterization, the catheter balloon is inflated manually and this causes heterogeneity in terms of pressure applied. Many studies have shown that application of higher pressures increases the restenosis rates [8]. Another advantage of the anastomosis model over balloon catheter is involvement of all layers of the vessel wall, thus – we think – forming more intimal injury. Therefore, we thought that balloon catheterization may cause problems in terms of standardization of the experimental model and preferred anastomosis model. Since estrogen diminishes the neointimal response,



**collagen*

Figure 1. Histological sections of the carotid artery (Masson trichrome stain). **A** – Group 1, **B** – group 2, **C** – group 3. Carotid artery sections obtained from control group showed thin and intact lamina and collagen-rich adventitia (A). Accumulation of intimal collagen in the remaining two groups (B, C)



I – intima, M – media

Figure 2. Histological sections of the carotid artery (hematoxylin and eosin stain). **A** – Group 1, **B** – group 2, **C** – group 3. The ratio of intimal thickness/media thickness was lower in group 3 than that of group 2. Arrowheads point at internal elastic laminae

we preferred male rabbits [9]. In our study, we observed that intimal hyperplasia developed in both anastomosis groups when compared with the control group with no anastomosis. This demonstrated that the anastomosis model created a successful vascular injury. Almost all surgical interventions of the arterial system cause vascular injury, thus leading to proliferation of the smooth muscle cells and intimal hyperplasia. After endothelial injury, platelet adhesion and aggregation take place at the site of injury. Growth factors and cytokines secreted by platelets, macrophages and activated endothelial cells cause proliferation of medial smooth muscle cells. Proliferated smooth muscle cells then migrate into the intima. Intimal hyperplasia develops after smooth muscle cell proliferation, and synthesis and accumulation of extracellular matrix in the tunica intima. In brief, the first stage of intimal hyperplasia is proliferation of smooth muscle cells and the second stage is migration of these cells into the tunica intima [5, 10-13].

In the light of all this knowledge, inhibition of smooth muscle cell proliferation would also diminish intimal hyperplasia. It was previously postulated that vitamin D and its metabolites interfered with cell proliferation, thus regulating apoptosis and controlling malignant cell growth and proliferation. These effects are seen on several cell types possessing vitamin D receptors. G1/S phase of the cell cycle forms the center of the antiproliferative effect of 1,25 $(OH)_2D_3$. Proliferating cells undergo a cycle: G0/G1 phase (most differentiated and non-dividing cells are in G1 phase), S phase (DNA synthesis takes place), G2 phase and the subsequent division (mitosis) phase (M phase). There are many studies published indicating that at pharmacological doses, 1,25 $(OH)_2D_3$ plays an inhibitory role at G1/S phase and interferes with cell division completely [14-16].

Although most of the studies related to the antiproliferative effect of vitamin D are associated with oncological issues, there are several studies investigating vascular smooth muscle cells. In one of these studies, Carthy *et al*. examined the effect of 1,25 $(OH)_2D_3$ on vascular smooth muscle cells and epidermal growth factor (EGF) in cell culture media. They demonstrated that 1,25 $(OH)_2D_3$ inhibited growth of the vascular smooth muscle cells. Likewise, 1,25 $(OH)_2D_3$ suppressed the mitogenic effect of EGF on these cells [4]. Another study showed that 1,25 $(OH)_2D_3$ inhibited expression of the elastin gene in smooth muscle cells and proliferation of the smooth muscle cells. This effect was mediated by increasing the number of immature elastic fibers, thus relatively decreasing the number of mature elastic fibers [17]. Consistent with the literature data, in our study, it was demonstrated that intimal hyperplasia was diminished significantly in the group of animals administered 1,25 $(OH)_2D_3$ in comparison with the sham group due to inhibition of smooth muscle cell proliferation. To our knowledge, this study is the first study in the literature at tissue level demonstrating that 1,25 $(OH)_2D_3$ diminishes intimal hyperplasia developing after the anastomosis.

Consequently, inhibition of proliferation of smooth muscle cells also inhibits intimal hyperplasia. Via its antiproliferative characteristics, 1,25 $(OH)_2D_3$ inhibits proliferation of smooth muscle cells and therefore diminishes development of intimal hyperplasia.

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