Inhibitory effects of ticlopidine and clopidogrel on the intimal hyperplastic response after arterial injury

Tiklopidin ve klopidogrelin arteryel hasar sonrası intimal hiperplastik yanıtına baskılayıcı etkileri

Tuğrul Göncü, Osman Tiryakioğlu, Ayhan Özcan, Hasan Arı, Mustafa Sezen, Hakan Vural, Ahmet Bayer**, Serhat Yalçınkaya***, Şenol Yavuz, Ahmet Özyazıcıoğlu*

From Departments of Cardiovascular Surgery, *Cardiology, **Pathology and ***Thoracic Surgery, Bursa Yüksek İhtisas Education and Research Hospital, Bursa, Turkey

ABSTRACT

Objective: The purpose of this study was to compare the effects of ticlopidine and clopidogrel on the development of neointimal hyperplasia after experimental arterial injury.

Methods: This experimental, prospective, randomized controlled study was performed on twenty-seven rabbits, which were divided into three groups, each of which contained nine subjects. Following the development of a balloon catheter injury in the iliac artery, no drugs were administered to Group 1 (control). Group 2 was given ticlopidine, while Group 3 was given clopidogrel. At the end of the 21-day experimental period, arterial sections were evaluated histomorphologically and immunohistochemically with staining using antibodies against platelet derived growth factor β and basic fibroblast growth factor. Statistical analyses were performed using Chi-Square, Mann Whitney U and one-way ANOVA tests.

Results: At the end of study period, ticlopidine and clopidogrel strongly reduced the development of intimal hyperplasia after arterial injury (54.1%, $p<0.001$, 53.2%, $p<0.001$, respectively). No significant difference was observed in terms of intimal and medial areas between the drug-treated groups. Expressions of the basic fibroblast growth factor and platelet derived growth factor β were significantly lower in the intima of drug treated groups with respect to the control group ($p<0.05$).

Conclusion: The results of our study suggest that ticlopidine and clopidogrel, which are widely used in antiplatelet treatment in clinics, can similarly prevent the development of intimal hyperplasia after experimental arterial injury. *(Anadolu Kardiyol Derg 2010; 10: 11-6)*

Key words: Ticlopidine, clopidogrel, intimal hyperplasia, restenosis, iliac artery

ÖZET

Amaç: Tiklopidin ve klopidogrelin, deneysel arteryel hasar sonrası neointimal hiperplazi gelişimi üzerindeki etkinliklerinin karşılaştırması amaçlandı.

Yöntemler: Bu deneysel, randomize, prospektif, kontrollü çalışmada, 27 tavşan her grupta 9 denek olacak şekilde 3 gruba ayrıldı. İliyak arterde oluşturulan balon kateter hasarı sonrası; Grup 1 deneklere (kontrol grubu) hiçbir ilaç verilmedi, Grup 2 deneklere tiklopidin, Grup 3'e klopidogrel verildi. Yirmi bir günlük deney süresi sonunda arteryel kesitler histomorfolojik olarak ve temel fibroblast büyüme faktörü (bFGF) ve trombosit kaynaklı büyüme faktörü beta (PDGF-β) antikorları ile boyama yapılarak immunohistokimyasal olarak değerlendirildi. İstatistiksel analizde Ki-kare, Mann Whitney U, ve tek yönlü ANOVA testleri kullanıldı.

Bulgular: Deney süresi sonunda, tiklopidin ve klopidogrel grubunda, intimal hiperplazi kontrol grubuna göre anlamlı olarak daha azdı (sırası ile % 54.1 $p<0.001$ ve % 53.2 $p<0.001$). İlaç verilen gruplar arasında ise intimal alan ve mediyal alanlar yönünden anlamlı bir fark saptanmadı ($p>0.05$). İlaç verilen gruplarda, neointimal tabakadaki bFGF ve PDGF-β ekspresyonu, kontrol grubuna oranla daha azdı ($p<0.05$).

Sonuç: Çalışmamızın sonuçları klinikte antiplatelet tedavide yaygın olarak kullanılan tiklopidin ve klopidogrelin; deneysel arteryel hasar sonrası intimal hiperplazi gelişimini benzer şekilde engelleyebileceğini göstermektedir. *(Anadolu Kardiyol Derg 2010; 10: 11-6)*

Anahtar kelimeler: Tiklopidin, klopidogrel, intimal hiperplazi, restenoz, iliyak arter

Address for Correspondence/Yazışma Adresi: Osman Tiryakioğlu MD, Bursa Yüksek İhtisas Education and Research Hospital, Department of Cardiovascular surgery, Bursa, Turkey Phone: +90 224 360 50 50 Fax: +90 224 360 50 55 E-mail: osmantiryaki@gmail.com

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Introduction

The long-term benefits of vascular reconstructive procedures, such as surgical arterial bypass, endarterectomy or balloon angioplasty, are significantly limited by restenosis secondary to neointimal hyperplasia (1, 2). Neointimal hyperplasia is the pathologic consequence of the vascular healing response. Despite intensive investigations, the mechanism controlling intimal thickening and the factors causing uncontrolled intimal hyperplasia are not yet clearly understood. Based on known physiopathological characteristics of restenosis, many pharmacological agents have been employed to prevent restenosis in human and animal models. However, no effective and definitive treatment that prevents intimal hyperplasia has been found yet (2-4).

Because platelets are one of the main factors in the initiation and maintenance of the intimal hyperplasic response after arterial injury (5, 6), it is highly rational to use drugs that strongly inhibit platelet functions, such as adhesion and/or aggregation, to reduce the related response.

Ticlopidine and clopidogrel are two thienopyridines with potent and apparently irreversible platelet inhibitory properties. The antiplatelet effects are mainly directed against ADP-induced stimulation of platelet function, in particular ADP-induced inhibition of adenylyl cyclase stimulation. Ticlopidine and clopidogrel are very similar in their structures but have widely differing side effect profiles and pharmacological properties (7, 8).

In many previous studies, both drugs particularly clopidogrel, were demonstrated to reduce the development of intimal hyperplasia following vascular injury (2, 9, 10). However, there are not sufficient comparative studies performed between ticlopidine and clopidogrel with respect to this effect.

It has not been completely understood how these drugs, which are frequently used for the prevention of restenosis following vascular interventions in clinics, exhibit their restenosis reducing effects. However, Walksman et al. (9) suggested in an experimental study, which they recently performed that clopidogrel may be effective due to its anti-inflammatory characteristics besides its inhibitory effects against proliferation of vascular smooth muscle cell (SMC).

Many growth factors such as platelet derived growth factor β (PDGF-β) and basic fibroblast growth factor (bFGF) modulate the vascular injury response. These growth factors are responsible for SMC proliferation and migration and act as survival signals preventing initiation of the apoptotic cascade (6, 11).

The purpose of this study was to compare the effects of ticlopidine and clopidogrel on the development of neointimal hyperplasia and PDGF and b-FGF, which have a significant role in SMC proliferation and migration following experimental arterial injury.

Methods

This experimental, prospective, randomized controlled study was performed on 27 New Zealand male white rabbits with 2625±225 grams mean weight and 10±1 months mean age. Throughout the course of this study, we abided by the principles outlined by the "Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care)" and obtained approval from the Ethics Committee of Bursa Uludağ University.

During the course of the study, all rabbits were routinely fed with standard rabbit diet. All rabbits were kept in a room with a ventilation system and sun exposure at a temperature of 20±2°C. The balloon catheter injury model, which has been previously described by Hamon et al. (12), was applied with some modifications. Variations from this model included the following: replacing the Fogarty catheter with a balloon angioplasty catheter for the formation of arterial injury and using a balloon inflation device instead of setting the balloon inflation pressures manually.

Twenty-seven rabbits were randomized into three groups according to a computer-generated random sequence, each of which included nine subjects. The groups were designated as follows:

Group 1 (control group, n=9): No drug was given to the subjects of this group throughout the duration of the study, and an angioplasty balloon catheter was used to induce an injury in the right external iliac artery. For the subjects in this group, the left external iliac artery, to which no surgical procedure was applied, was used for the evaluation of the normal arterial sections.

Group 2 (ticlopidine group, n=9): The study group in which ticlopidine (Ticlocard, Koçak, Turkey) was applied at a dose of 200 mg/kg/day after the right external iliac artery balloon catheter injury.

Group 3 (clopidogrel group, n=9): The study group in which clopidogrel (Karum, Sanovel, Turkey) was applied at a dose of 20 mg/kg/day after the right external iliac artery balloon catheter injury.

Ticlopidine or clopidogrel was administered orally via gavage, applied once each day for a treatment period of 21 days by dissolving the medication in water. Drug treatment was started during vascular intervention.

Anesthesia was applied by intramuscular (IM) ketamine hydrochloride (Ketalar, Eczacıbaşı, Istanbul-Turkey) at a dose of 100 mg/kg and IM xylazine hydrochloride (Rompun, Bayer, Istanbul-Turkey) at dose of 14.2 mg/kg. Following induction of anesthesia, venous cannulation was carried out using the dorsal ear vein of subjects. Five minutes prior to surgical intervention, 50 U/kg IV amoxicillin (Alfasilin, Fako, Turkey) and 300 IU/kg IV heparin sulfate (Liquemine, Roche, Turkey) were administered. Then, the superficial femoral artery was mobilized in a sterile manner. By applying vertical femoral arteriotomy, a balloon angioplasty catheter (balloon size: 2.5 mm diameter and 20 mm length) (Cordis-Europa, Netherlands) was advanced to the abdominal aorta inside of the femoral artery in a retrograde fashion. The balloon of the angioplasty catheter was inflated up to an atmospheric pressure of 5 using a balloon inflation device (Medtronic, Minneapolis, USA) filled with saline and was slowly pulled towards the femoral artery. This procedure was repeated three times by rotating the catheter 120°. Thus, an injury was induced in the vascular wall, and the foundation for stenosis development in the future periods was established. The catheter was pulled, and the femoral artery was ligated both distally and proximally to the arteriotomy. The skin incision was closed using absorbable suture material.

Sampling vessels and histomorphological analysis

At the end of the study (the 21st day), anesthesia was administered to all subjects as described above. Then, the abdomen was opened by a midline incision the aorta was cannulated with a 22G branule (Bıçakçılar, Istanbul, Turkey) after releasing it just below diaphragm. The inferior vena cava was also cut at this level, and drainage of venous blood was achieved. In order to preserve the *in vivo* size of arteries during analysis, saline was administered via the cannula placed in the aorta, and this procedure was continued until clear fluid was obtained from the inferior vena cava. A 10% formalin solution was infused for 20 minutes in order to fix the vessels. Then, the abdominal aorta was removed by dissecting the common iliac, superficial iliac artery, and femoral arteries localized before the ligated portion, and it was kept in a 10% formaldehyde solution until the time of histological analysis. Three samples, each 0.5 cm in length, were obtained by starting just below the internal iliac artery, and those sections were embedded in paraffin. Sections with 5 µm thickness were cut from those paraffin blocks with the help of a microtome. After deparaffinizing the sections and placing them onto glass slides, the sections were stained with hematoxylin-eosin and elastin Van Gieson stains. For morphological evaluation, preparations that had been stained with elastin Van Gieson stain, according to the method used previously by Petrik et al. (13), were examined under an Olympus x40 BH-2 photomicroscope and photographed. Intimal and medial areas were calculated in those photos using a digital planimeter (Placaom KP-90 N Sokusha, Japan). Subsequently, the intima to media ratio was calculated by dividing the total intimal areas of each section by the total medial area.

Immunohistochemical analysis

Immunohistochemical staining was performed using the avidin–biotin–peroxidase complex technique (14). Paraffin-embedded external iliac artery tissues were sectioned at 5 µm, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked in three 5-minute baths of H_2O_2 at 3% in distilled water. The sections were incubated with rabbit polyclonal antibodies against PDGF-β (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and bFGF (1:2000; Chemicon International, Temecula, CA) for 30 minutes at 37°C. Then, the sections were washed with 0.01 M phosphate-buffered saline with a pH of 7.4 and incubated with avidine-biotine peroxidase for 30 minutes.

Finally, the sections were incubated with 3-amino-9-ethylcarbazole substrate-chromogen and counterstained with Mayer's hematoxylin. The preparations that were subjected to immunohistochemical staining were evaluated under a light microscope.

The intensity of the staining of the samples was scored from 0 to 3 as follows: 0-no visible staining; 1-cells with faint staining; 2-moderate intensity with multifocal staining, and 3-intense diffuse staining.

Statistical analysis

All values obtained were evaluated as "mean±standard deviation," and the statistical significance of differences between mean results was determined using the SPSS for Windows version 11.0 statistical program (Chicago, IL, USA). The normal distribution suitability of the groups was assessed using the Shapiro-Wilk test. Categorical data were evaluated with Chi-Square and non-parametric continuous variables were compared using Mann Whitney U-test, whereas parametric continuous variables were evaluated with the one-way ANOVA test. Study groups were compared with the control group by applying the Dunnett posttest. For all statistical evaluations, p values <0.05 were recognized as statistically significant.

Results

Whereas the intimal area was <0.01±0.003 mm^2 in non-injured arteries, it was 0.387±0.021 mm^2 in injured arteries of the control group on the 21st day after injury (p<0.001), (Fig. 1A, 1B). The neointimal area was reduced compared to the control group by 54.1% in the ticlopidine group (p<0.001) and by 53.2% in the clopidogrel group (p<0.001). Mean intimal area was 0.387±0.021 mm^2 in the control group, 0.178±0.032 mm^2 in the ticlopidine group, and 0.181±0.033 in the clopidogrel group (Table 1, Fig. 1C, 1D).

In terms of the decrease in neointimal area, the ticlopidine and clopidogrel groups exhibited no statistically significant difference (p>0.05), (Table 1, Fig. 1C, 1D). The media layer was thinner in the ticlopidine and clopidogrel groups with respect to the control group. However, this difference was not statistically significant. The mean medial area in the control group was 0.404±0.037 mm^2, compared to; 0.398±0.035 mm^2 in the ticlopidine group and 0.401±0.033 mm^2 in the clopidogrel group (p>0.05), (Table 1).

Comparison of the control group and the two study groups in terms of intimal/medial area ratios revealed a significant drop in both study groups. The intima/media ratio was 0.94±0.04 in the control group, 0.44±0.09 (p<0.001) in the ticlopidine group, and 0.45±0.07 (p<0.001) in the clopidogrel group. When the intima/media ratios of the ticlopidine and clopidogrel groups were

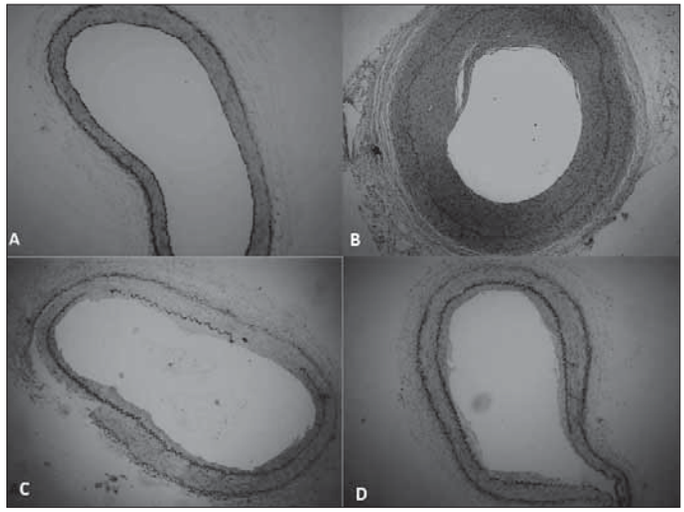

Figure 1. Reduction of intimal hyperplasia with either ticlopidine or clopidogrel treatment, as indicated by elastin van Gieson stain (x40): A) non-injured; B) Control; C) Ticlopidine; D) Clopidogrel

compared, no significant difference between them was detected (p>0.05), (Table 1).

Expression of PDGF-β and bFGF was lower in the intimal area in the clopidogrel and ticlopidine groups with respect to the control group. The staining scores for PDGF-β and bFGF were 2.44±0.72 and 2.33±0.86, respectively, in the control group versus 1.22±0.66 p=0.005 and 1.11±0.78; p=0.01, in the ticlopidine group and 1.11±0.78 (p=0.004) and 1.00±0.70 (p=0.006) in the clopidogrel group. The mean staining scores for PDGF-β and bFGF were similar in clopidogrel and ticlopidine groups (p>0.05), (Table 2, Fig. 2).

Discussion

The present study showed a 53.2% reduction in neointimal area in the clopidogrel group and a 54.1% reduction in the ticlopidine group when compared to that of the control group. A comparison of neointimal area reduction between the clopidogrel group and the ticlopidine group exhibited no statistically significant difference.

Cortelekoglu et al. (10) demonstrated that clopidogrel significantly reduced the development of neointimal hyperplasia in an experimental model, which is similar with the one we used in our study. In a recent study, Waksman et al. (9) demonstrated systemic clopidogrel administration attenuated reduced neointima formation by 37% in balloon-denuded iliac arteries of hypercholesterolemic rabbits. A study conducted by Herbert et al. (15) investigated the effects of clopidogrel, ticlopidine, and acetylsalicylic acid on intimal smooth muscle hyperplasia in rabbits that had been subjected to damage of the carotid artery endothelium by air. Following a 16-day treatment period, clopidogrel (25 mg/kg given on a daily basis) reduced the development of intimal thickening by 48% (p<0.01), ticlopidine

Table 1. Histomorphometric assessment of arterial sections

Variables	Group 1 (Control) (n=9)	Group 2 (Ticlopidine) (n=9)	Group 3 (Clopidogrel) (n=9)	F *	p**		
					Group 1-Group 2	Group 1-Group 3	Group 2-Group 3
Mean intimal area, mm^2	0.387±0.021	0.178±0.032	0.181±0.033	52.67	<0.001	<0.001	0.52
Mean medial area, mm^2	0.404±0.037	0.398±0.035	0.401±0.033	6.00	0.08	0.14	0.36
Intima/media ratio	0.94±0.04	0.44±0.09	0.45±0.07	56.71	<0.001	<0.001	0.78

Data in the table are expressed as mean±standard deviation
* - one-way ANOVA test **-posthoc Dunnett test

Table 2. Immunostaining assessment of arterial sections

Variables	Group 1 (Control) (n=9)	Group 2 (Ticlopidine) (n=9)	Group 3 (Clopidogrel) (n=9)	p		
				Group 1-Group 2	Group 1-Group 3	Group 2 Group 3
PDGF-β						
0 (No visible staining)	-	1	2			
1 (Cells with faint staining)	1	5	4			
2 (Moderate intensity with multifocal staining	3	3	3	0.034*	0.032*	>0.05*
3 (Intense diffuse staining)	5	-	-			
Total staining score	22	11	10	0.005Ω	0.004Ω	>0.05Ω
Mean staining score	2.44±0.72	1.22±0.66	1.11±0.78			
bFGF						
0 (No visible staining)	-	2	2			
1 (Cells with faint staining)	2	4	5			
2 (Moderate intensity with multifocal staining	2	3	2	0.04*	0.04*	>0.05*
3 (Intense diffuse staining)	5	-	-			
Total staining score	21	10	11	0.01Ω	0.006Ω	>0.05Ω
Mean staining score	2.33±0.86	1.11±0.78	1.00±0.70			

The staining scores in the table are expressed as mean ± standard deviation whereas subject numbers as per staining densities are expressed numerically
*- Chi-square test, Ω- Mann-Whitney U test,
bFGF - basic fibroblast growth factor, PDGF-β - platelet derived growth factor β

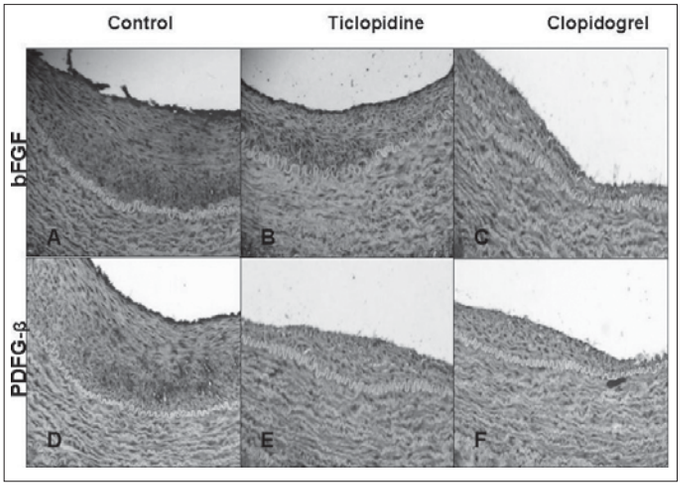

Figure 2. Immunohistochemical staining for bFGF and PDGFβ (x200): A, D) Control; B, E) Ticlopidine; C,F) Clopidogrel. Expressions of PDGF-β and bFGF were lower in the intimal area in the drug treatment groups with respect to the control

bFGF - basic fibroblast growth factor, PDGF-β - platelet derived growth factor β

(200 mg/kg given on daily basis) reduced it by 57% ($p<0.001$), and acetylsalicylic acid was ineffective. Our results support the results obtained in that study. However, unlike the study by Herbert et al. (15), our study found no significant differences between ticlopidine and clopidogrel in terms of intimal hyperplasia prevention.

Because smooth muscle cell (SMC) proliferation and the subsequent formation of extracellular matrix deposition by SMCs are the core factors playing a role in the development of intimal hyperplasia, using specific inhibitors of SMC proliferation is a reasonable approach for preventing restenosis. In order to evaluate this approach, the effects of many drugs on arterial injuries have been tested in animals and in patients who have been exposed to transluminal angioplasty for coronary arteries (2-5).

To simulate these clinical conditions, considerably different experimental models have been developed to elucidate the mechanism of intimal thickening. However, there are no animal models reflective of the formation of restenosis in humans (3, 16).

In the present study, we used the catheter injury model employed by Hamon et al. (12) and Ohkawa et al. (17), applying some modifications to it. In our study, we preferred the same type and sizes balloon angioplasty catheters, in place of a Fogarty catheter in order to standardize the severity of the damage. Aiming to minimize the differences between animals in terms of vascular diameters and age, we tried to include rabbits of the same weight and age. Because neointimal formation in rat carotid arteries following balloon injury differs in males and females and because estrogen reduces the neointimal response forming against balloon injury (18), we included only male rabbits in our study. Generally, rabbit models fed with cholesterol-rich diets are preferred for restenosis and atherosclerosis studies conducted after angioplasty. However, serum cholesterol levels in such models are known to be above 2.000 mg/ml (18). This hypercholesterolemia causes diffuse cholesterol deposition in the subendothelium and principally mimics lipid storage diseases. In such models intimal lesions typically contain a high quantity of macrophages (~%50) and are dissimilar to the restenosis seen in humans (18). Due to these disadvantages of the cholesterol-rich dietary model and because our primary aim in this study was to observe the acute effects of balloon injury causing hyperplasia rather than the arterial restenosis seen in atherosclerosis, we employed an animal model fed by a routine diet. Because time is considered an important factor for development of intimal hyperplasia (19), the study period following the vascular injury was limited to 21 days.

The integrity of smooth muscle cells and endothelial cells plays a critical role in the maintenance of the structural and functional properties of the arterial wall (20). When the vascular surface is de-endothelialized, the de-endothelialized areas are immediately covered by a set of platelets. The platelets are replaced by regenerated endothelium that moves toward the arterial lumen within the following days. Concurrently, SMCs that began to proliferate in the media then migrate toward the intima. In the meantime, these cells synthesize and secrete a huge amount of extracellular matrix in addition to proliferation (20, 21). Platelets directly relate to intimal proliferation after arterial injury, and severe thrombocytopenia inhibits intimal thickening, an effect that is correlated with the degree of thrombocytopenia (22). After arterial injury, platelets rapidly adhere to the site of injury via several adhesion receptors: thromboxane A_2 is generated, changes in the glycoprotein (GP) IIb/IIIa complex occur, the GP IIb/IIIa complex then binds to fibrinogen, and, subsequently, platelets aggregate and are activated (23). Activated platelets release PDGF, a potential smooth muscle cell mitogen (6, 24). This close interaction between the platelets and the response to the vascular injury demonstrates that drugs that strongly inhibit platelet function should reduce the pathological intimal response observed after vascular injury.

As reported previously (15), the interaction between subendothelial connective tissue and platelets could be almost completely prevented by daily doses of ticlopidine (200 mg/kg) or clopidogrel (25 mg/kg). Based on these results, we chose drug levels within this range for our study.

A number of studies suggest that PDGF and bFGF are involved both in the initial wave of medial SMC proliferation as well as SMC migration to the intima (25, 26). A neutralizing antibody against bFGF significantly reduces intimal SMC proliferation and migration (11). PDGF is the most important growth factor released by activated platelets. Besides inducing proliferation, the primary effect of PDGF on vascular smooth muscle cells could be the induction of migration, as PDGF is the strongest reported chemo attractant for vascular SMCs (27).

In our study, the intensity of bFGF and PDGF-β expression were evaluated with immunohistochemical staining in arterial sections, and expression of bFGF and PDGF-β were lower in the ticlopidine and clopidogrel groups with respect to the control group. There were no significant differences between the two treatment groups in terms of the concentration of these factors.

Our findings confirm that ticlopidine and clopidogrel are effectively reduced these mitogenic factors and hereby may affect intimal hyperplasia by decreasing both proliferation and migration of smooth muscle cells.

Study limitations

There are some potential limitations of this study. This is a short-term study, the number in the study groups was low, and statistical analysis was limited. The pathophysiology of restenosis in a human artery is probably more complex than the arterial response to injury in animal models, and may in fact represent a different process. Therefore, the extrapolation of data from an animal model to human requires caution.

Conclusion

In conclusion, clopidogrel and ticlopidine, which are potent ADP-selective platelet inhibitors, can significantly reduce the effects of factors such as thrombosis, intimal thickening, and luminal stenosis that hamper the success rate of arterial reconstructions. We recommend the usage of both of these compounds after vascular reconstructive interventions.

Conflict of interest: None declared

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