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Vascular smooth cell proliferation in perfusion culture of porcine carotid arteries

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ABSTRACT

Objective of this study was to develop a novel *in vitro* artery culture system to study vascular smooth muscle cell (SMC) proliferation of porcine carotid arteries in response to injury, basic fibroblast growth factor (FGF2), and FGF2 conjugated with cytotoxin saporin (SAP). Perfusion-cultured porcine carotid arteries remained contractile in response to norepinephrine and relaxant to acetylcholine for up to 96 h. SMC proliferation of cultured arteries was detected by bromodeoxyuridine incorporation in both non-injured and balloon-injured arteries. In the inner layer of the vessel wall near the lumen, SMC proliferation were less than 10% in uninjured vessels, 66% in injured vessels, 80% in injured vessels with FGF2 treatment, and 5% in injured vessels with treatment of FGF2-SAP. Thus, the cultured porcine carotid arteries were viable; and the injury stimulated SMC proliferation, which was significantly enhanced by FGF2 and inhibited by FGF2-SAP.

Proliferation of smooth muscle cells (SMCs) is a major event in the development of atherosclerosis and is thought to contribute to restenosis of arteries following balloon angioplasty [1–3]. During the course of atherosclerosis progress, growth factors and cytokines promote the migration and proliferation of vascular SMCs to contribute to the neointima formation [4]. Among the several growth factors involved in these processes, basic fibroblast growth factor (FGF2) appears to be a major factor to stimulate SMC proliferation [5,6]. Animal studies have shown that the combination of FGF2 with saporin (SAP) or platelet derived growth factor – BB (PFGF-BB) could reduce neointimal hyperplasia and lead to the formation of stable vessels [7,8]. However, detail mechanisms of regulation of SMC proliferation are not fully understood yet.

Numerous experimental approaches have been described to understand the pathogenesis of SMC proliferation. Among them are cell culture, animal models, and vascular tissue culture models. *In vitro* artery culture including static culture and perfusion culture models represents an alternative technique for studying vascular structure and function. It is more physiological than cell culture and less costly and more easily controlled (hemodynamically and biochemically) than *in vivo* studies. The static organ culture of the saphenous vein to model human vein graft neointimal hyperplasia was described by Soyombo and colleagues in 1990 [9] and Angelini and colleagues in 1991 [10]. Using this *in vitro* model, Allen et al have shown that the endothelium produces a paracrine mediator of SMC proliferation [11]. The major disadvantage of static organ culture is that it represents the response of a vein segment to culture in the absence of flow. Perfusion vascular tissue culture models combine many of the benefits of both cell culture and animal models. The chemical and mechanical environment of the vascular tissue can be precisely regulated in the perfusion culture systems. This allows true single factor to be studied, which would be impossible in the complex physiologic environment in animal models.

Our group has developed a simple technique for *in vitro* artery perfusion culture to study vascular biology under steady or unsteady flow conditions for up to several days [12,13]. Objective of this study was therefore to investigate SMC proliferation regulated by injury, FGF2 and SAP in porcine carotid arteries by using our *in vitro* vascular culture system.

Materials and methods

Perfusion culture system. We have developed a simplified blood vessel perfusion culture system [12,13], which consists of the medium chamber and gas exchange enhancer, the peristaltic pump, the compliance chamber, the pressure transducer, the vessel chamber, and the flow meter (Fig. 1A). Circulating cell culture medium from the medium chamber was driven by the peristaltic pump (Cole-Parmer) with controlled perfusion rates.

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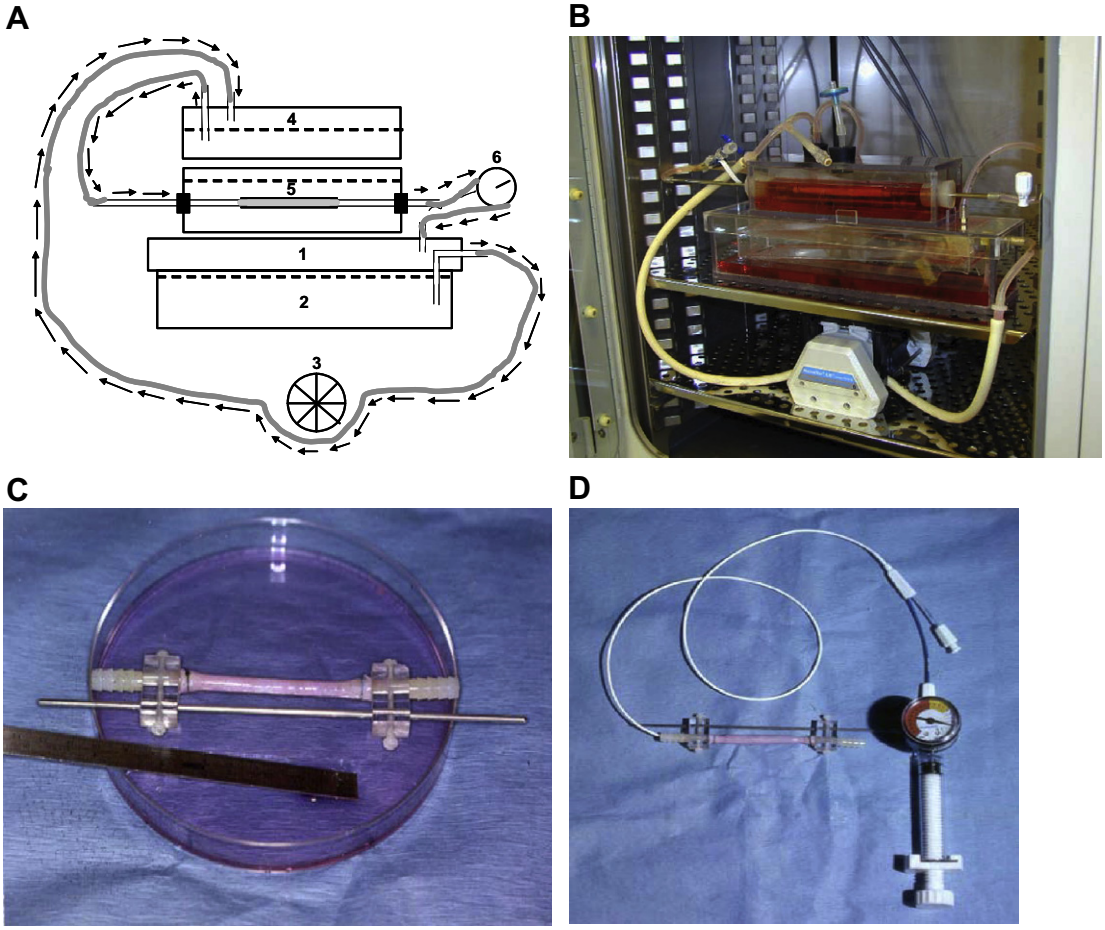

Fig. 1. Artery perfusion culture system. (A) Schematic showing: 1. Aeration enhancer; 2. Perfusion chamber, which contains circulating culture medium; 3. Roller pump; 4. compliance chamber; 5. vessel chamber, which contains static culture medium and a vessel; and 6. flowmeter. Arrows indicate the direction of flow. (B) The entire system was placed inside a cell culture incubator.

Laminar flow was achieved by connecting a compliance chamber in the fluid circuit. The porcine carotid artery was connected to the specially designed cannula and housed in a separated chamber (vessel culture chamber) with static cell culture medium. Flow rate and pressure were monitored by a flowmeter and a pressure transducer, respectively, which were connected in the fluid circuit. Returning medium was aerated by a specially designed gas exchange enhancer and returned back to the medium chamber. The entire system was relatively small and placed inside a cell culture incubator for temperature control and gas and moisture supplement (Fig. 1B).

Artery procedure and culture conditions. Porcine common carotid arteries were harvested from 7- to 9-month-old farm pigs weighing 250–290 lb at a local slaughterhouse. The carotid arteries were dissected out and prepared under sterile conditions, gently removing the excess fat and adventitia and keeping the endothelial surface moist at all times as well as to avoid damage to the surface or the intima. The 6–8 cm long vessel was then mounted onto a fitted cannula (internal diameter 2.75 mm, and external diameter 3.75 mm) at each end. The carotid segment was placed in the vessel chamber containing 50 ml of DMEM supplemented with 5% antibiotics (penicillin and streptomycin). The circulating medium was DMEM supplemented with 10% calf bovine serum (CBS) and bromodeoxyuridine (BrdU 5 μg/ml). The constant flow rate of 100 ml/min and the intraluminal pressure of 100 mm Hg were maintained. Pressure was maintained by a distal resistant clamp. Vessels were kept at 37 °C in an incubator under humidified air gassed with 5% CO_2 for different periods of time.

Four groups of cultures were run at the same flow and pressure conditions. First group was normal arteries that were cultured for 24, 48, 72, and 96 h, respectively. The second group was the arteries, which undergone balloon injury first and then cultured for 24, 48, 72, and 96 h, respectively. The clinic angioplasty balloon (5 cm long, 6 mm i.d.) was inserted into the lumen of vessels and inflated at 1 atmosphere pressure for 1 min before fixing arteries on the cannulas (Fig. 1C and D). The third group was balloon-injured arteries that were cultured for 96 h with intravascular medium supplemented with FGF2 (4×10^{-10} M). The fourth group was balloon-injured arteries that were cultured for 96 h with intravascular medium supplemented with FGF2-SAP (4×10^{-10} M). FGF2 and FGF2-SAP were the gift from Prizm Pharmaceutics In. (San Diego, CA).

Vessel viability. Vascular viability was confirmed by vasomotor function analysis in response to norepinephrine (NE), acetylcholine (ACh) or nitroglycerine challenges in selected arteries at different time points of perfusion culture. The outer diameters of the arteries were captured by a video camera and recorded on a videotape. After the outer diameters were recorded as initial control, NE was added and mixed with the perfusion medium in the medium chamber to a final concentration of 10^{-5} M., and then the outer diameter was recorded for 20 min. ACh (10^{-5} M) or nitroglycerine (400 μg/ml) was then added to the perfusion medium and the diameter was recorded for another 20 min. The video images were imported into a personal computer, and the outer diameters of cultured vessels were measured with the NIH Image program (National Institute of Health, MD). Arterial vasoconstriction in

response to NE, and vasodilatation in response to a subsequent dose of acetylcholine or nitroglycerine were measured and expressed as the percentage of diameter change. Percent contraction was calculated as $(D_i - D_c)/D_i \times 100$, where D_i was the initial diameter and D_c was the contracted diameter. Percent relaxation was calculated as $(D_r - D_c)/(D_i - D_c) \times 100$, where D_r was the relaxed diameter.

Immunohistochemistry. The arteries were harvested after the proper period of time of culture and fixed in 10% buffered formalin for overnight for further immunohistochemistry analysis. Immunostaining was carried out according to the avidin–biotin complex immunoperoxidase procedure (LSAB Kit, Dako Co., Carpenteria, CA) to identify proliferating cells with anti-BrdU monoclonal antibody (Dako). SMCs and endothelial cells were identified by immunostaining of α-actin and factor VIII related antigen, respectively. Antibodies for α-actin (HHF35) and factor VIII related antigen (N1505) were purchased from Dako Company. BrdU-positive cells were quantified manually using a cell counting technique per high power field (X400) on a micrometer grid. In each field, all cells were counted and the number of positively stained cells was expressed as a percentage of total cells to arrive at the BrdU index. Eight evenly divided locations from luminal side to adventitia side in each section were selected for cell counting. At each location, the vessel wall was divided into four layers from the luminal side to the adventitial side for separate expression of cell proliferation rates.

Statistical analysis. Comparisons among groups were made using an Analysis of Variance (ANOVA, single factor) with Microsoft Excel (2003). Values are given as means ± standard error (SE). Results were considered significant if the p value was less than 0.05.

Results

Vessel viability

The vessel viability was demonstrated by vasomotor activity after perfusion culture (Fig. 2). In response to NE, normal arteries contracted and the vessel diameter was decreased by 7%, 5%, 4%, 4%, 3.5% at 0, 24, 48, 72 and 96 h of perfusion culture, respectively. SMC contractility of the vessels was decreased slightly over the time. In response to ACh, normal arteries relaxed and the vessel diameter was increased by 15%, 15%, 10%, 9%, and 5% at 0, 24, 48, 72 and 96 h of perfusion culture, respectively. Endothelial cell-dependent vasorelaxation were also slightly decreased over the time. Selected arteries that were undergone balloon injury and perfusion culture for 96 h showed the decrease of their diameter by 6.87 ± 3.12% in response to NE and the increase of their diameter by 5.34 ± 2.56% in response to nitroglycerine. ACh had no effects on vasorelaxation in the balloon-injured vessels. Thus, porcine carotid arteries remained viable and responsible for vasomotor activities after perfusion culture up to 96 h in this study.

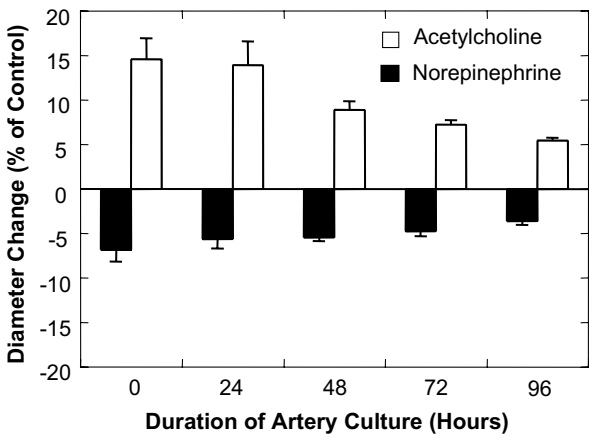

Fig. 2. Contraction and relaxation of normal porcine carotid arteries perfusion-cultured for 0, 24, 48, 72 and 96 h. Contraction was due to vasomotor challenge with 10^{-5} M norepinephrine (NE) and relaxation was due to vasomotor challenge with 10^{-5} M acetylcholine (ACh). $n = 6$. Error bars indicate the standard error for each group of measurements.

SMC proliferation in the time course study

Proliferating cells of the cultured vessels were studied by BrdU incorporation and immunohistochemistry. SMCs in the vessel media were counted in the four layers from the lumen side to the adventitia side. In general, BrdU-positive cells increased as culture time increased, and cell proliferation response occurred in a higher extend in the inner layer of the media near the luminal side than in the middle or the outer layers near the adventitia side. Normal arteries showed 10%, 13%, 18%, and 19% of SMC proliferation in the inner layer of the media after 24, 48, 72, and 96 h culture, respectively (Fig. 3A). These cell proliferation rates were significantly decreased across the medial layer towards the adventitia. In contrast, balloon-injured arteries showed significantly higher magnitudes of SMC proliferation at all time points in four layers of the vessel wall compared with normal arteries (Fig. 3B). These data indicate that balloon injury significantly induce SMC proliferation of porcine carotid arteries in this *in vitro* artery perfusion culture model.

Effects of FGF2 and FGF2-SAP on SMC proliferation

In order to apply the perfusion culture model for testing biological or pharmacologic agents, arteries were cultured with or without FGF2 or FGF2-saporin for 96 h. SMC proliferation showed significant differences among the four groups of arteries after 96 h perfusion culture (Fig. 4A and B). Again, cell proliferation response occurred strongly in the luminal side of vessel wall with the effects tapering toward the adventitia. In the inner layer of the vessel wall near the lumen, SMC proliferation were less than 10% in non-injured vessels, 66% in balloon-injured vessels, 80% in injured vessels with FGF2 treatment, and only 5% in injured vessels with treatment of FGF2-SAP ($p < 0.05$ using AVONA). The middle layer and outer layer near adventitia, SMC proliferation was significant lower than the inner layer in all groups except in FGF2-SAP treated group. Thus, FGF2 significantly increased SMC proliferation while FGF2-SAP significantly decreased SMC proliferation in balloon-injured porcine carotid arteries in the *in vitro* artery perfusion culture model.

Discussion

In the current study, we have designed an *in vitro* artery perfusion culture model to allow more precise control over the chemical and mechanical environments of the tissue than that in animal models. Unlike cell culture models, the vascular tissue culture model retains the *in vitro* cellular phenotypes and matrix architecture as assessed by a physiologic contraction and relaxation assay and histological examination and is thus clinically more relevant than cell culture models. Porcine carotid arteries were able to show contraction and endothelial-dependent relaxation for at least 96 h in culture. Vascular SMC proliferation was detected in response to vascular injury and biological factors such as FGF2 and mitotoxin FGF2-saporin. Thus, this artery perfusion culture model represents an alternative research approach to study the mechanisms of vascular lesion formation.

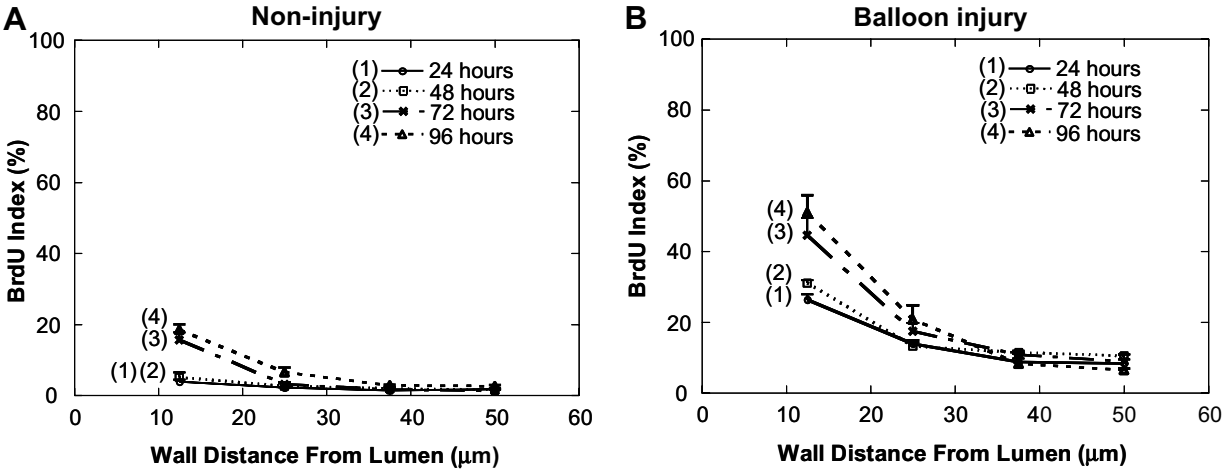

Fig. 3. The time course of SMC proliferation in porcine carotid arteries. After 24, 48, 72 or 96 h of vessel culture, the arteries were fixed and subjected to BrdU immunostaining. SMC proliferation in two groups of perfusion-cultured arteries was quantified by direct cell counting on tissue slides with BrdU immunostaining. The vessel media was divided into four layers from the luminal side to the adventitial side. There was a significant difference in BrdU indices in the luminal layer of vessels between the normal non-injured arteries (A) and balloon angioplasty-injured arteries (B) (ANOVA, $n = 6$, $p < 0.05$).

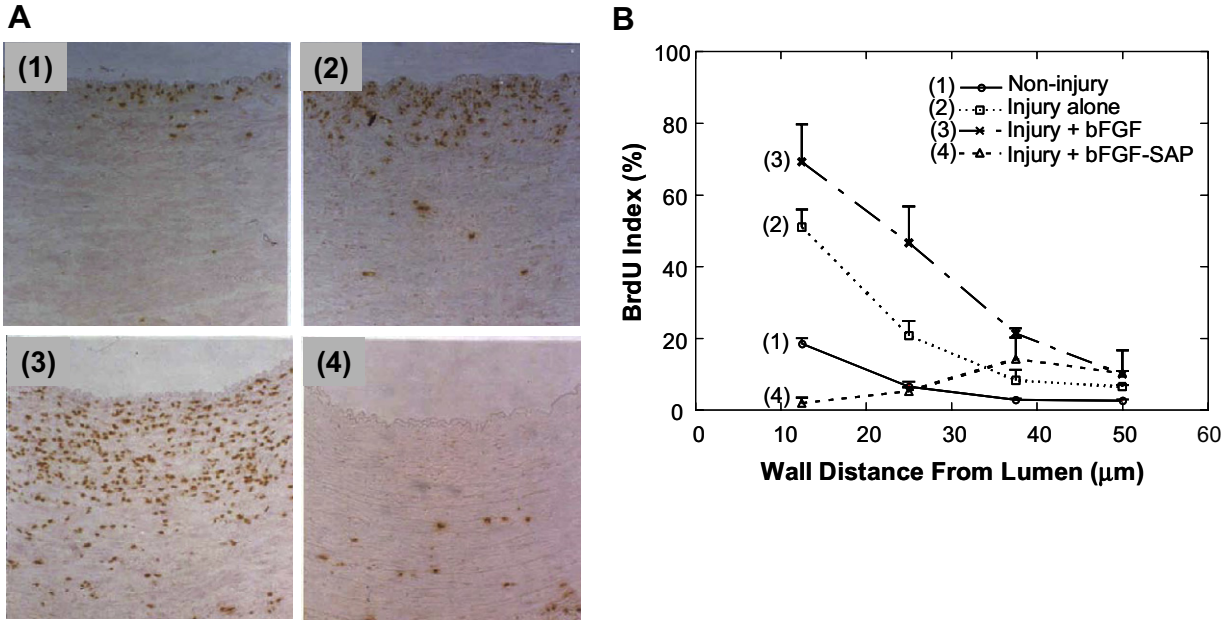

Fig. 4. The effects of EGF2 and FGF2-SAP on SMC proliferation in porcine carotid arteries. Four groups of porcine arteries were perfusion-cultured for 96 h at 100 mm Hg pressure and 100 ml/min flow rate. Cell proliferation was assayed by BrdU incorporation and immunostaining. (A) Representatives of microscopic images of cultured arteries. Dark brown color indicates proliferating cells (BrdU-positive staining). (1) Normal non-injured artery; (2) balloon angioplasty-injured artery; (3) balloon-injured artery plus FGF2; (4) balloon-injured artery plus FGF2-SAP. Original magnification was 100×. (B) SMC proliferation in four groups of perfusion-cultured arteries was quantified by direct cell counting on tissue slides with BrdU immunostaining. $n = 6$.

The system described here offers several advantages over previously published vessel perfusion culture systems. First, unlike the systems reported by Labadie et al. and Golledge et al. [14,15], our system is self-contained and compact and fits easily into a standard-sized CO_2 incubator. Four vessels can be easily accommodated in one incubator, offering the possibility of culturing up to 16 vessels at a time with our set up of four stacked incubators. Second, the volume of circulating medium can be lowered substantially when needed, which reduce the cost when studying hard-to-produce proteins or particles. Also, our system offers the capability to perform real-time noninvasive assessment of both endothelial cell and smooth muscle layers. Some authors must remove the cultured vessels and slice the vessel into rings to perform ring assays of contraction and relaxation [15,16]. An enhancement to the system is the ability to generate physiologic pulsatile flow [12]. This is accomplished with a second pump attached to a computer-designed cam that actuates a 5-ml syringe to create the pulsatile component of the flow. A check-valve is added along with a second compliance chamber distal to the vascular segment. Cams have been designed to simulate femoral, carotid, and coronary artery waveforms precisely. Pressure and flow can be changed independently, allowing single factor studies. Again, this system fits into a standard incubator and the simplicity in this design is an advantage over other published systems [14–16]. Different flow waveforms can be easily designed by changing the cam in the system. This allows investigating changes in vascular biology resulting

from hemodynamics that would be impossible to control and quantify in animal models.

Vasomotor function in response to NE and nitroglycerine were studied in the normal non-injury porcine carotid arteries and balloon-injured arteries. The major purpose of this assay was to demonstrate vessel viability after perfusion culture for 96 h. Both groups of vessels were responsive to NE and nitroglycerine after perfusion culture for 96 h. We did not find a significant difference in vasomotor function in response NE and nitroglycerine between these two groups.

Proliferation of SMCs is a major event in the development of atherosclerosis and complications of procedures used to treat atherosclerotic diseases, including postangioplasty restenosis, vein graft failure, and transplant vascular pathology [1]. The rat study by Dr. Clowes has shown that SMC proliferation reached a maximum at 48 h in the media (46%) and at 96 h in the intima (73%) after carotid arterial injury [17]. Similar SMC proliferation kinetics was observed in our *in vitro* tissue culture system and balloon injury substantially increased SMC proliferation compared with non-injured vessels. Thus, the *in vitro* system represents a valuable approach to study the pathophysiologic changes in the vessel wall.

Previous experimental studies have suggested that FGF2 is an important mediator of the vascular response following arterial injury [5,6]. Injury results in release of FGF2, which then stimulates SMC proliferation [5]. Furthermore, injury also upregulates the expression of the FGF2 receptors on the SMCs, which in turn promotes the FGF2 effect [18,19]. FGF2 binding to its cellular receptor transduces signals to the nucleus to initiate DNA replication and cell division via a mechanism of internalization and nuclear translocation of surface-bound growth factor [20]. Infusion of FGF2 enhances SMC proliferation after vessel injury, whereas neutralizing antibodies to FGF2 inhibit DNA synthesis [5,6,21]. In accordance with animal studies, our results have shown that exogenous FGF2 also significantly induced SMC proliferation in the balloon-injured porcine carotid arteries in the *in vitro* artery perfusion culture model.

SAP is a powerful ribosome-inactivating protein isolated from the seeds of the plant *Saponaria officinalis* [22]. It has been used to make potent and effective immunotoxins and ligand toxins [23,24]. FGF2 and SAP have been conjugated and characterized as a mitotoxin to selectively kill proliferating cells that express the FGF receptors [25]. FGF2-SAP enters target cells via the FGF receptor, inhibits protein synthesis, and elicits cell death [26,27]. Furthermore, our previous work has shown that FGF2-SAP inhibits neointimal proliferation without apparent effect on the uninjured artery in a dog model [7]. This study demonstrated that FGF2-SAP significantly decreased SMC proliferation in balloon-injured porcine carotid arteries in the *in vitro* artery perfusion culture model, which supported the conclusion of animal study and suggested a new strategy for reducing neointimal hyperplasia by the selective killing of proliferating SMCs with a potent chimerical mitotoxin.

In summary, we have designed and implemented a novel perfusion culture system that permits the survival of intact porcine carotid arteries for up to 96 h. The balloon angioplasty injury stimulated SMC proliferation, which was significantly enhanced by FGF2 and inhibited by FGF2-SAP. This study suggests that this novel artery culture system is a useful model to evaluate the differential effects of isolated factors on SMC proliferation. Furthermore, this model has applications in the study of the molecular mechanisms of vessel biology and pathology and also in screening of drugs designed to affect vessel walls.

Acknowledgments

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