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Morphological heterogeneity with normal expression but altered function of G proteins in porcine cultured regenerated coronary endothelial cells

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1 Experiments were designed to investigate whether the pertussis toxin-dependent endothelial dysfunction following balloon injury is due to a reduced expression or an insufficient function of G-proteins.

2 Endothelium-dependent responses of porcine coronary arteries were examined *in vitro* by use of conventional organ chambers. Morphological analysis was performed by isolating and culturing the endothelial cells from these arteries. The expression of Gi-proteins in regenerated endothelial cells was measured by Western blots and immunolabelling. The function of G-proteins was assessed by measuring the GTPase activity of cultured endothelial cells.

3 Eight days following denudation, endothelial regrowth was confirmed by histological examination and by demonstrating the presence of endothelium-dependent relaxations to bradykinin and 5-hydroxytryptamine (5-HT). In primary culture, the regenerated endothelial cells displayed a 'cobblestone' pattern as seen with native endothelial cells.

4 Twenty eight days after denudation, the endothelium-dependent relaxations induced by 5-HT were impaired, but those to bradykinin were maintained. However, the latter were reduced when endothelium-dependent hyperpolarization was prevented.

5 Twenty eight days after denudation, multinucleated giant cells were present in the regenerated but not in the native cultured endothelial cell populations. These regenerated endothelial cells incorporated less tritiated thymidine than native endothelial cells.

6 The intensities of the bands on the immunoblot of the regenerated endothelial cells, when several antibodies against Giα1/α2/α3 were used, were the same as those obtained in native endothelial cells. The immunolabelling with the same antibodies was similar between the giant cells and the regenerated endothelial cells of normal size. The hydrolysis of GTP was lower in regenerated than in native endothelial cell membranes.

7 In conclusion, endothelium-dependent relaxations mediated by Gi-proteins are impaired in balloon denuded coronary arteries. This dysfunction following regeneration cannot be explained by a reduced expression of Gi proteins but rather reflects an abnormal function of the G-proteins in the regenerated endothelium.

Keywords: Regenerated endothelium; endothelial dysfunction; Gi-protein; pertussis toxin; bradykinin; NaF; 5-hydroxytryptamine

Introduction

In the pig, angioplasty induces endothelial denudation of the blood vessel wall and leads to proliferation of the underlying smooth muscle cells (Steele *et al*., 1985; Schwartz *et al*., 1990). The ability to repopulate the area denuded endothelium (endothelial regeneration) permits restoration of the role of the endothelial cells to modulate the contractile responsiveness of the underlying vascular smooth muscle cells and inhibit their proliferation (Haudenschild & Schwartz, 1979; Furchgott, 1983). Scanning electron microscopy suggests that in the pig, regenerated endothelial cells *in situ* are morphologically different from native cells as they are more numerous, elongated and irregularly oriented (Shimokawa *et al*., 1987; Niimi *et al*., 1994; Azuma *et al*., 1995). Following balloon injury of porcine coronary arteries the regenerated endothelial cells selectively lose their ability to release endothelium-derived-relaxing factor (EDRF) (Shimokawa *et al*., 1987) in that endothelium-dependent relaxations evoked by 5-hydroxytryptamine (5-HT) or α_2-adrenoceptor agonists are impaired, whereas the response to adenosine diphosphate (ADP) or bradykinin is maintained (Shimokawa *et al*., 1989). The common feature between 5-HT and α_2-adrenoceptor agonists is that they act on receptors coupled to pertussis-toxin sensitive G proteins (Dolphin, 1987; Flavahan *et al*., 1989; Shimokawa *et al*., 1991). Arteries previously submitted to balloon denudation exhibit reduced relaxations to sodium fluoride (NaF), a direct activator of G-proteins (Shibano & Vanhoutte, 1994). Furthermore, pertussis toxin caused an ADP-ribosylation of only certain types of G-protein such as Gi or Go. Gi-protein has been detected in endothelial cells (Flavahan & Vanhoutte, 1990; Liao & Homcy, 1992; Shibano *et al*., 1992; 1994). The other pertussis-toxin sensitive endothelium-dependent relaxations are also impaired in the regenerated endothelium compared to the native endothelium (Shimokawa *et al*., 1989). Taken in conjunction, these observations suggest that the selective endothelial dysfunction of the regenerated endothelium may, in part, be due to abnormalities at the level of the Gi-protein.

The present study was designed to compare the morphology and Gi-protein function in native and regenerated endothelial cells of the porcine coronary arteries. Specifically, the time-course of the endothelial regeneration and the function of the regenerated endothelium were studied together with the mor-

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phology, and the expression and function of Gi-protein in regenerated endothelial cells in primary culture. DNA synthesis and GTPase activity of the G-proteins of regenerated endothelial cells from coronary arteries were determined twenty eight days following balloon denudation. The data demonstrate the presence of a morphological heterogeneity in cultured regenerated endothelial cells, as well as an abnormal function rather than an abnormal expression of Gi-protein.

Methods

Coronary endothelial denudation

The experiments were performed on Large-White pigs of either sex weighing 18 to 25 kg and were carried out in accordance with the guidelines of the French Ministry of Agriculture for the use and care of animals. The animals were anaesthetized with an intramuscular anaesthetic mixture injection of tiletamine and zolazepan (15 mg kg^{-1}) and maintained with infusion of sodium thiopentone (8 mg kg^{-1} h^{-1}). They were intubated and ventilated with a respirator (Mark 8; Bird Co. Palm Springs, CA, U.S.A.). Under fluoroscopy, a guide catheter (model AR1, 7F, Baxter, Maurepas, France) was introduced via the femoral artery into the left coronary ostium. Heparin (10 μg kg^{-1}) and bretylium tosylate (7 mg kg^{-1}) were administered intravenously to prevent extensive thrombus formation and cardiac arrhythmias, respectively. Then a balloon dilatation catheter (model 72-QK, Baxter France; 3 or 3.5 mm according to the size of the coronary artery) was introduced into the left anterior descending (LAD) coronary artery through the guide catheter. The LAD coronary artery was denuded by inflating the balloon catheter three times for 30 s. Terramycine LA (20 mg kg^{-1}) was given intramuscularly as a prophylactic antibiotic. The animals were under observation until they recovered from the anaesthetic. They were then housed in individual cages.

Organ chamber experiments

The animals were sedated with an intramuscular injection of tiletamine and zolazepam. Evan's Blue dye of 0.5% was injected intravenously 30 min before the animals were killed, 1, 8 or 28 days following balloon denudation, to check the presence of endothelium. After heart explantation, the left anterior descending (LAD) and the left circumflex (LCX) coronary arteries were excised and immersed in cold physiological salt solution of the following composition (mM): $CaCl_2$ 2.5, EDTA 0.016, NaCl 118, $NaHCO_3$ 24.8, KH_2PO_4 1.18, KCl 4.7, $MgSO_4$ 1.2 and glucose 11, pH = 7.4 (control solution). The rings were dissected free of loose connective tissue and suspended in organ chambers filled with 20 ml of control solution (37°C), aerated with 95% O_2-5% CO_2. The preparations were connected to a strain gauge to record isometric force. The rings were stretched progressively until the contractile response evoked by 20 mM KCl was maximal. To study relaxation, the rings first were contracted with prostaglandin $F_{2\alpha}$ or KCl (to produce 30–70% of the maximal contraction induced by 60 mM KCl) in the presence of propranolol (10^{-7} M; to prevent β-adrenoceptor activation). Concentration-response curves for 5-HT, bradykinin or sodium fluoride were obtained in a cumulative fashion in the presence of indomethacin (10^{-5} M; to inhibit cyclo-oxygenase), for 5-HT in the presence of ketanserin (10^{-6} M; to prevent the activation of 5-HT_2 receptors) and for NaF in the presence of $AlCl_3$ (10^{-5} M; to allow the formation of a fluoroaminate complex, AlF_4^-). In some experiments, rings were incubated with pertussis toxin (300 ng ml^{-1}) one hour before exposure to prostaglandin $F_{2\alpha}$.

Morphometric study

Rings used in the organ chamber experiments were fixed under tension in 10% formaldehyde in phosphate buffer. They were processed by means of a standard paraffin histological technique. Different nonserial cross sections (5 μm length, 200 μm apart) were prepared from the paraffin blocks and stained with haematoxylin-eosin-safran for light microscopy analysis. The light microscopical pictures were analysed by use of a computerized image-analysis system with 256 levels of gray and a 512×512 pixel grid (Histo Software, Biocom, Les Ulis, France). This system allows an overall analysis of the vascular cross sections. The internal elastic lamina was used as the border to distinguish the intima from the media. For each ring of coronary arteries, intimal, medial and lumen cross-sectional areas were averaged from the analysis of 2 cross sections. Four to six rings were analysed per type of coronary artery for each pig ($n = 3$). The index of intimal thickening was defined as the ratio of the intimal to medial cross-sectional area. The endo-

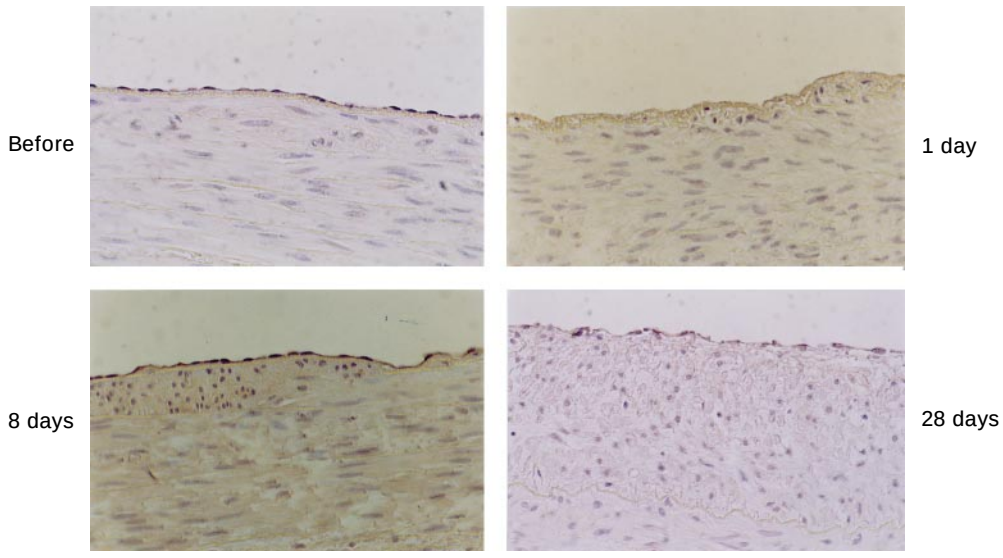

Figure 1 Light micrograph of an immersion fixed cross section of a porcine left descending coronary artery before, 1 day, 8 days and 28 days following endothelial balloon denudation. The preparation was fixed after use in an organ chamber study. The endothelial cells layer was stained with rabbit anti-human von Willebrand factor (1/200), then biotinylated anti-rabbit IgG was added (1/300). The immunostaining was revealed by DAB chromogen system (1/400). The visualization was aided by counter staining with haematoxylin.

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Primary cell culture

Porcine coronary endothelial cells were harvested by gently scraping the intima of the arteries with a rubber policeman, and placed in a culture medium containing Eagle's minimal essential medium (MEM) supplemented with 5 mM L-glutamine, 10% foetal calf serum (FCS) and penicillin-streptomycin (100 u ml^{-1}). Heparin (0.5 mg ml^{-1}) was added to the medium in order to inhibit the proliferation of smooth muscle cells. The primary culture (passage zero) of endothelial cells were characterized by optical microscopy, stained with haemacolour reagents (Keisari, 1992) and immunofluorescence staining with anti-Von Willebrand factor (1 : 200) and anti-α smooth muscle actin (1 : 200) antibodies. The vascular smooth muscle cells (VSMC) were prepared from explants of medial layer placed into collagen precoated Petri dishes, the VSMC migrate and proliferate after one or two weeks in culture, they were stained in passage 1. At confluence, the endothelial cells were washed with Earle's balanced salt solution (EBSS) and collected by scraping with a rubber policeman in a buffer containing 25 mM Tris base, 5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), and a mixture of protease inhibitors, leupeptine (10 μg ml^{-1}), phenylmethylsulphonyl fluoride (0.2 mM), pH = 7.4 at 4°C. For cell membrane preparation, the cells were centrifuged (120 *g* for 10 min) and the pellet was resuspended in the same buffer. The suspension was sonicated (20 strokes) on ice and centrifuged again (120 *g* for 10 min). The supernatant was then centrifuged at 100 000 *g* (30 min). The pellet was resuspended and the protein content was measured with bovine serum albumin as standard (Bradford, 1976).

DNA synthesis

Cells from control and previously (28 days) denuded coronary arteries in passage zero (P0) were subcultured into 96 well plates. Cells were kept quiescent for 48 h in culture medium containing only 0.5% FCS. Then, the cells were placed in culture medium containing either FCS 0.5% or 10% for one day. The incorporation of tritiated thymidine was measured by the addition of 1 μCi of thymidine per well for 4 h in the incubator (5% CO_2-95% O_2, 37°C). The reaction was stopped by removing the medium and the addition of NaOH 0.1 N. The cells were collected on filter paper, after incubation with EDTA-trypsin, by an automated cell harvester (Skatron, Dolasletta, Norway). Radioactivity was measured by liquid scintillation spectroscopy (Beckman, Gagny, France). In parallel, after treatment with trypsin the cell number was determined from 10 wells of cell culture by use of a Coulter counter. DNA synthesis was expressed in d.p.m. per 1000 cells and represented the difference between the d.p.m. obtained in culture medium with 10% versus 0.5% FCS. The results are expressed as the means ± s.e.mean for six wells per type of cell from four pigs.

Immunoblots

Membranes of native or regenerated endothelial cells in primary culture (passage P0) were subjected to a 10% polyacrylamide gel (30 μg protein/lane) and the proteins were transferred by electrophoresis onto nitrocellulose membranes (Towbin *et al*., 1979). After transfer, the nitrocellulose membranes were blocked for non specific sites with 5% bovine serum albumin (BSA) in Tris-buffered saline and incubated overnight at room temperature with the following dilution of Gi antisera: anti-Giα3, sc 262 (1 : 500); anti-Giα1, sc 391 (1 : 500); anti-α family, sc 386 (1 : 200) and anti-Giα1 and α2, AS7 (1 : 200). The blots were washed and treated with biotinylated anti-rabbit IgG as the second antibody. The immu-

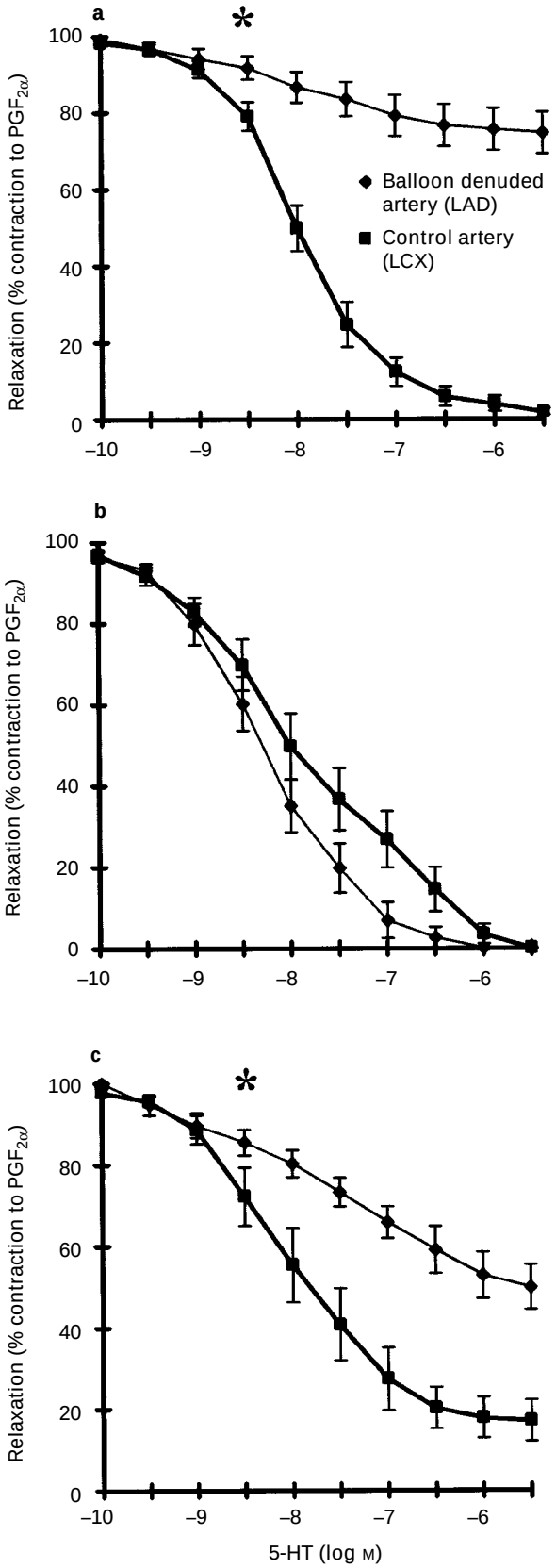

Figure 2 Cumulative concentration-response curves to 5-HT in rings (contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) from the left anterior descending (LAD) coronary arteries or the left circumflex artery (LCX) (a) 1 day, (b) 8 days or (c) 28 days following balloon injury. The relaxations are expressed as percentage of maximal contraction induced by prostaglandin $F_{2\alpha}$. Results are presented as means ($n = 3$); vertical lines show s.e.mean. The asterisks indicate statistically significant differences ($P < 0.05$) between the two types of preparations from the indicated concentration.

noreactive proteins were detected with streptavidin and biotinylated alkaline phosphatase followed by substrate.

Acetylated low density lipoprotein uptake

Acetylated low density lipoproteins (LDL), labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate were used. Cells grown on 96 well plates were incubated in culture medium with Dil-acetylated LDL 1 μg ml^{-1} per well for 4 h in an incubator (5% CO_2-95% O_2, 37°C). Then, they were washed twice with PBS and fixed with paraformaldehyde 2%.

Indirect immunofluorescence

Cells grown on 96 well plates, were washed with a phosphate buffer (PBS). They were then fixed and permeabilized with ethanol at −20°C for 1 min. The cells were incubated for 20 min with PBS containing 3% BSA. All subsequent incubations with antibodies and washes were performed in PBS containing 1% BSA. The anti-peptide antisera sc386 (1 : 50 dilution), sc391 (1 : 30 dilution), sc262 (1 : 50 dilution) and AS7 (1 : 30 dilution), and the antisera anti-von Willebrand factor (1 : 200) and anti-smooth muscle α-actin (1 : 200) were added subsequently to the wells and incubated for one hour at room temperature. The adsorption of the antisera with the antigen (peptide) was performed by incubating the antisera with an excess of peptide (10 μg ml^{-1} overnight. The primary antibodies were visualized with a goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (1 : 300 dilution) or a goat anti-mouse IgG conjugated to FITC (1 : 200). The wells were then rinsed, exposed to Citifluor and the fluorescence was visualized by fluorescence microscopy (wavelength; excitation: 485 nm and emission: 530 nm).

GTPase activity

Cell membranes from control and previously (28 days) denuded coronary arteries were obtained as described above. The assay was conducted at 25°C for 15 min in a reaction mixture (0.15 ml final volume) containing: 0.4 μM [γ-^{32}P]-GTP (30 Ci mmol^{-1}), 1 mM creatine phosphate, 50 u ml^{-1} phosphocreatine kinase, 0.25 mM ATP, 1 mM dithiothreitol, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM EGTA in 10 mM Tris buffer, pH = 7.4. Guanosine 5′-triphosphate (GTP) hydrolysis was initiated by adding the membranes to the reaction mixture. The assay was terminated with 15 μl of $HClO_4$ (11.6 M) at 4°C. Then, 35 μl of K_3PO_4 were added. The mixture was centrifuged at 1000 *g* for 10 min. The supernatant was analysed by high-performance liquid chromatography (h.p.l.c.) (Bernocchi *et al.*, 1994) to determine the percentage of [γ-^{32}P]-GTP hydrolysed. High affinity GTPase was calculated as the difference between total and non specific hydrolysis (measured in the presence of 1 mM unlabelled GTP). The results are expressed as means ± s.e.mean of triplicate measurements (n = 6).

Table 1 Effects of endothelium removal in left anterior descending coronary arteries on intimal and medial areas

	8 days		*28 days*	
	LAD	*LCX*	*LAD*	*LCX*
Neointima area (μm^2)	54 ± 14	23 ± 5	368 ± 96	67 ± 34
Media area (μm^2)	1084 ± 73	917 ± 54	1294 ± 55	1075 ± 226
Lumen area (μm^2)	6143 ± 526	6373 ± 486	5677 ± 681	6504 ± 816
I/M (%)	5 ± 1	3 ± 1	28 ± 6*	7 ± 3

Data are expressed as means ± s.e.mean (n = 3). LAD = left anterior descending coronary artery; LCX = left circumflex coronary artery, I/M = neointima: media ratio. The asterisk indicates a statistically significant difference (P < 0.05) between LAD and LCX.

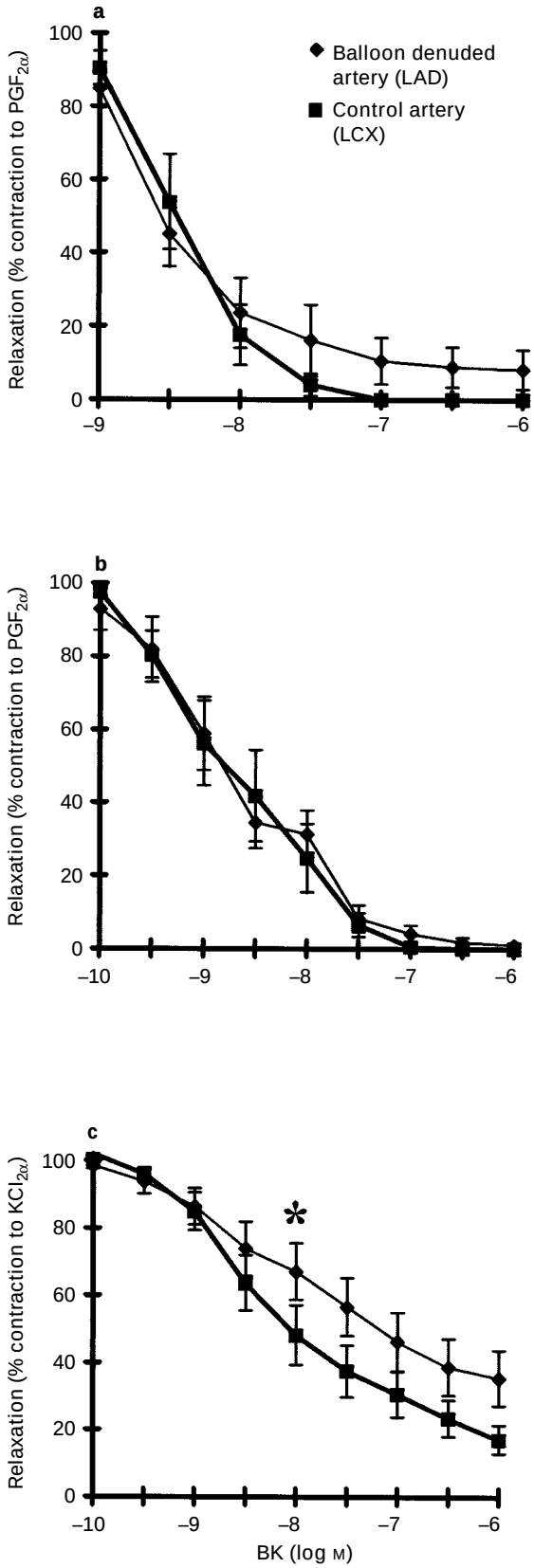

Figure 3 Cumulative concentration-response curves to bradykinin (BK) in rings (contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) from LAD and LCX coronary arteries (a) 8 days or (b) 28 days following balloon injury. (c) Cumulative concentration-response curves to bradykinin (BK) on rings (contracted with KCl 20 mM) from the LAD coronary artery or the circumflex artery, 28 days following balloon injury. The relaxations are expressed as percentage of maximal contraction induced by prostaglandin $F_{2\alpha}$ or KCl. Results are presented as means (n = 3); vertical lines show s.e.mean. The asterisks indicate statistically significant differences (P < 0.05) between the two types of preparations from the indicated concentration.

Drugs and reagents

Anti-von Willebrand factor, anti-smooth muscle α-actin antisera, anti-rabbit or anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated, bradykinin, citifluor, indomethacin, ketanserin, prostaglandin $F_{2\alpha}$, dithiothreitol, phosphocreatine, creatine phosphate, ATP, 5-HT and sodium fluoride were purchased from Sigma Chemical CO (Saint Quentin Falavier, France). Anti-rabbit IgG, biotinylated antibody (from goat) and streptavidin-horseradish peroxidase conjugate were obtained from Amersham. DAB chromogen system (3,3-diaminobenzidine tetrahydrochloride) was purchased from Immunotech (Marseilles, France). Minimal essential medium (MEM), penicillin-streptomycin, glutamin and trypsine-ethylene diamine tetraacetic acid (EDTA) were purchased from Gibco BRL (Cergy Pontoise, France). Foetal calf serum was purchased from Dutscher (Brumath, France). Hemacolor reagents were obtained from Merck (Darmstadt, Germany). Human acetylated low density lipoproteins labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylondocarbocyanine perchlorate (Dil-Ac-LDL) were obtained from Biomedical technologies (Stoughton, Massachusetts, U.S.A.). Anti-Gi-proteins antibodies sc262, sc386, sc391 were obtained from Tebu (Le Perray en Yvelines, France). Anti-Gi-proteins antibodies AS/7 were purchased from ICN (Orsay, France). [γ-^{32}P]-GTP (1.11 TBq mmol^{-1}) was obtained from Dupont NEN (Boston, MA, U.S.A.). [^{3}H]-thymidine (4.33 TBq mmol^{-1}) was purchased from Amersham Life Sciences (Amersham, U.K.).

Statistical analysis

Data are expressed as means ± s.e.mean; *n* refers to the number of animals studied. Statistical evaluation of the data was carried out by three way analysis of variance for the endothelium-dependent relaxations, by paired Student's *t* test for GTPase activity studies and by a one way analysis of variance for the uptake of thymidine studies. Differences were considered to be statistically significant when P was less than 0.05.

Results

Day 1

One day following balloon injury, the intimal surface of the vascular wall of the LAD stained with Evan's blue, whereas the control LCX remained unstained. No endothelial cells were observed on cross sections of the LAD (Figure 1). Endothelium-dependent agonists such as 5-HT failed to induce relaxations in the previously denuded artery (Figure 2).

Day 8

Histomorphometry Eight days after balloon injury, the LAD no longer stained with Evan's blue. Light microscopy of cross sections of the LAD revealed the presence of endothelium (Figure 1). There was no statistically significant difference either in the intimal and medial areas or in their ratio between the LAD and the LCX (Table 1).

Endothelium-dependent relaxations 5-HT and bradykinin produced concentration-dependent relaxations in rings from LAD and LCX coronary arteries contracted with prostaglandin $F_{2\alpha}$. Maximal relaxations were similar in the control arteries and the LADs (Figures 2 and 3).

Morphology of cultured endothelial cells Primary cultured endothelial cells derived from the LCX displayed a polygonal shape and contained one nucleus. These cells were packed tightly and presented a characteristic 'cobblestone' appearance (Figure 4). Their size was fairly uniform. The cultured regen-

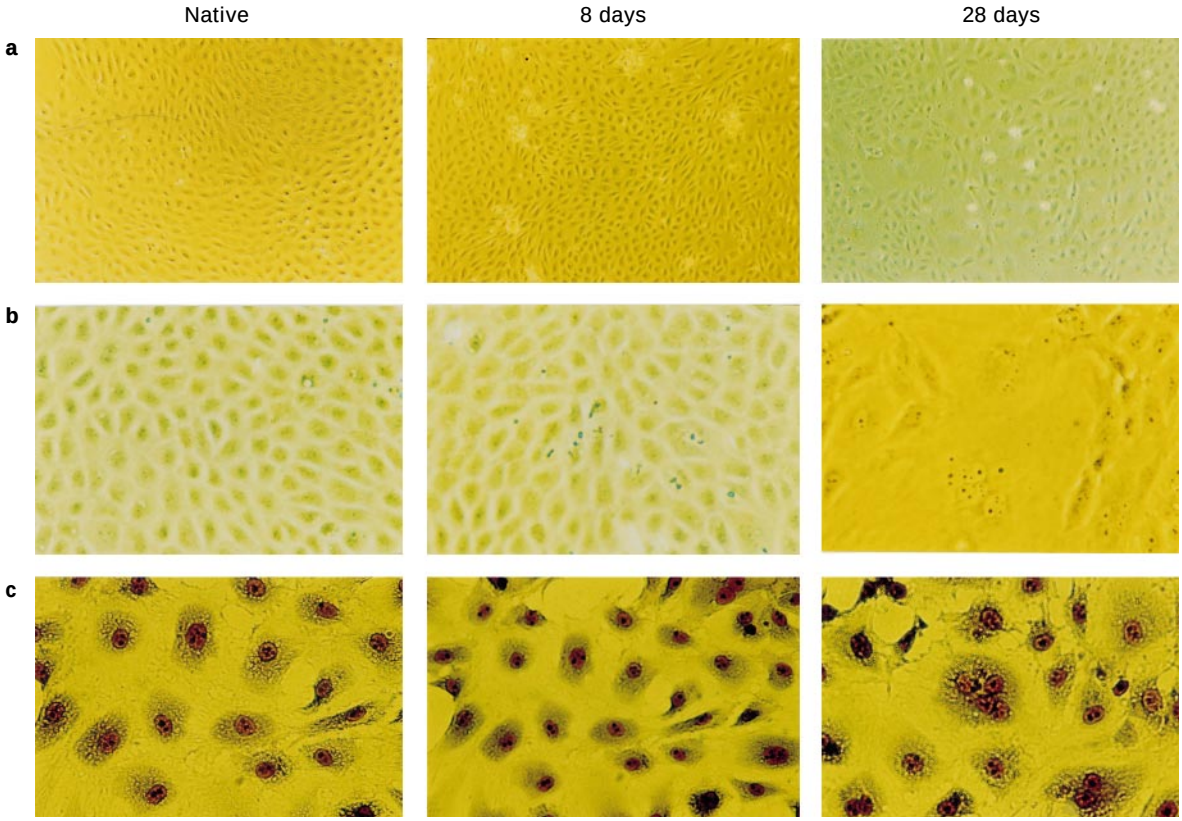

Figure 4 Phase contrast pictures of primary cultures of native endothelial cells and regenerated endothelial cells 8 days and 28 days following balloon injury. Colorimetric labelling with haemacolour reagents of the endothelial cells fixed and permeabilized (×400). (a) Phase contrast (×125); (b) phase contrast (×400); (c) haemacolour labelling (×400).

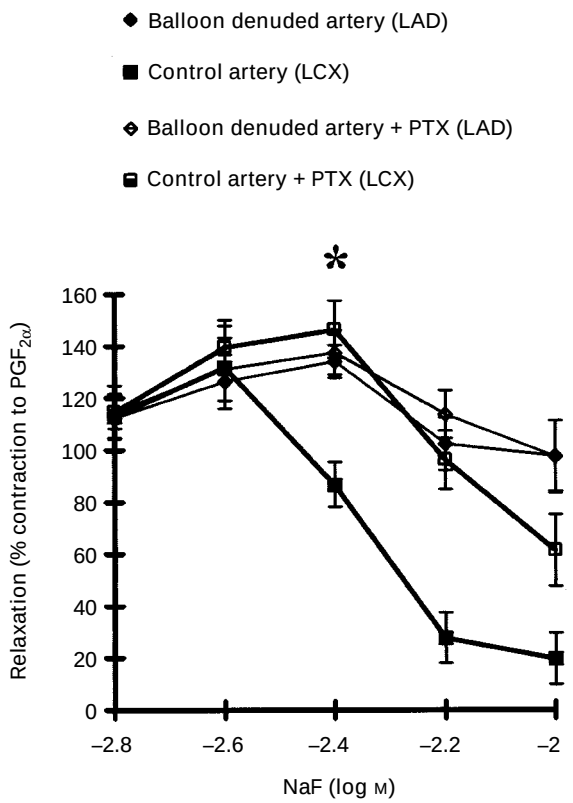

Figure 5 Cumulative concentration-response curves to sodium fluoride (NaF, $n=3$), in rings (contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) from LCX or LAD coronary arteries that underwent balloon injury 28 days before the experiment. Some rings were treated with pertussis toxin (PTX, 300 ng ml^{-1}). The relaxations are expressed as percentage of maximal contraction induced by prostaglandin $F_{2\alpha}$. Results are presented as means and vertical lines show s.e.mean. The asterisks indicate statistically significant ($P<0.05$) effect of the treatment with pertussis toxin on the control coronary arteries from the indicated concentration.

erated endothelial cells derived from the previously denuded LAD coronary artery, displayed a similar morphology (Figure 4).

Day 28

Histomorphometry Twenty eight days following balloon injury light microscopy of a cross section of the LAD demonstrated the presence of a neointima (Figure 1). The intima to media ratio was $28\pm6\%$ ($n=3$), which was significantly different from the value in the LCX (Table 1).

Endothelium-dependent relaxation In the previously denuded LAD contracted with prostaglandin $F_{2\alpha}$, the concentration-dependent, endothelium-dependent relaxations to 5-HT were impaired compared to those observed in the LCX (Figure 2). The concentration-response curves to bradykinin (10^{-10} to 10^{-6} M) were not significantly different between previously denuded and control arteries (Figure 3). In the presence of a depolarizing solution (20 mM KCl) (to inhibit reponses to endothelium-derived hyperpolarizing factor (EDHF), (Mombouli *et al*., 1992), the concentration-relaxation curve to bradykinin of the previously denuded LAD was shifted to the right of that obtained in the control LCX (Figure 3). Sodium fluoride (a direct activator of G-proteins) caused concentration-dependent relaxations of the LCX (Figure 5). These relaxations were reduced significantly in the LAD after previous balloon denudation (Figure 5). Pertussis toxin (300 ng ml^{-1}; to ADP-ribosylate Gi-proteins) inhibited the relaxations to NaF only in the control artery (Figure 5).

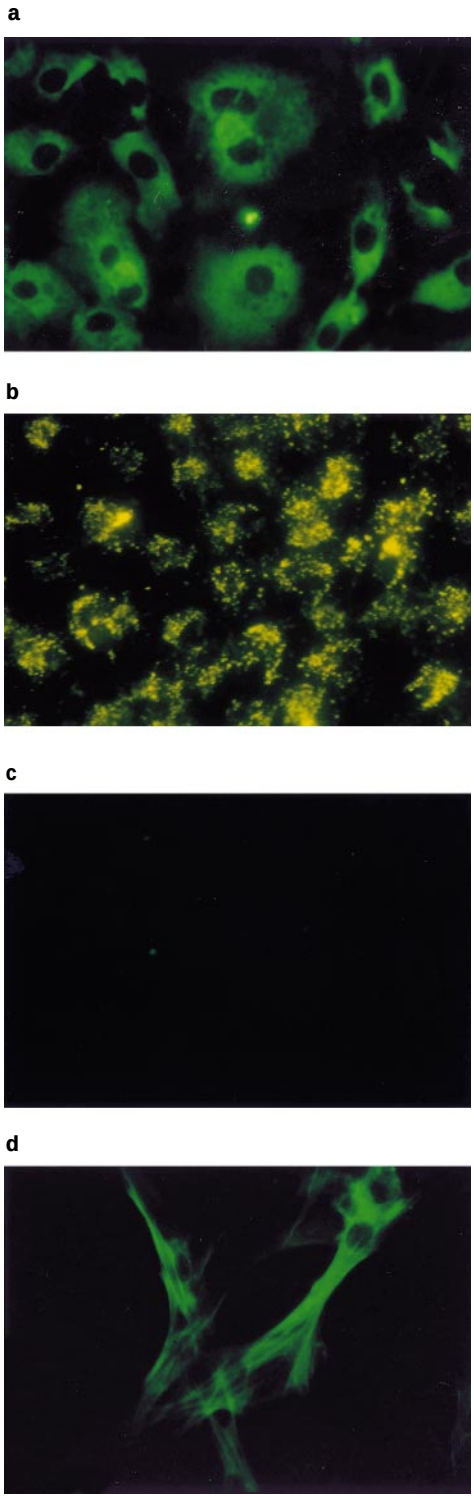

Figure 6 (a) Indirect immunofluorescence of regenerated endothelial cells 28 days following balloon injury with a rabbit anti-human von Willebrand factor (1 : 200), (b) labelling with Dil-Ac-LDL (1 μg ml^{-1}) (orange) coupled with staining with anti-smooth muscle α-actin (1 : 200) (green), (c) smooth muscle cells labelling with Dil-Ac-LDL and (d) smooth muscle cells staining with anti-smooth muscle α-actin (1 : 200). Cells were used as described in Methods and probed with each antibody or Dil-Ac-LDL. FITC-conjugated goat anti-rabbit IgG was used as secondary antibodies ($\times400$) for the immunostaining of the von Willebrand factor.

Morphology of cultured endothelial cells Native cells in primary culture presented the typical morphology of endothelial cells (Figure 4). Cultured regenerated cells varied in size and shape (Figure 4). Giant endothelial cells were intermingled

among cells of normal size. These giant cells were multinucleated in primary culture (Figure 4). These cells displayed a positive immunofluorescent staining to von Willebrand factor-related antigen and an uptake of Dil-Ac-LDL but a negative immunofluorescent staining to α-smooth muscle actin-related antigen (Figure 6). Such giant multinucleated cells were observed in all primary cultures from previously denuded arteries, although the population varied between animals. The number of giant cells was determined under the microscope. In the regenerated endothelial cells $11.6 \pm 4.1\%$ ($n = 3$) of the cells were larger than native endothelial cells.

DNA synthesis DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation at the same subconfluent state of primary culture of native and regenerated endothelial cells. The uptake of thymidine was significantly lower in regenerated than in native cells (Figure 7).

Expression of Gi proteins Membranes of primary cultured regenerated and native endothelial cells were prepared and the expression of Giα protein was assessed by immunoblot with several anti-Gi antibodies. In membrane fractions of regenerated endothelial cells, Giα1, Giα2 and Giα3 were labelled by different antibodies as illustrated by bands with an apparent molecular weight of 40–41 kDa (Figure 8). The labelling revealed on the immunoblot was similar to that obtained from membrane fractions of native endothelial cells. Since the regenerated endothelial cell population contained multinucleated giant cells among cells of normal size, immunolabelling was also performed to verify whether or not the Gi-protein expression in these giant cells is the same as in normal cells. For immunofluorescence microscopy, the endothelial cells were

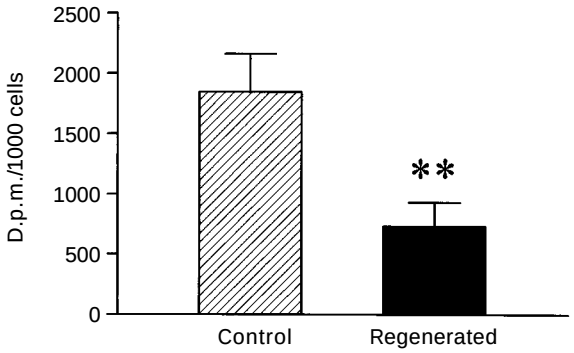

Figure 7 Uptake of tritiated thymidine by control left circumflex artery (LCX) cells and regenerated endothelial cells 28 days following balloon injury of left anterior descending (LAD). The incorporation of 1 μCi of thymidine per well was measured after an incubation of 4 h in three to six wells per each type of cell culture (regenerated or native) from four pigs. The asterisks indicate statistically significant differences ($P < 0.01$) between the two types of preparations.

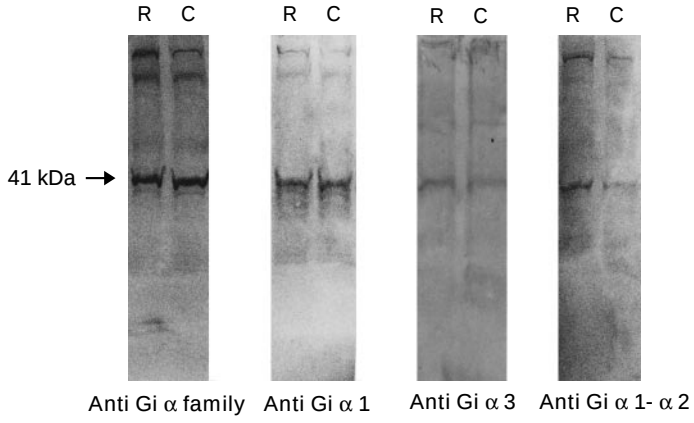

Figure 8 Immunoblot of membranes of regenerated (R) or native (C) endothelial cells with sc391, sc262, sc386 and AS7. Antibodies against: anti-Giα3, sc262 (1 : 500); anti-Giα1, sc391 (1 : 500); anti-α family, sc386 (1 : 200) and anti-Giα1 and α2, AS7 (1 : 200). Proteins were subjected to SDS–PAGE, transferred onto nitrocellulose membranes, and immunoreacted with the indicated antibodies. Representative immunoblot of three separative experiments.

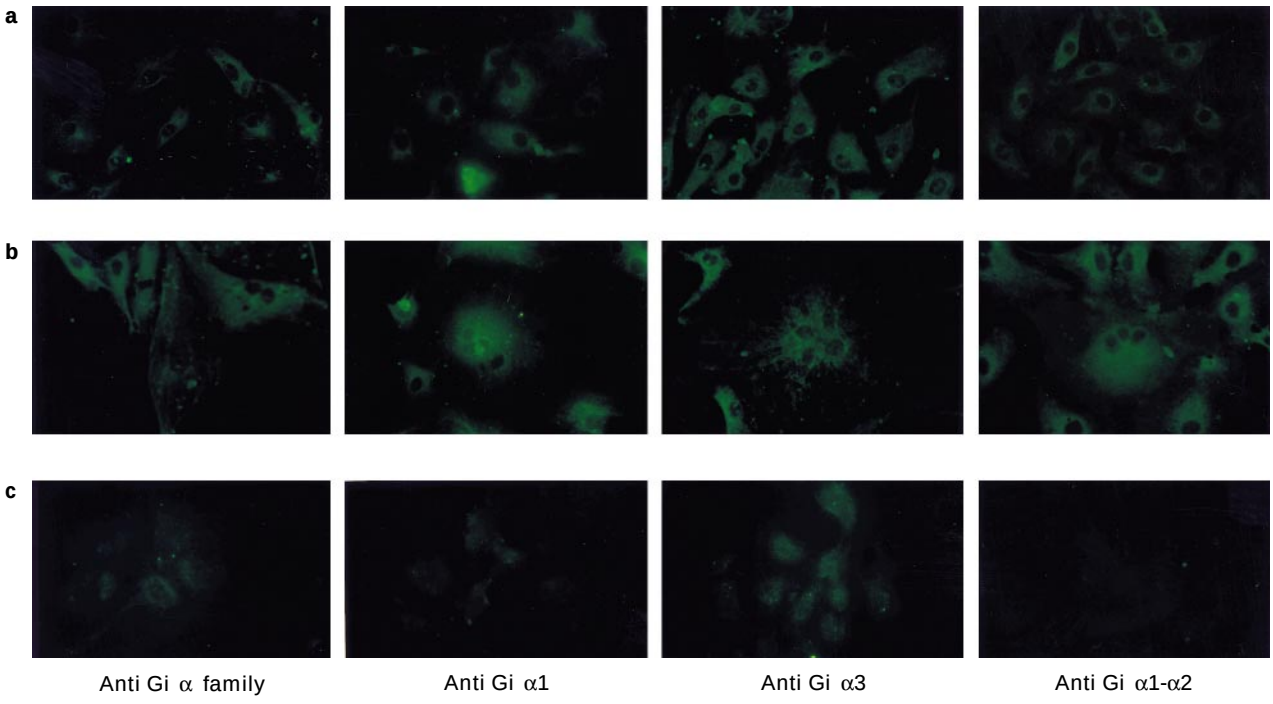

Figure 9 Indirect immunofluorescence of (a) native or (b) regenerated endothelial cells with sc386, sc391, sc262 and AS7. Antibodies against: anti-Giα3, sc262 (1 : 500); anti-Giα1, sc391 (1 : 500); anti-α family, sc386 (1 : 200) and anti-Giα1 and α2, AS7 (1 : 200). Cells were fixed and permeabilized and probed with the different antibodies. FITC-conjugated goat anti-rabbit IgG was used as the second antibody. (c) Control, labelling pattern of native endothelial cells with the antibody preadsorbed with its antigen.

fixed and permeabilized with ethanol. Whereas no staining was observed when the primary antibody was omitted (data not shown), typical endothelial cells were stained with the same pattern with each anti-Gi antibody. The specificity of the immunofluorescence signal of the endothelial cells was examined further by adsorbing these antibodies with antigens before staining. The intensity of the labelling was reduced by adsorbing the antibodies with their respective antigen (Figure 9). The labelling of Giα1, Giα2, or Giα3 on the regenerated endothelial cells was similar to that observed in cultured native endothelial cells (Figure 9). The giant cells were labelled with a similar intensity as that of regenerated cells of normal size.

GTPase activity The GTPase activity (V_{max}) and the Michaelis constant (K_m) of G-proteins of membranes from native endothelial cells were 1.12 ± 0.10 pmol min^{-1} mg^{-1} prot. and 0.61 ± 0.06 μM ($n=4$), respectively. The basal GTPase activity of membranes from regenerated endothelial cells was significantly lower than that observed with membranes of native endothelial cells (Figure 10).

Discussion

Balloon injury results in denudation of the endothelial surface of the vascular wall (Haudenschild & Schwartz, 1979) and afterwards the endothelial cells present at the edge of the denuded area proliferate and the artery quickly regains an endothelial cell lining. Eight days following balloon injury, endothelial cells were observed by light microscopy (Shimokawa *et al*., 1989; Berdeaux *et al*.,1994). The newly formed endothelium functions normally. Twenty eight days following balloon denudation, regenerated endothelium selectively lost the pertussis-toxin sensitive G-protein coupled responses to 5-HT, but those of bradykinin were maintained, which is consistent with earlier studies (Shimokawa *et al*., 1987; 1989). A selective endothelial dysfunction implicating abnormalities at the level of the Gi-protein has been observed in atherosclerotic coronary arteries. Indeed, studies performed on arteries of pigs fed with high-cholesterol diet, demonstrate that the ADP-ribosylation induced by pertussis toxin is less pronounced in regenerated endothelial cells compared to native endothelial cells, suggesting either a decreased amount or a reduced function of Gi-proteins (Shibano *et al*., 1992). On the basis of these results, the expression and function of G-proteins in regenerated endothelial cells was assessed in the present study.

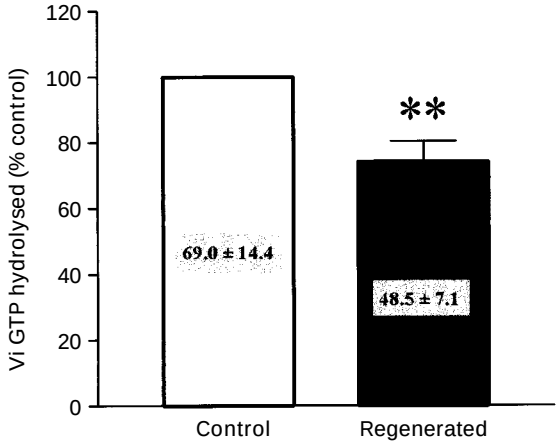

Figure 10 GTPase activity of the G-proteins of native and regenerated endothelial cells 28 days following balloon injury as measured by the hydrolysis of [γ-^{32}P]-GTP by membranes of endothelial cells. The assay was initiated by adding the membranes (25 μg) to the reaction mixture and incubating 15 min at 25°C. The reaction was stopped at 4°C by addition of $HClO_4$. The percentage of [γ-^{32}P]-GTP hydrolysis was determined by h.p.l.c. The results are expressed as means ± s.e.mean ($n=6$) in pmol min^{-1} mg^{-1} protein. The columns represent the evolution of the activity against the GTPase activity from membranes of native endothelial cells. The asterisks indicate statistically significant differences ($P<0.01$) between the two types of cell cultures.

The expression of three different α-subunits of the pertussis-sensitive Gi-protein was determined in regenerated endothelial cells in primary culture. A comparison of the intensity of the proteins labelled in Western blot led to the conclusion that the expression of each subunit of Gi-protein is not decreased in the regenerated compared to the native endothelial cell membrane preparation. The immunofluorescent studies in the giant multinucleated endothelial cells and those of normal size, demonstrated that the atypical regenerated endothelial cells expressed each subunit of the Gi-protein (Giα1, Giα2, Giα3). The intensity of the immunofluorescent labelling was similar to that observed in the cells of regular size. Taken in conjunction, these findings indicate that the impaired function of the regenerated endothelium, 28 days following balloon denudation, cannot be attributed to the loss of the Gi-protein α subunit. However, in human coronary arteries from atherosclerotic patients the expression of endothelial Gi-protein is reduced (Tsutsui *et al*., 1994). This alteration may be associated with atherosclerosis rather than the regeneration of endothelium.

The characteristic parameters of G-protein activity were determined and are in agreement with previous findings (Brandt & Ross, 1985; Liao & Homcy, 1993). The basal GTPase activity of membrane fractions of regenerated endothelial cells was reduced compared to that of native endothelial cells in primary culture. These observations strongly suggest that the function of the G-proteins is curtailed in regenerated endothelial cells. The basal GTPase activity accounts for the function of the overall G-proteins present in the endothelial cells and does not distinguish between the different families of G-proteins. The relaxation induced by the direct activator of G-proteins, NaF, was prominently reduced by an inhibitor of the Gi-protein, pertussis toxin, suggesting a major role for the Gi-protein of the G-proteins present in endothelial cells (Flavahan & Vanhoutte, 1990). Moreover, the present study confirmed that the relaxations to NaF are reduced in arteries with regenerated endothelium (Flavahan & Vanhoutte, 1990). Thus, it appears likely that the endothelial dysfunction seen with regeneration of the endothelium involves the function of the pertussis toxin-sensitive Gi-protein. Alternative mechanisms could underly the endothelial dysfunction of the regenerated endothelium. In particular, following balloon denudation of the rabbit carotid artery an accumulation of endogenous inhibitors for nitric oxide synthesis occurs (Azuma *et al*., 1995). These findings are not in disagreement with the present results. Indeed, on previously denuded coronary arteries, bradykinin-induced relaxations are not altered, whereas they are blunted in depolarizing conditions. In the porcine coronary arteries, bradykinin causes the release of both nitric oxide and endothelium-derived hyperpolarizing factor (EDHF; Mombouli & Vanhoutte, 1995). In depolarizing solution, only the nitric oxide pathway can be activated. Therefore one could postulate that the lack of impairment of bradykinin-evoked relaxations under control conditions illustrates a greater contribution to the EDHF-pathway to compensate for the failing release of nitric oxide from 28 days regenerated endothelium.

In earlier work (Shimokawa *et al*., 1989), no *in situ* morphological differences were noticed between early regenerated endothelial cells and native cells (eight days following balloon injury), with scanning electron microscopy. In primary culture, the early regenerated cells displayed a typical endothelial cell morphology similar to that observed in cultured native endothelial cells. Twenty eight days following balloon denudation, intimal hyperplasia developed under the regenerated endothelium and was accompanied by a morphological heterogeneity of the regenerated endothelial cells, whereby giant multinucleated cells were intermingled with 'typical' endothelial cells. These 'atypical' cells were endothelial in nature since they contain von Willebrand factor and accumulated Ac-LDL. Several investigators have stated, on the basis of *in situ* scan-

ning electron microscopy, that the regenerated cells from different species are elongated compared to native endothelial cells (Shimokawa *et al*., 1987; Niimi *et al*., 1994; Bannykh *et al*., 1994; Azuma *et al*., 1995). During the regeneration process, there is a close relationship between endothelial dysfunction and appearance of giant multinucleated endothelial cells. However, the presence of these atypical endothelial cells is unlikely to be solely responsible for the endothelial dysfunction since, they represent a modest percentage (less than 15%) of the total cell population. The morphology of these cultured giant regenerated endothelial cells resembles closely that described for the giant multinucleated cells, observed *in vitro* and *in vivo*, with endothelium denudation in the rabbit (Poole *et al*., 1958), haemodynamic stress in the rabbit (Fallon & Stehbens, 1972), atherosclerosis in rabbits and in man (Efskind, 1941; Sinapius, 1952; Tokunaga *et al*., 1989; Tashiro *et al*., 1994), and with senescence in bovines and man (Togunaga *et al*., 1989; Augustin-Voss *et al*., 1993). The appearance of these giant cells may indicate a change in endothelial cell phenotype involved in the phenomena of endothelial alteration. The observation that the DNA synthesis decreased confirms a phenotypic change of the regenerated endothelial cells. Bovine aortic endothelial cells maintained in culture for up to 45 passages (100 population doublings) become senescent and display phenotypic changes, cells becoming larger, multinucleated and slow growing (Augustin-Voss *et al*., 1993). After balloon injury, the endothelial cells must migrate and proliferate to reline the vascular wall. Thus, they are involved *in vivo* in multiple population doublings which could explain the appearance of the observed phenotypic change (Fallon & Stehbens, 1972; Tokunaga *et al*., 1989; Augustin-Voss *et al*., 1993; Tashiro *et al*., 1994). It is conceivable that the capacity of the endothelium to regenerate is limited, since cells proliferating in the lesion might eventually become senescent. Hence, the occurrence of these giant cells may be a marker of endothelial dysfunction.

In conclusion, the present findings support the concept that endothelial dysfunction observed, twenty eight days following balloon injury, in regenerated endothelium is not due to a reduced expression of the Gi-protein but rather is due to depressed function of G-proteins, probably the pertussis-toxin sensitive Gi-protein.

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