Explants of porcine coronary artery in culture: A paradigm for studying the influence of heparin on vascular wall cell proliferation

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Abstract

Explant cultures of porcine coronary artery provided a coculture model, used as a paradigm of arterial wall in contact with vascular prosthesis which allowed the study of spatial and temporal changes in cell phenotype. First cells emerging from the explant had an endothelial phenotype monitored by cytoimmunostaining. Percentages of anti-smooth muscle α -actin labelled cells were assessed at early and late phase by flow cytometric analysis to control the effect of heparin. At $100 \mu\text{g ml}^{-1}$, no effect on α -actin labelled cell growth has been detected. This result contrasted with the inhibition of monolayer cell cultures. At $500 \mu\text{g ml}^{-1}$, the proliferation of smooth muscle cells was reduced. This explant system should be useful for testing drugs susceptible to interfere with restenosis.

Introduction

A major complication of angioplasty to treat diseased small diameter vessels is restenosis. Vessel obstruction is mainly due to the dysfunction of vascular cells. Many reports have examined the interactions between smooth muscle cells (SMC) and endothelial cells (EC) which are fundamental for regulating cell functions (Abramovitch et al., 1998; Malinda et al., 1999). Endothelial cells secrete growth factors acting in agreement with cytokines released by activated blood cells to inhibit or to promote smooth muscle cell growth (Xu et al., 1993). Several cell coculture methods have been used to study these cell–cell interactions (Asakawa et al., 1999; Christ et al., 1992; Fillinger et al., 1993). The cell types have generally been separated in space and/or in time so that soluble mediators can diffuse from cell to cell through an artificial microporous membrane (Fillinger et al., 1993; Kinard et al., 1997) or in the culture medium (Dodge et al., 1993). We have developed a culture method using explants of circular cross-sections of coronary arteries. These explants retain the integrity of the arterial structure and the diversity of their cell phenotypes. This

organotypic culture model, largely developed in our laboratory, differed from cell cocultures (Fillinger et al., 1993; Kinard et al., 1997) in that cells were released from explants as a function of time and their growth was controlled by reciprocal cell interactions. It also differed from the organ culture described by Finking et al. (2000) in that cells grew over substratum and gave rise to cell layers. The *in vitro* contact between arterial wall tissues and culture substratum mimics the *in vivo* junction of host vascular tissues with vascular prostheses.

However difficulties in obtaining fresh biopsies of human coronary arteries led us to look for an animal model which had histological, physiological and pathophysiological properties, similar to those of human patients. The pig cardiovascular system appears the suitable model (Christen et al., 1999; Gross, 1985).

Therapeutic treatments like coronary balloon angioplasty and stenting can severely damage the artery wall, leading to restenosis due to SMC proliferation and to the deposition of extracellular matrix proteins (Zubilewicz et al., 2001). Grafting of active molecules such as heparin, is an alternative to improve the biocompatibility of vascular prostheses (Kang et

al., 1995) or stents (Ahn et al., 1999). The increase of cationic charges on prosthetic surfaces reduced the risk of thrombosis and improved their haemocompatibility (Hardhammer et al., 1996; Seifert et al., 1996). However poor information can be found to explain the effect of heparin on smooth muscle cell proliferation in coculture models. In single cell cultures, heparin inhibited the proliferation of SMC (Fager et al., 1992; Ishihara et al., 2000; Laemmel et al., 1998).

In the present report, we describe a culture system of pig coronary artery which maintains the integrity of vessel wall tissues. We have monitored the outgrowth of cells from sections of pig arteries incubated in growth medium. Their phenotypes were described by cytoimmunostaining for EC and SMC specific markers and the uptake of acetylated-low-density-lipoprotein (diI-Ac-LDL). We then evaluated the percentage of smooth muscle cell phenotype at different periods of the culture, first under control conditions, and then, in the presence of different concentrations of heparin.

Material and methods

Explant culture

Coronary arteries from 1-year old pigs were obtained within 10–15 min of slaughter at the Compiègne abattoir. They were dissected clear of myocardial and adipose tissues and placed for 1–2 h in phosphate-buffered saline (PBS pH 7.4) containing antibiotics and fungicide to prevent contamination. The narrower parts of arteries were then cut into 1 mm thickness rings with Pascheff-Wolff scissors. These explants were everted so that the endoluminal surface faced outward. Ten explants were placed in a 35 mm culture dish containing 0.5 ml culture medium, Iscove modified Dulbecco's medium (GibcoBRL) + 10% fetal calf serum (Eurobio) and antibiotics (penicillin 100 U ml⁻¹, streptomycin 100 µg ml⁻¹, Eurobio). Cultures were incubated at 37 °C in a humidified 5% CO₂ incubator and the medium was changed every 2–3 days.

Cell cultures

EC were removed from the endoluminal surface by collagenase treatment. Coronary arteries were opened along their long axis and 1–2 cm long fragments were placed, luminal surface down, on filter papers impregnated with 0.2% collagenase (Boehringer) in 1 mM

phosphate buffered saline (0.14 M NaCl, 4 mM KCl and 11 mM glucose pH 7.4) for 5 min at 37 °C. The cells were then scraped off, diluted in Iscove modified Dulbecco's culture medium and seeded in culture dishes. The remaining deendothelialized fragments of artery were cut into 1 mm² squares, placed in culture dishes and incubated as above to yield SMC cultures.

Confluent cell monolayers were harvested by a brief incubation with 0.1% trypsin (Sigma-Aldrich Chimie, France) and 0.1% EDTA and cells were resuspended in Iscove modified Dulbecco's medium. Primary cultures of EC and SMC were used to preserve their original phenotype.

The effect of heparin (Sigma-Aldrich Chimie, France) was tested on both artery explants and dissociated cell cultures by adding 100, 200 or 500 µg ml⁻¹ to the culture medium.

Cell counting and viability

Cells were removed from explants with trypsin and counted in a Coulter® counter. Viability was assessed by Trypan blue exclusion.

Acid phosphatase assay

EC and SMC were seeded at 4000 cells per well in 96-well plates and grown for 4–8 days. Acid phosphatase activity was assayed according to Yang (1996). The culture medium was removed, cells were washed with 200 µl PBS and 100 µl 0.1 M sodium acetate (pH 5.0), 0.1% Triton X-100 plus 5 mM p-nitrophenylacetate was added to each well. The plates were incubated for 2 h at 37 °C and the reaction was stopped by adding 10 µl 1 N NaOH. Absorbance at 415 nm was recorded using a microplate reader (Bio-Rad Model 550).

Statistical analysis

Results are expressed as means ± standard deviation. Differences between absorbances within experiments and cell counts were analysed by ANOVA ($n > 30$) and the Student-Newman-Keuls multiple comparison test. The Kruskal-Wallis test was used as a non-parametric test ($n < 30$) followed by a Mann-Whitney test. Results were considered significant at $p < 0.05$.

Cell phenotypic characterization

Cell cultures were fixed in absolute methanol at –20 °C and incubated for 30 min with one of the primary antibodies: (i) monoclonal mouse anti-smooth

Figure 1. Phase contrast micrographs of explants of pig coronary artery: endothelial cells with their apposed organization (arrow) in 4-day-old culture (a) $\times 200$; smooth muscle cells in 7-day-old culture (b) $\times 100$; multilayer cells in 14-day-old culture (c) $\times 100$; hills (arrow) and valleys (arrowhead) pattern in 14-day-old culture (d) $\times 100$. Explants show as a black spot.

muscle (SM) specific α -actin (Sigma) diluted 1:50, (ii) polyclonal rabbit anti-porcine angiotensin converting enzyme (ACE) (gift from Dr. Baudin, Paris) diluted 1:100, (iii) polyclonal rabbit anti-human von Willebrand factor (vWF) (Sigma) diluted 1:50, and then for 30 min with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse immunoglobulin antibody (Sigma) diluted 1:100, or with FITC or rhodamine conjugated goat anti-rabbit immunoglobulin antibody (Sigma) diluted 1:100. They were washed with PBS plus 1% BSA between each immunoreaction. In each case, negative controls were incubated with nonimmune serum instead of the primary antibody.

EC were identified by the selective uptake of diI-Ac-LDL. Cells were incubated with $5 \mu\text{g ml}^{-1}$ acetylated lipoprotein labelled with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-

indocarbocyanine perchlorate (DiI-Ac-LDL) (Bio-medical Technologies, Inc., Stoughton MA), at 37°C for 30 min (Voyta et al., 1984). The samples were examined with an Olympus microscope equipped with epifluorescence illumination.

Fluorescence-activated cell sorting (FACS)

Cells from the explant cultures were dissociated with trypsin-EDTA fixed in cold absolute methanol and immunolabelled with a primary antibody and a fluorescent secondary antibody as above. SMC α -actin immunofluorescence was analysed using a Fluorescence-Activated Cell Sorter (Ortho-Diagnostic Systems Inc.) equipped with a 488 nm (500 mW) line from an argon laser. Histograms of 256-channel resolution were collected for 14 000 cells for each sample.

Figure 2. Immunofluorescent labelling of cells and DiI-Ac-LDL uptake by cells growing out from 5-day-old coronary artery explants: fluorescent anti-vWF antibody labelling (a) $\times 1000$; fluorescent anti-ACE labelling (b) $\times 1000$; fluorescent anti-smooth muscle α -actin antibody labelling (c) $\times 1000$; rhodamine excitation fluorescence of DiI-Ac-LDL exposed cells (d) $\times 400$.

Figure 3. Immunofluorescent labelling of cells and DiI-Ac-LDL uptake by cells growing out from 10-day-old coronary artery explants: rhodamine excitation fluorescence of DiI-Ac-LDL exposed cells near the explant (a) $\times 400$; fluorescent anti-smooth muscle α -actin antibody labelling at the periphery of cell layer (b) $\times 1000$; fluorescent anti-vWF antibody labelling (c) $\times 1000$; fluorescent anti-ACE labelling (d) $\times 1000$; rhodamine excitation fluorescence of DiI-Ac-LDL exposed cells (e) $\times 1000$.

Scanning electron microscopy

Cell layers were fixed in 3% glutaraldehyde in Rembaum buffer (pH 7.4), dehydrated in graded alcohol series, critical-point dried from CO₂ (Polaron Instrument Inc.), sputter-coated with gold (Polaron Instrument Inc.) and examined in a Jeol (model JSM 840) scanning electron microscope.

Results

Cell phenotype

Apposed hexagonal cells grew out from the coronary artery explants incubated 4–6 day (Figure 1a). At this time of explant culture, immunofluorescence studies showed numerous bright fluorescent dots in the cytoplasm of the cells labelled with an anti-vWF antibody or anti-ACE antibody (Figures 2a, b). The labelled cells were in clusters or dispersed. Anti-SM α -actin antibody revealed no actin bundles (Figure 2c). Most of the cells released from the explants incorporated DiI-Ac-LDL into their cytoplasm and contained bright red fluorescent spots (Figure 2d). These three markers indicated the endothelial phenotype of all the newly grown cells.

Incubation for longer than 7–10 days caused changes in the anti-vWF antibody and DiI-Ac-LDL labelling patterns. EC in close contact with the explants were specially labelled but there were a few fluorescent single cells scattered over the cell layer (Figure 3a). Anti-SM α -actin antibody labelled most of the cells. Some contained numerous parallel bundles of actin while others were uniformly faintly labelled (Figure 3b). Cells incubated for 14 days were labelled by anti-SM α -actin, but they neither retained the anti-vWF antibody (Figure 3c) and the anti-ACE antibody (Figure 3d), nor incorporated DiI-Ac-LDL (Figure 3e). The microscopic observations showed a culture of elongated cells which became multilayered at 14 days (Figures 1b, c) with a hill and valleys pattern typical of SMC (Figure 1d).

Effect of heparin on single cell growth

Preliminary experiments were done to check the effect of heparin on the growth of SMC monolayers for 4–8 days. Cell growth was assessed by measuring acid phosphatase activity. Heparin inhibited the proliferation of SMC ($p < 0.001$) on days 4 and 7

(Figure 4). The acid phosphatase activity was reduced by $48.6 \pm 7.6\%$ on day 4 and $42.5 \pm 4.1\%$ on day 7 in cultures grown in the presence of heparin ($100 \mu\text{g ml}^{-1}$) (Figure 4). Statistical analysis showed a dose-dependent effect of heparin ($p < 0.01$).

Effect of heparin on explant cultures

The percentages of α -actin labelled SMC in the heterogeneous cell population of explants were determined by FACS at early (7 days) and late (14 days) stages of culture. The diagrams of fluorescent cells (Figure 5) showed that early emerging cells gathered in a weakly fluorescent cell peak (zone 1) which corresponded to $71.8 \pm 11.9\%$ (mean of 4 experiments) of the total number of the fluorescent cells (Figure 5a). Control cell labelling showed that this weak fluorescent peak contained non-specifically α -actin labelled cells and/or cells labelled by the secondary antibody in the absence of primary serum (Figure 5d). The zone 2 interval was confirmed by immunolabelling a SMC culture (Figure 5h). There were more α -actin labelled cells in zone 2 on day 14 (Figure 5e) which accounted for $53.2 \pm 10.2\%$ (mean of 7 experiments) of the total number of cells. The fluorescent patterns on days 7 and 14 were not substantially modified by heparin. The α -actin labelled cells accumulated in zone 2 of the diagram on day 7, accounted for $27.9 \pm 16.1\%$ of the cells grown in medium containing $100 \mu\text{g ml}^{-1}$ heparin and for $24.8 \pm 18.6\%$ of the cells grown in medium containing $500 \mu\text{g ml}^{-1}$ heparin (Figures 5b, c). These percentages increased to 60.8 ± 8.6 ($100 \mu\text{g ml}^{-1}$) and 63.7 ± 6.7 ($500 \mu\text{g ml}^{-1}$) by day 14 (Figures 5f, g).

We further quantified the effect of heparin on vascular cell growth by counting in a Coulter[®] counter, the number of cells released from 6 culture dishes each seeded with 10 explants. The cultures grown in medium containing 100 or $500 \mu\text{g ml}^{-1}$ heparin. The number of α -actin labelled SMC per explant was calculated by multiplying the mean number of cells released by explant by the percentage of α -actin labelled cells determined by FACS (Table 1). On day 7, neither the increase in cells released from explants grown with $100 \mu\text{g ml}^{-1}$ heparin, nor the decrease at $500 \mu\text{g ml}^{-1}$ were statistically significant. The average number of cells per explant was significantly reduced by both heparin concentrations on day 14 ($p < 0.01$) in a dose-dependent manner ($p < 0.05$). On day 7, counting of α -actin labelled cells showed that $100 \mu\text{g ml}^{-1}$ heparin has no effect on the cell number but $500 \mu\text{g ml}^{-1}$ heparin significantly reduced their proliferation

Table 1. Cell number per explant

		Heparin concentration		
		0	100 $\mu\text{g ml}^{-1}$	500 $\mu\text{g ml}^{-1}$
7 Days	Total cells	6154 $\pm$ 930	7209 $\pm$ 835	4822 $\pm$ 811
	α -actin +	997 $\pm$ 150	952 $\pm$ 110	559 $\pm$ 94 ^{a, c}
14 Days	Total cells	18198 $\pm$ 1185	14500 $\pm$ 870 ^a	10072 $\pm$ 1379 ^{a, b}
	α -actin +	7534 $\pm$ 490	7105 $\pm$ 426	5519 $\pm$ 755 ^{a, b}

Dose-effect of heparin on the number of α -actin labelled and α -actin unlabelled cells in explant cultures grown for 7 and 14 days. Cells released from the explants were harvested, counted and the percentages of α -actin labelled cells were assessed by FACS analysis. Absolute numbers of α -actin labelled and unlabelled cells per explant were calculated. Values are means $\pm$ SD of 6 determinations of one representative experiment.

^a $p < 0.01$ compared to control culture.

^b $p < 0.05$ compared to heparin 100 $\mu\text{g ml}^{-1}$.

^c $p < 0.01$ compared to heparin 100 $\mu\text{g ml}^{-1}$.

Figure 4. Dose response effect of heparin on the proliferation of single smooth muscle cell cultures. Cells were harvested on days 4 and 7 and acid phosphatase activity assayed. Absorption at 415 nm is plotted against heparin concentration. Values are means $\pm$ SD ($n = 6$). *** $p < 0.001$ compared to control culture. ## $p < 0.001$ between two heparin concentrations on day 4. ff $p < 0.01$ between two heparin concentrations on day 7.

($p < 0.01$). On day 14, significant inhibition of SMC growth required 500 $\mu\text{g ml}^{-1}$ heparin.

The percentages of viable cells in control cultures, grown for 7 and 14 days, and in the cultures supplemented with heparin, were higher than 96%. Only 500 $\mu\text{g ml}^{-1}$ heparin slightly reduced the cell viability to 93%.

Effect of heparin on cell α -actin fluorescence

The FACS patterns (Figure 5) showed the intensity of α -actin isoform fluorescence in labelled SMC (zone 2) (Table 2). The mean intensity was about 36.6 (arbitrary logarithmic unit), regardless of the heparin concentration. Prolonged incubation increased the α -actin fluorescence to 53.7 $\pm$ 9.6 in control cultures. The addition of heparin significantly strengthened the

Figure 5. FACS of arterial explant cultures grown for 7 days (a, b, c) and 14 days (e, f, g) without heparin (a, e) in medium containing $100 \mu\text{g ml}^{-1}$ (b, f) and $500 \mu\text{g ml}^{-1}$ (c, g) heparin. Cells from explants were harvested and immunolabelled with a monoclonal anti-smooth muscle α -actin antibody (primary antibody) and FITC-anti mouse IgG antibody (secondary antibody). Fluorescent cells were analyzed by cytofluorometry. The ordinate gives the number of cells per channel and the abscissa gives the relative fluorescence intensity (logarithmic scale). Zone 1 was determined by labelling explant culture cells by secondary antibody alone (d). Positive control was established by immunolabelling coronary SMC culture (P_3) (h).

Table 2. Mean fluorescent intensity of α -smooth muscle actin labelled cells in coronary explant cultures

Heparin concentration $\mu\text{g ml}^{-1}$	7-Day incubation (n = 4)	14-Day incubation (n = 7)
0	36.7 $\pm$ 16.3	53.7 $\pm$ 9.6
100	36.6 $\pm$ 17.7	78.2 $\pm$ 14.3 ^a
500	35.1 $\pm$ 17.2	103.6 $\pm$ 8.4 ^{a, b}

Effect of heparin on fluorescent intensity of α -actin in labelled SMC. Cells released from explant cultures performed in the presence of various concentrations of heparin were harvested at 7 or 14 days, immunolabelled with a monoclonal anti-smooth muscle α -actin antibody (primary antibody) and FITC-anti mouse IgG antibody (secondary antibody). Fluorescent cells were analyzed by cytofluorometry. The relative fluorescence intensity (logarithmic scale) was reported. Values are means $\pm$ SD of 4, 7 determinations.

^a $p < 0.05$ compared to control culture at day 14.

^b $p < 0.05$ compared to heparin 100 $\mu\text{g ml}^{-1}$ at day 14.

effect of time and the increase in fluorescence was proportional to the heparin concentration.

Discussion

The explant culture method used is well suited to analysis of the early stages of *in vitro* coronary artery cultures. Explants of chick embryo vessels generate a heterogeneous cell population (Dufresne et al., 1994), whose phenotypes change in a characteristic spatiotemporal manner. Our phenotypic analyses of cultures of human, canine, bovine and porcine artery explants (data not shown), all showed that the first cells to arise from vessel explants have the endothelial phenotype, vWF, ACE and the ability to incorporate DiI-Ac-LDL. The EC change shape from polygonal to elongated, 24–48 h following cell emergence, whereas SMC start to migrate and/or proliferate. A few EC subsequently round up and become detached from the cell layer. EC are similarly lost from pig in organ culture (Koo et al., 1992). This may be due to injury of EC and/or to the presence of dysfunctional cells. Cells grew out of our explants and the decrease in EC proliferation occurred at the same time as the increase in SMC growth. One hypothesis is that newly produced SMC might release cytokines to inhibit EC growth as in bovine coculture models (Saunders et al., 1992). By contrast, EC activated by injury might promote

SMC growth. Deendothelialized arterial explants were cultured to yield SMC, providing further evidence for interactions between both phenotypes. There was a delay of about 1–2 days before the outgrowth of SMC, compared with cultures of intact explants. Thus, explants of coronary artery rings in which structure of vessel wall is preserved, allow cells to proliferate and migrate over substratum, involving complex interactions of activated vascular cells. Prolonged incubation (14–15 days) gave rise to multilayered outgrowth of cells containing SM α -actin and cells which did not. Severe injury of pig coronary arteries result in the translocation of adventitial fibroblasts to the neointima (Shi et al., 1996). Since our culture technique preserves the adventitia, it would allow fibroblasts to migrate and proliferate.

Heparin is widely experimented for treating restenosis in porcine models (Ahn et al., 1999; Goodwin et al., 2000) because of the inhibiting effect on the proliferation of SMC. The inhibition of primary cultures of porcine SMC proliferation agreed with previous findings for rat and human SMC cultures (Bono et al., 1997). The percentage of inhibition of SMC growth by 100 $\mu\text{g ml}^{-1}$ heparin is similar to that found by Laemmel (1998) for bovine SMC.

Heparin had a more complex effect on explants. Flow cytofluorometry was used to quantify its effect on the dynamics of α -actin labelled cells. The growth of these cells was not affected by 100 $\mu\text{g ml}^{-1}$ heparin for up to 14 days in contrast to the cell monolayer culture conditions. The resistance of SMC to heparin may be due to the release of antagonists of heparin by explants or to heparin-resistant SMC (Delmolino et al., 1997).

The FACS patterns showed that the intensity of α -actin fluorescence of SMC increased with time in culture and was enhanced by heparin after 14 day incubation. Others studies have correlated the increase in α -actin production with inhibition of SMC proliferation (Fager et al., 1992). During the early growth phase of explant culture, the fluorescent intensity of α -actin remained constant even in the presence of 500 $\mu\text{g ml}^{-1}$ heparin which reduced cell proliferation. Prolonged incubation of the culture allowed increase of α -actin, the synthesis of which was enhanced by both concentrations of heparin, higher effect produced by higher concentration. Therefore we conclude that heparin activated the expression of SMC α -actin which accumulated during the late growth phase in stress fibers, whereas the inhibitory effect on SMC proliferation was effective at high concentration of heparin,

regardless of the incubation time.

In summary, coronary artery explant culture appears to be a useful system for following the behavior of SMC in coculture condition. It was also suitable for monitoring changes induced by pharmacological agents. To date, a range of low molecular weight heparins, which are distinct non interchangeable drugs and endowed with more sustained anticoagulant properties than unfractionated heparin, is available (Gulba, 1999; Shulman, 2000). However, clinical tests carried out on different animals and on different implantation sites provided opposite results on restenosis (Oberhoff et al., 1998; Baumbach et al., 2000). Preliminary experimental tests as herein described, could orient the choice of drugs or combined molecules in further clinical trials.

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