Cell cycle and apoptosis genes in atherosclerosis

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Chapter 5

Conditional gene targeting in atherosclerosis

Local Cre-mediated gene recombination in vascular smooth muscle cells in mice

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ABSTRACT

Here we describe a means to conditionally modify genes at a predefined and localized region of the vasculature using a perivascular drug delivery device (PDD). A 4-hydroxytamoxifen (4-OHT)-eluting PDD was applied around the carotid or femoral artery of a mouse strain, carrying both the tamoxifen-inducible and smooth muscle cell (SMC)-specific Cre-recombinase (SM-Cre-ER^{T2}) transgene and a stop-floxed -galactosidase gene in the Rosa26 locus: the SM-CreER^{T2}(ki)/rosa26 mouse.

A dose and time curve of 0-10% (w/w) 4-OHT and 0-14 days application of the PDD in SM-CreER^{T2}(ki)/rosa26 mice showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application (carotid artery 2.4±1.8%; femoral artery 4.0±3.8% of SMCs). The unique 4-OHT-eluting PDD allowed us to achieve SMC-specific recombination in the same order of magnitude as compared to systemic tamoxifen administration. In addition, recombination was completely confined to the PDD-treated vessel wall segment.

Thus, local application of a 4-OHT-eluting PDD results in vascular SMC-specific Cre-mediated recombination in SM-CreER^{T2}(ki)/rosa26 mice without affecting additional SMCs.

Pathological processes, such as atherosclerosis and post-angioplasty restenosis, occur in highly localized regions of the vasculature.[1] Studying these processes using genetic modification may thus require a restriction to the area that is conditionally gene targeted. Moreover, some conditional alterations to smooth muscle cells (SMCs) of the vasculature as a whole may not be compatible with life, but should be addressed in a limited area of a vessel.To temporally and conditionally modify genes in a predefined and localized region of a blood vessel, we used a perivascular drug delivery device (PDD). The perivascular drug-eluting cuff has been used to study the effect of pharmaceutical compounds on neointima formation or restenosis.[2] The PDD is very suitable for local drug delivery and can simultaneously induce neointima formation.[2]

Via the release of 4-hydroxytamoxifen (4-OHT) from the PDD we studied the temporal and conditional modification of genes in a predefined and localized region of a blood vessel.The PPD was applied in a mouse strain that carried both the mutant estrogen receptor ligand binding domain, responsive to tamoxifen and SMC-specific Cre-recombinase (SM-Cre-ER^{T2}) transgene[3] and a stop-floxed β-galactosidase gene in the Rosa26 locus:[4] the SM-CreER^{T2}(ki)/ rosa26 mouse.

The unique 4-OHT-eluting PDD allowed us to achieve SMC-specific recombination in the same order of magnitude as compared to systemic tamoxifen administration. In addition, recombination was completely confined to the SMCs of the PDD-treated vessel wall segment.These data indicate that the novel 4-OHT-eluting PDD is an efficient tool to specifically induce highly localized Cre-mediated recombination in the SM-CreER^{T2}(ki)/rosa26 mouse.

METHODS

Transgenic mice

Mice that carry a tamoxifen-inducible Cre-recombinase under control of the smooth muscle cell (SMC)-specific SM22 promoter (SM-CreER^{T2}(ki) mice)[3] were crossed with the rosa26 reporter mouse line[4] to generate SM-CreER^{T2}(ki)/rosa26 mice. SM-CreER^{T2}(ki)/rosa26 mice were genotyped for the SM-CreER^{T2}(ki) promoter[3] and the rosa26 transgene.[4] Homozygous SM-CreER^{T2}(ki)/rosa26 littermates 8-10 weeks of age were compared in experiments. All animal work was approved by the regulatory authority of the institutional experimental animal committee.

Perivascular delivery device

The Poly(ε-caprolactone)-based perivascular delivery devices (PDD) were manufactured as previously described.[5,6] In brief, 4-hydroxytamoxifen (4-OHT, Sigma-Aldrich Chemicals BV, Zwijndrecht, The Netherlands) was first blended with PEG before this blend was mixed with molten PCL at 70 °C.The PCL:PEG ratio was 4:1 (w/w). Drug-loaded PDD were made from the blended molten 4-OHT–polymer mixture and designed to fit around the femoral and carotid arteries of mice (Figure 1). Drug-eluting PDD had the shape of a longitudinal cut cylinder with an internal diameter of 0.5 mm, an external diameter of 1 mm, a length of 2 mm, and a weight of approximately 5 mg. PDD were loaded with 1%, 2.5%, 5% and 10% (w/w) n=5 and *in vitro*

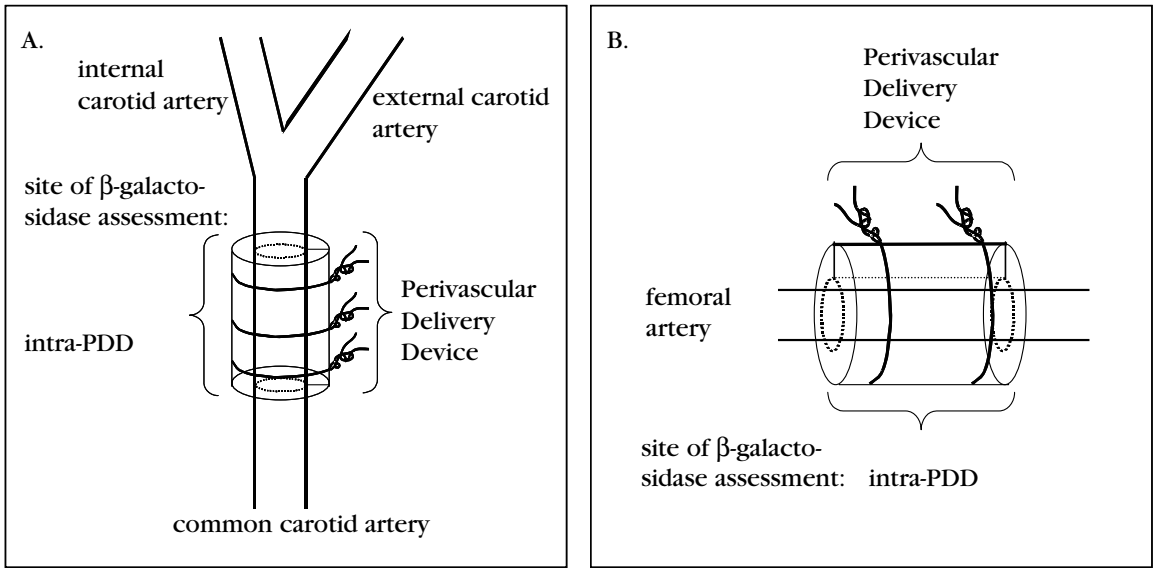

Figure 1. Local 4-hydroxytamoxifen (4-OHT) application using the perivascular delivery device (PDD) at the level of (A.) the carotid and (B.) femoral arteries.

release profiles were performed as previously described.[2,7] PDD of each composition were placed in 20 ml glass scintillation vials and cooled to 4°C. Five milliliters of iced-cold PBS pH 7.4 containing 0.2% bovine serum albumin (fraction V, Roche Diagnostics, Mannheim, Germany) were placed on top of the cuffs followed by 5 ml of n-octanol. The n-octanol formed an upper immiscible phase on top of the PBS. 4-OHT is far more soluble in n-octanol than in PBS (log Poctanol/water=3.2), which ensured rapid partition into the octanol phase. The vials were capped and incubated at 37°C. The concentration of 4-OHT in the octanol phase was analyzed by UV-VIS absorbance methods (Pharmacia LKB Ultrospec III, Peak Tek Inc., Glenside, USA) at a 4-OHT-specific wavelength (247 nm) using a double beam UV/VIS spectrophotometer (UVIKON 933, Kontron Instruments Ltd, Milan, Italy). This octanol phase was replaced back into the vial. A calibration graph of 4-OHT in n-octanol was established by measuring the absorbance of a 0-50 μg/ml range of standards in n-octanol.

Conditional gene targeting, histology and quantification of recombination

To achieve local recombination, SM-CreER^{T2}(ki)/rosa26 and control littermate rosa26 mice were anaesthetized and a PDD was placed around the carotid or femoral arteries as described,[2,8,9] containing vehicle, 0.1, 0.3, 1, 3 or 10% (w/w) 4-OHT for 7 (n=6 arteries/group) or 14 (n=8 arteries) continuous days. In order to achieve systemic recombination of the rosa26 transgene, SM-CreER^{T2}(ki)/rosa26 and control rosa26 mice (n=5/group) were injected intraperitoneally with 100 μl 20 mg tamoxifen (TMX, Sigma) for 7 continuous days. Next to carotid and femoral arteries, several SMC-rich organs were harvested from SM-CreER^{T2}(ki)/rosa26 and control rosa26 mice, including aorta, stomach, intestines and bladder to evaluate the site-specificity of 4-OHT induced recombination. β-Galactosidase activity was demonstrated as described[10] by staining of 20 μm cryosections. β-Galactosidase positive cells were counted and expressed as a percentage of the total number of morphologically identified SMCs. Antibodies against PECAM-1 (CD31, 1:200, Sigma, St. Louis, USA) were used to stain endothelial cells.

Statistical Analysis

All data are represented as mean±SD. Data were analysed using the non-parametric Mann-Whitney rank sum test. P-values less than 0.05 were regarded as statistically significant.

RESULTS

In vivo application of the perivascular delivery device

We developed a perivascular poly-(ε-caprolactone)-based delivery device (PDD) loaded with a tamoxifen derivative 4-hydroxytamoxifen (4-OHT) to restrict conditional recombination to a predefined and localized region of the vasculature in a susceptible mouse strain. PDDs loaded with a dose range of 4-OHT were generated to make a release profile *in vitro*. 4-OHT release from the PDDs was sustained and dose-dependent for at least 3 weeks.

To determine the optimal loading concentration of 4-OHT in the PDDs, leading to the highest levels of recombination *in vivo*, PDDs were placed around carotid and femoral arteries with a dose range from 0 to 10% (w/w) 4-OHT for 7 days. Arteries were examined for β-galactosidase-positive SMCs and morphology. At a loading of 0.1% 4-OHT recombination was hardly detectable, while at 0.3% 4-OHT 2.2±2.1% SMC-recombination for carotid and 1.5±1.5% for femoral arteries was found (Figure 2). At a loading concentration of 1%, 4-OHT recombination was increased to 4.0±3.8% for femoral and 2.4±1.8% for carotid arteries (Figure 2, Figure 3B and F, Table 1) and was not significantly different between both arteries (P=0.361). At a loading of 3 or 10% 4-OHT recombination was approximately 3-fold decreased as compared to 1% loaded PDDs (Figure 2, Figure 3C, G and D, H). Increasing the application time of the 1% 4-OHT-loaded PDDs around carotid and femoral arteries from 7 to 14 days did not affect the percentage of SM-recombination (data not shown). Importantly, no

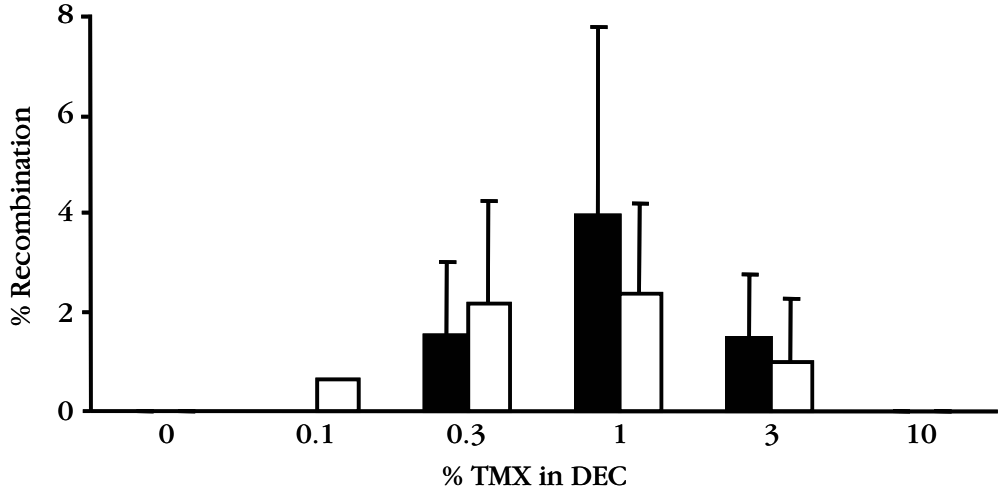

Figure 2. Percentage of medial gene recombination in femoral artery (black bars) and carotid artery (white bars) after incubation with PDDs containing 0, 0.1, 0.3, 1, 3, 10% (w/w) 4-OHT for 7 days. Success of recombination is shown as the number of β-galactosidase-positive SMCs as a percentage of the total number of SMCs.

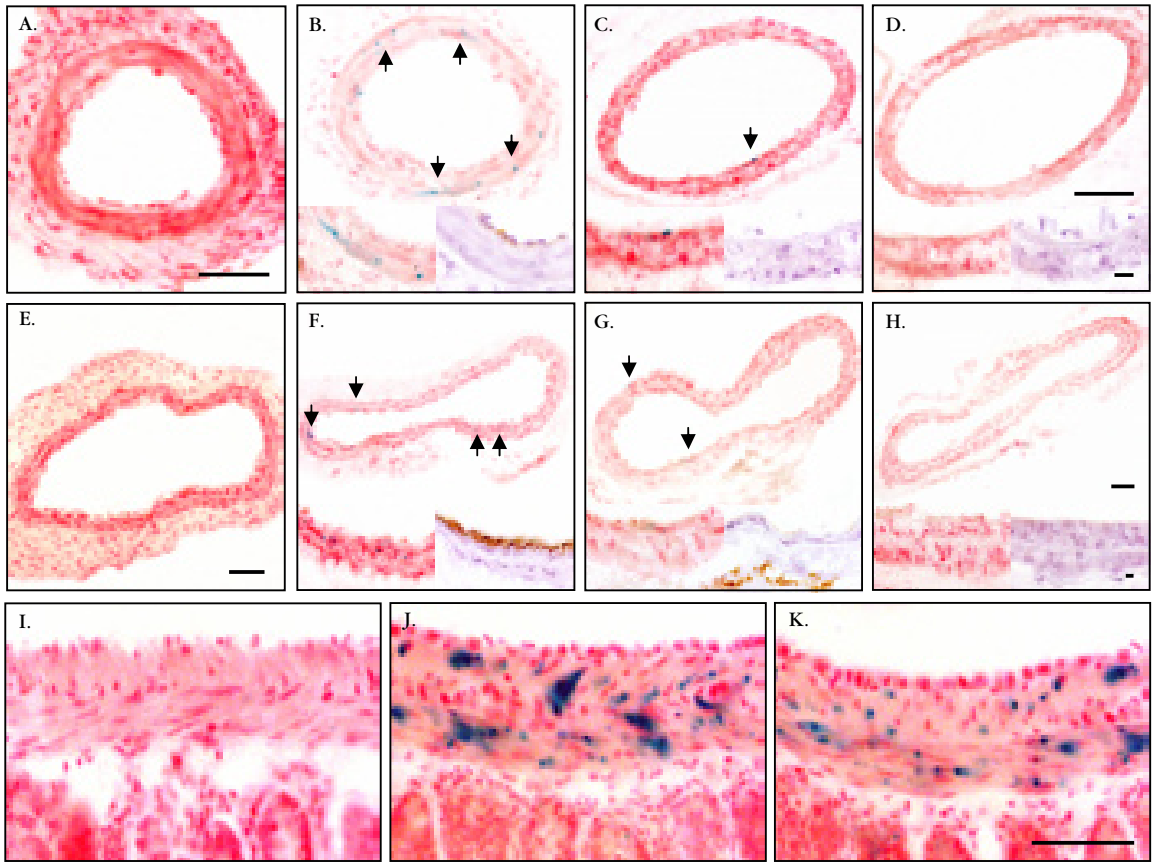

Figure 3. (A-H.) Microscopic images of β-galactosidase (top row, counterstained with nuclear fast red, magn. 200x and left insert, magn. 600x) and PECAM-1 staining (right insert, magn. 600x) of representative cross-sections of (A-D.) femoral and (E-H.) carotid arteries of SM-CreERT2(ki)/rosa26 mice treated with a (A, E.) 0, (B, F.) 1, (C, G.) 3 or (D, H.) 10% (w/w) 4-OHT-loaded PDD for 7 days. (I-K.) Microscopic images of β-galactosidase stained cross-sections of the intestines (I.) without 4-OHT, (J.) 1% 4-OHT-loaded PDD and (K.) systemic 4-OHT administration. Arrows indicate β-galactosidase-positive cells. Scale bar= 50μm.

β-galactosidase positive cells were detected in the aorta, stomach, intestines or the bladder (both 0.0±0.0%), indicating that recombination was restricted to the site of PDD application. No recombination was observed in SM-CreER^{T2}(ki)/rosa26 mice treated with empty PDDs, neither in control rosa26 mice receiving a 4-OHT loaded or empty PDDs. In conclusion, 1% (w/w) 4-OHT-loading for PDDs and application for one week yielded the highest percentage of SM-recombination.

Systemic application of tamoxifen via intraperitoneal (IP) injection for 7 days resulted in β-galactosidase positive staining in cryosections of several SMC-rich organs (aorta 6.7±3.1, femoralis 7.3±1.3, carotis 2.1±0.5 (Table 1) vs. stomach 61.5±5.8, intestines 47.6±9.4 (Figure 3I, J and K), bladder 92.5±4.2). In the femoral artery, systemic tamoxifen administration resulted in a 1.8-fold higher level of SMC recombination as compared to the 4-OHT-eluting PDD. In the carotid artery, the 4-OHT-eluting PDD allowed us to achieve similar levels of SMC-specific recombination as compared to systemic tamoxifen administration.

Table 1. Recombination in vascular SMCs of SM-CreER^{T2}(ki)/rosa26 mice after 7 days of local 4-OHT or systemic tamoxifen administration.

Organ	**Administration**	
	Local	Systemic
	0.05 mg/PDD	*2 mg/day*
	Recombination (%)	
Femoralis	4.0±3.8	7.3±1.3
Carotis	2.4±1.8	2.1±0.5
Aorta	n.d.	6.7±3.1

n.d. = not determined

Morphological analysis

After application of the 1% 4-OHT PDD to carotid and femoral arteries for 7 days no morphological abnormalities were observed. However, using 3% 4-OHT-loaded PDDs we observed medial thickening as a result of massive increase of medial SMCs and using 10% PDDs this coincided with occasional hemorrhage of the media, as derived from presence of red blood cells in the media (Figure 3 C, G and D, H). Furthermore, the CD31-positive endothelial lining was affected (Figure 3, right insets), as compared to 1% loaded PDDs. In conclusion, local application of PDDs loaded above 1% 4-OHT hampers SM-recombination of both the carotid and femoral vessel wall, as a result of toxic side-effects.

DISCUSSION

In the present study, we describe the means to conditionally modify genes at a predefined and localized region of a blood vessel using a perivascular drug delivery device (PDD). When applied to SM-CreER^{T2}(ki)/rosa26 mice, a dose and time curve of 4-OHT released from the PDD showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application (carotid artery 2.4±1.8%; femoral artery 4.0±3.8% of SMCs). No gene recombination could be detected in vehicle treated SM-CreER^{T2}(ki)/rosa26, 4-OHT treated control rosa26 mice, gastrointestinal SMCs or other regions of the vasculature (0.0±0.0%). Thus, local application of a 4-OHT-eluting PDD results in highly localized SMC-specific Cre-mediated recombination in SM-CreER^{T2}(ki)/rosa26 mice at levels that are in the same order of magnitude to systemic tamoxifen administration, but without affecting additional SMCs.

The efficiency of systemic versus local application of 4-OHT in carotid and femoral arteries is similar at 2-7%. This efficiency could neither be increased by loading more 4-OHT in the PDD (Figure 2), nor by increasing the exposure time of the PDD (data not shown). In contrast, higher 4-OHT dosages in the PDD actually resulted in vascular toxicity (Figure 3). The dose-response curve of locally delivered 4-OHT to the vessel wall and the results of systemic 4-OHT administration seems to justify the notion that the efficiency of SMC recombination in carotid and femoral arteries of SM-CreER^{T2}(ki)/rosa26 mice is maximal at 2-7%.

In our experiments, we observed a more than 10-fold difference in recombination in vascular versus gastrointestinal SMCs. This difference in susceptibility to recombination has also been observed by Feil et al.[3] One explanation for this phe-

nomenon may be that the activity of the SM22 promoter fragment used in the SM-CreER^{T2}(ki) construct is decreased in vascular SMCs versus gastrointestinal SMCs. However, indirect analysis of SM22 promoter activity by measuring Cre mRNA levels using quantitative real-time PCR did reveal relatively high expression levels in both vascular and gastrointestinal SMCs in our mice (data not shown). Alternatively, the difference in recombination efficiency between vascular and gastrointestinal SMCs could be caused by differences in accessibility of the loxP sites for the Cre enzyme.[11] In the present study we did not further address this topic.

The present study indicates that, using the SM-CreER^{T2} mouse model, vascular recombination efficiency does not exceed 8% in femoral and 4% in carotid arteries. These levels are not sufficient to study genes that potentially show a mild phenotype upon activation or deletion. The SM-CreER^{T2}(ki) mice would be more suitable for loss-of-function or gain-of-function experiments of targets, that will have a dramatic impact upon subtle presence or absence. Examples of such targets are specific secretory proteins (cytokines, chemokines, enzymes) and oncogenes. In the case of secretory tissue inhibitor of metalloproteinase-3 (TIMP-3) recent data showed that a 8-10% adenoviral transduction efficiency resulted in potent effects on gelatinolytic activity, apoptosis and vascularization of melanomas.[12] Apoptosis and matrix breakdown are important processes implicated in local vascular diseases such as atherosclerosis and restenosis. In addition, studies aiming at the investigation of the vasculature using systemic TMX treatment could result in lethality as a result of whole body SMC targeting. The induced lethality can be circumvented by local TMX application using the PDD. Thus, the limited recombination levels achieved with the PDDs in the SM-CreER^{T2}(ki) model could still be sufficient when the right target genes are considered.

The application of a 4-OHT-eluting device to locally induce the ER^{T2}-driven Cre-recombinase gene is particularly useful in case the applied tissue-specific promoter does not display a sufficiently narrow expression pattern. In this respect, it is noteworthy to mention that the 4-OHT-eluting polymer PDD, when size adapted and placed at the gastrointestinal tract, can also be used to induce local gene recombination in SMCs of the stomach and intestine (data not shown). Thus, this technology enables physical limitation to the 4-OHT exposed area that can subsequently undergo Cre-mediated recombination.

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