II. Materials and Methods

1. Study animals

Twenty-four Juvenile domestic eight-week-old swine weighing from 20 to 25 kg were used in this study. All animals received ticlopidine and aspirin continuously for 60 days. At the age of 8 - 9 weeks old, animals were treated with angioplasty and/or brachytherapy and they were scarified with anesthesia at 1 day and 6 weeks after surgical treatments and coronary arteries were injected with potassium chloride (Sigma-Aldrich, St. Louis, MO, USA). Besides, the coronary arteries post-treatments were perfusion-fixed and placed in 4% formalin (Sigma-Aldrich, St. Louis, MO, USA) at 100 mmHg for 24 hours.

2. Radiation and porcine balloon injury

The left anterior descending artery (LAD), the left circumflex coronary (LCX), and the right coronary artery (RCA) were assigned by randomization to receive doses of 20 Gy, 14 Gy and sham control (n = 8). Sham operated porcine underwent the same surgical procedure with the exception of coronary angioplasty, called sham control group (arteries). There were normal coronary arteries treated the combination of the balloon injury and radiation. Balloon catheters filled with liquid radioisotopes provided excellent dose homogeneity for intracoronary radiation therapy and liquid-filled balloons with rhenium-188 (^{188}Re) have been used in a cost-effective way. ^{188}Re-liquid-filled balloon device was delivered using a Tungsten-188/Rhenium-188 (^{188}W-^{188}Re) generator. This β emitter with a maximum energy ($E_{\beta}max$) of 2.12 MeV, and a 16.9 hours half-life is attractive for the applications of internal radiotherapy. A balloon dilatation catheter was inserted into carotid and filled with the radioisotope. The image is monitored by carotid angiography (CAG). The dose activity had been estimated and the measurement of dosage level was cumulated by exposure time. The cycle of inflation/deflation was three until the prescribed dose was administered. The

PTCA/Re group was involved in angioplasty treatment prior to radiation after 10-20 minutes (n = 8). The Re/PTCA group was involved in the exposure of radiation immediately before the treatment of angioplasty (n = 8). During the treatment of angioplasty, an estimated balloon: artery ratio of 1.3:1 was used. At 6 weeks after operation, the animals were scarified and the segments of LAD, LCX and RCA were dissected at the sites of balloon injury and radiation with a β-source. The purpose of angioplasty at this ratio was to damage intima cells. There was zero percent of the porcine death after angioplasty.

3. Histopathologic analysis

6 weeks after balloon injury, follow-up angiography was performed on porcine coronary arteries to assess the degree of luminal narrowing in the injured segments just before the porcine was anesthetized by using a lethal dose of intravenous thiopental sodium (Sigma-Aldrich, St. Louis, MO, USA). The heart was then isolated and perused at 100 mmHg for 24 h with 4% neutral buffered formalin. The LAD, including the injured segment, was isolated and paraffin-embedded for sectioning. Each artery was cut into four equal segments. From each segment, 10 sections (each 6 μm thick) were collected at 3 mm intervals for histological analysis. The sections were stained with hematoxylin-eosin (H&E) for qualitative and quantitative measurements, respectively. Histological analysis of intimal thickening was performed by using computerized digital microscopic planimetry. Measurements of the following parameters were made on the vessel cross-sections and used for further calculations: lumen area, and neointimal area.

Table 7. List of the design of experiments associated with other conditions.

Experiments	Surgical treatments	The number of arteries	Number of coronary arteries		
			LAD[a]	LCX[b]	RCA[c]
1 Gene expression					
At 1 day after surgical treatments					
	Control[d]	6	3	0	3
	PTCA[e]	6	3	0	3
	Re-188[f]	6	3	0	3
	PTCA/Re[g]	6	3	0	3
	Re/PTCA[h]	6	3	0	3
2 Histopathologic analysis					
At 6 weeks after surgical treatments					
	Control	6	2	**2**	2
	PTCA	6	4	2	2
	Re-188	6	3	0	3
	PTCA/Re	6	2	2	2
	Re/PTCA	6	2	2	2

[a] LAD, left anterior descending coronary artery; [b] LCX, left circumflex coronary; [c] RCA, right coronary artery; [d] Control, sham control group; [e] PTCA, PTCA treatment group alone; [f] Re188, brachytherapy treatment group alone (20 Gy); [g] PTCA/Re, angioplasty prior to brachytherapy; [h] Re/PTCA, angioplasty after brachytherapy

4. Cell culture

Human vascular smooth muscle (HVSMC), isolated from human normal aorta smooth muscle, was obtained from Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). Cell were grown as adherent cells at 37 °C in the presence of 5% CO_2 in nutrient mixture F-12 Ham Kaigan's Modification (Hsm's F-12K medium, Sigma) supplemented with 10% FBS (Gibco BRL, Grand Island, NY, USA), 10 mM HEPES Buffer (Biological Industries Ltd. Kibbutz Beit Haemek), 10mM TES, 0.05 mg/ml ascorbic acid, 0.01 mg/ml insulin, 0.01 mg/ml transferrin, 0.03 mg/ml ECGS, 10 ng/ml sodium selenite, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were lysed trypsinized using 0.25% trypsin/1 mM EDTA. Cells were grown in Ham's F-12K for one or more passages. All experiments were done in 8 to 9 passages. TES, ascorbic acid, insulin, transferrin, sodium selenite, penicillin, streptomycin, and trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). ECGS was purchased from Upstate Biotechnologies (Lake Placid, NY, USA). EDTA was purchased from Merck (Darmstadt, Germany).

5. γ-Radiation of VSMCs

HVSMC were irradiated with 2 and 20 Gy at a dose rate of 0.2153 Gy/min in T_{25} flask. GHS-2, γ emitter from cobalt-60 (^{60}Co), was supported by National Radiation Standard Laboratory in Institute Nuclear Energy Research. After irradiation, new medium were immediately changed in T_{25} flasks so as to avoid the contamination of free radicals. Cells were harvested at 1, 2, and 3 day for analysis of mRNA expression after radiation and nonirradiated cells were harvested at the same time point.

6. Reverse transcription-polymerase chain reaction

Total RNA was extracted from segments of total coronary arteries, including LAD, RCA,

and LCX applying a Trisolution reagent (Genemark Technology Co., Ltd., Taiwan), according to the manufacturer's instructions. Segments of total coronary arteries were followed by phenol/chloroform extraction and ethanol precipitation in turn. The quality of the isolated RNA was verified by 1% agarose gel electrophoresis. Porcine ICAM-1, VCAM-1, and GAPDH expression were evaluated by semiquantitative RT-PCR. The complementary deoxyribonucleic acid (cDNA) was produced from 3μg of total RNA by reverse transcription with 500 U of Superscript reverse transcriptase (HT Biotechnology Ltd., Cambridge, England) in a 20μl reaction solution containing a final concentration of 1X SUPER reaction buffer (HT Biotechnology Ltd., Cambridge, England), 1 mM dNTPs (deoxy-CTP, -GTP, -ATP, -TTP), 0.5 μg oligo (dT) primer (Protech Technology Enterprise Co., LTD, Taiwan), and 25 U of human placental ribonuclease inhibitor (HT Biotechnology Ltd., Cambridge, England). Commercially available PCR primers for ICAM-1, VCAM-1 and GAPDH were purchased from BioBasic, Inc. (Markham, Canada). The specific-primer sequences and sizes of the amplified products were shown in Table 8. PCR was performed with 250 U Taq Polymerase (Protech Technology Enterprise Co., Ltd., Taiwan), and the number of cycles producing a 'linear' increase in products was determined. 1 μl cDNA product was used and the PCR reaction mixture contained a final concentration of 1X PCR buffer, 200 μM dNTPs, 0.2 μM each 5'and 3' primer and 2.5 U of Taq 3 min, PCR amplification was performed under the following condition: 94℃ for 30s, 55℃ for 30s, and 72℃ for 30s in each cycle performed by a PTC-100 thermal cycler (MJ Research Inc., Watertown, MA, USA). PCR fragments were separated by gel electrophoresis and visualized with an UVB-101 transilluminator (Apices Scientific Co., Ltd, MA, USA) following ethidium bromide staining. ICAM-1 and VCAM-1 mRNA expression were expressed as the ratio of specific to GAPDH mRNA. The product of GAPDH estimated by RT-PCR was used for normalization of bands. PCR products were separated on a 2.5%

agarose gel. Bands were scanned and quantified using Scion Image (NIH, USA).

HVSMCs were harvested and total RNA was prepared using as described previously. 3 μg total RNA was used in one reaction of RT. Human NF-κB was amplified by RT-PCR. The protocol was previously described as above and primers were shown in Table 8.

Table 8. List of primers used for PCR

Genes	GenBank Accession No.	Organisms	forward primers (5' → 3')	Reverse primers (5' → 3')	Product size (bp)
GAPDH[a]	BC023632	Human and Pig	GCC AAA AGG GTC ATC ATC TC	ATG ACC TTG CCC ACA GCC T	320
NF-κB[b]	NM003998	Human and Pig	CCT GGT GGA GAA CTT TGA GC	CCA GAG ACC TCA TAG TTG TC	362
ICAM-1[c]	NM000201	Human	GGC TGG AGC TGT TTG AGA AC	CTG GCT TCG TCA GAA TCA CG	378
	AF156712	Pig	TCA ATG GAA CCG AGA AGG AG	CCT GGG TCT GGT TCT TGT AT	341
VCAM-1[d]	NM080682	Human	CGA GAC CAC CCC AGA ATC TA	CTA TCT CCA GCC TGT CAA ATG	369
	L43124	Pig	AAG CTG AGG GAT GGG AAT CT	GGC CCT GTG GAT GGT ATA TG	330

[a] GAPDH, glyceraldehyde-3-phosphate dehydrogenase; [b] NF-κB, nuclear factor kappa B; [c] ICAM-1, intercellular adhesion molecule-1; and [d] VCAM-1, vascular adhesion molecule-1

7. Post-processing and hybridization of cDNA microarray

Array post processing can be divided into three steps: re-hydration, blocking, and denaturing. Since the DNA solution dries to the outer edge of the spot during the printing process, the goal of re-hydration is to allow the DNA a chance to more evenly distribute across the surface of the spot. This will help increase the amount of total DNA bound after processing. Selecting array slides and marking the boundaries of the array slide on backside of the slide are to be used for the first step in the post-processing. After processing, the arrays will not be visible. 100 ml 1X SSC (AMRESCO, Inc. USA) was prepared into hydration humid chamber (Sigma-Aldrich, St. Louis, MO, USA) and was to warm on slide warmer. We set the slide array side down over 1X SSC and observed spots until full hydration was achieved. Re-hydration will slightly expand the size of the spots which can lead to actually touching spots together. Upon reaching full hydration, array slide should be dried, then we removed the slide and subsequently slide were received the UV crosslink for 65 mJ used by Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, NY, USA). Slides were plunged in 0.2% SDS (Riedel-de Häen, Seelze, Germany) in the shaking rack for 10 min. After blocking process, washing the slide was to remove contaminant up and down in ddH_2O for several seconds. Plunging slides in 100℃ water (just stopped boiling) for 2 min was to denature the double stranded DNA molecules and thus enhanced hybridization availability. After boiling step, slides were set into cold 100% ethanol chamber to stop the reaction. We centrifuged slides and racked for 5 min at 800 rpm. Array slides were immediately stored in slide box in order to avoid the light and dash.

The fluorescent cDNA probes were prepared through reverse transcription (RT). Aliquots of 10-15 μg of each amplified cDNA were labeled with Cy3-dUTP and with Cy5-dUTP. In order to stop reaction of RT, Cy5 and Cy3 probes were added 15 μl of 0.1 N NaOH, 2 mM EDTA to the original mixture and incubated at 70℃ for 30 min. After

stopping step, 15 μl of 0.1 N HCl was added to Cy5 and Cy3 probes in order to neutralize the previous step. The mixture of Cy5 and Cy3 probes was purified by using QIAquick PCR purification kit (QIAGEN Inc., CA, USA). Mixture of Cy5 and Cy3 probes were precipitated with ethanol and dissolved in 32 μl of hybridization solution the amount of which was dependent on requirement of each experiment design.

To limit nonspecific binding of the probe, 20 μg of human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), 20 μg polyA RNA (Sigma-Aldrich, St. Louis, MO, USA) and 20 μg tRNA (Gibco BRL, Grand Island, NY, USA) were added as blocking reagents. Probes were added to a hybridization solution (3X saline sodium citrate (SSC), and 0.1% SDS). Denature probes were in hot water at 100℃ for 2 min, and prepared probes were spinning at 13000 rpm for 5 min to remove water. The purified and denatured probes were then added and hybridized at 60°C for 16 - 18 h in the incubator. After removing from the chamber, slides were washed twice in a solution containing 2X SSC, 0.3% SDS for 20 min at 50°C. In the next 3 washing steps, slides were inverted in 1X SSC for 5 min, in 0.2X SSC for 5 min, and in 0.1X SSC for 5 min, respectively. Thereafter, slides were dried by spinning for 3 min at 800 rpm in a Beckman GS-6 tabletop centrifuge. Each RNA sample was labeled and hybridized on independent replicate microarray. Slides were scanned at 532 nm (Cy3-dUTP) and 635 nm (Cy5-dUTP) wavelengths with a GenePix 4000A scanner (Axon Instruments, Union City, CA, USA) to generate 16-bit gray scale pixel image files of the arrays.

8. Global normalization

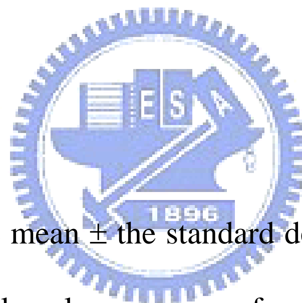
An MA-plot, as described in Dudoit and Fridlyand, is used to represent the (R,G) data. Normalization is usually applied to the log-ratios of expression, which will be written $M = \log_2 R - \log_2 G$. The log-intensity of each spot will be written $A = (\log_2 R + \log_2 G)/2$. A represents the measure of the overall brightness of the spot. It is convenient to use base-2

logarithms for M and A so that M is units of 2-fold change and A is in units of 2-fold increase in brightness. On this scale, M = 0 represents equal expression, M = 1 represents a 2-fold change between the RNA samples, M = 2 represents a 4-fold change. Two of normalization methods are below, including global normalization and intensity-dependent normalization.

Global methods assume that the red and green intensities are related by a constant factor, i.e. R = kG, and the center of the distribution of log ratios is shifted to zero

$$\mathrm{Log}_2 R/G \rightarrow \log_2 R/G - c = \log_2 R/(kG)$$

A common choice for the location parameter $c = \log_2 k$ is the median or mean of the intensity log ratios M for a particular gene set.



9. Statistical analysis

All results represented the mean ± the standard deviation (SD) for each treatment group in each experiment. Statistical analyses were performed on data in an EXCEL spread sheet using Student's *t*-test (two-sided, unequal variances). Values with $p < 0.05$ were considered to be significantly different.