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ORIGINAL ARTICLE

Genetic profiling of aortic allografts: prothymosin alpha as potential target?

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Summary

Transplant arteriosclerosis is the result of intima proliferation in large vessels upon organ transplantation. Obliteration of the vascular lumen will ultimately lead to ischemia and late graft failure. Gene array analysis was performed to identify factors involved in the pathogenesis of transplant arteriosclerosis. Aortic transplants from Dark Agouti to Wistar Furth rats were performed to identify potential target genes. Hierarchical clustering of genes specifically upregulated in allogeneic but not in syngeneic aortas revealed 19 genes. A gene that fulfilled these criteria is prothymosin alpha (PTMA), a regulator of estrogen receptor transcriptional activity. PTMA gene and protein expression levels were confirmed by PCR and immunohistochemistry. Estrogen receptor staining was increased in allogeneic aortas. Furthermore, cyclin D1 a downstream target of PTMA, was also up regulated in allogeneic aortas. In conclusion, PTMA was identified as potential candidate gene involved in transplant arteriosclerosis.

Introduction

Chronic allograft nephropathy (CAN) is the major cause of late renal transplant loss and is characterized by glomerular and/or vascular lesions in a background of interstitial fibrosis and tubular atrophy [1]. Vascular lesions comprise obliteration of the vascular lumen as a result of proliferation of the intima and adventitia together with deposition of extracellular matrix proteins [2]. The decrease in vascular lumen ultimately leads to ischemia and late graft failure.

The pathogenesis of these vascular abnormalities is unknown, although alloimmune factors seem to play a role. Various molecular pathways have been implicated in this process and include growth factors and cytokines. Downstream of vascular injury these signaling pathways result in a final common pathway and accumulation of damage.

Estrogen receptor (ER) signaling has been linked to the development of vascular lesions. In general, estrogens are vascoluprotective [3] and specific targeting of the ERβ both in rats and man has provided vasculoprotection [4]. As estrogen signaling is important in the development of vascular lesions, alterations in the signaling might play a role in the development of vascular lesions upon transplantation.

Experimental models for transplant arteriosclerosis consist of aorta transplantations (Tx) from Dark Agouti

(DA) to major histocompatibility complex (MHC) incompatible Wistar Furth (WF) recipients [5]. Syngeneic transplantations from DA to DA can serve as controls.

In the present paper we used micro-arrays to investigate the gene expression profile in an experimental model of transplant arteriosclerosis. Prothymosin α (PTMA), a modulator of ER transcriptional activity, is identified as one of the genes involved in transplant arteriosclerosis.

Materials and methods

Aorta transplantations were performed using male DA ($RT1^a$) and WF ($RT1^u$) rats (Harlan Winkelmann Gmbh Borchen, Germany; Harlan Nederland, Horst, The Netherlands) as donors and recipients respectively ($n = 6$ for day 7, 14, 30) [5]. Syngeneic DA to DA transplantations were performed as controls. An aortic segment (10–15 mm) was anastomosed end-to-end to the recipient aorta in the orthotopic position below the renal arteries above the aorta bifurcation. Animals received 1 mg/kg body weight of temgesic (buprenorphine-hydrochlorid, Schering-Plough B.V., Amstelveen, The Netherlands) subcutaneously for pain relief. Aortic grafts were removed at 7, 14 and 30 days after Tx. Animal care and experimentation were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Aorta grafts were stored in RNAlater solution (Ambion, Sanbio B.V., Uden, The Netherlands) at −20 °C. Directly before RNA-isolation aorta's were snap-frozen in N_2 (l) and homogenized in RNazol B (Campro, Veenendaal, The Netherlands) using an ultra turrax (IKA Labortechnik, Staufen, Germany). Subsequently RNA was isolated using Trizol (Life Technologies Inc, Rockville, MD, USA) according to instructions of the manufacturer. Prior to ethanol precipitation isolates were treated with DNase (Promega Benelux B.V., Leiden, The Netherlands).

For gene array expression analysis total RNA of two aorta's was pooled and 4 μg RNA was reverse transcribed into cDNA in the presence of ^{32}P-labeled dATP (Amersham, Eindhoven, The Netherlands) using a primer mix supplied with the gene array. Labeled cDNA was hybridized to Atlas cDNA Expression arrays (Clontech, Palo Alto, CA, USA: http://bioinfo.clontech.com/atlasinfo/array-info-action.do?catalog_no=7738-1) containing 588 known rat cDNAs (no ESTs) spotted in duplicate on nitrocellulose filters. Hybridization was performed according to instructions of the manufacturer; filters were exposed to storage phosphor plates (Eastman Kodak Co, Scientific Imaging Systems, New Haven, Connecticut, USA) and quantified using a phosphor imager (Storm, Molecular Dynamics, Sunnyvale, CA, USA). For all time-points three separate hybridizations were performed, all using RNA from two independent aortic transplants (six aortas in total). Local median background correction was performed on all spot intensities, with a minimum value set to 10. The sum of signals on each blot was normalized, and all filters were corrected for GAPDH content. Ratios of average-transplant-data to normal-tissue-data were calculated per time-point in GeneSpring 7.0 (Silicon Genetics, Redwood City, CA, USA). Clustering on genes (normalized to median values) was performed using standard correlation.

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using primers specific for rat prothymosin alpha (PTMA; upstream: 5′-CTTACGCACC GTGACCTAT-3′; downstream: 5′-TCCTCCATGAATCAT GT-3′), GAPDH was used as a control. PCR reactions were performed under standard conditions using 40 cycles.

Paraffin sections of aortic grafts were stained for PTMA (1–30; Prof. F. Dominguez, Santiago de Compostela, Spain), ERβ protein (PAI-310B, Affinity BioReagents Inc., Paris, France) and cyclin D1 (Santa Cruz, Biotechnology Inc., Santa Cruz, CA).

Results

Transplantation of DA aortas in WF recipients results in transplant arteriosclerosis with its characteristic concentric intimal thickening. Syngeneic DA grafts do not show intimal proliferation. Gene expression profiling was performed on allogeneic and syngeneic aortic grafts. Normal aortas expressed 488 of the 588 genes present on the array. Hierarchical clustering was performed, comparing allogeneic and syngeneic aortas on day 7, 14 and 30 after transplantation to normal, nontransplanted aortas (Fig. 1a). A cluster of 19 genes that showed upregulated expression in allogeneic but unchanged expression in syngeneic aortas was selected (Fig. 1b). Prothymosin α (PTMA) was also identified as an potential candidate gene as it is abundantly expressed in normal aortas, increased in allogeneic aortas at all time-points (days 7, 14 and 30) and remains stably expressed in syngeneic aorta grafts (data not shown). PTMA clustered with thyrotropin releasing hormone (TRH), a molecule known as one of the hypothalamic hormones that exerts its functions predominantly within the central nervous system. A direct link between PTMA and TRH has not yet been described. However, TRH results in the release of thyrotropin and the latter can increase PTMA expression in certain cell types [6].

A few other genes also had increased expression in allogeneic aorta grafts, including cathepsin C, interleukin-7 and proton-coupled dipeptide cotransporter (Fig. 1b). However, we decided to look to PTMA in more detail

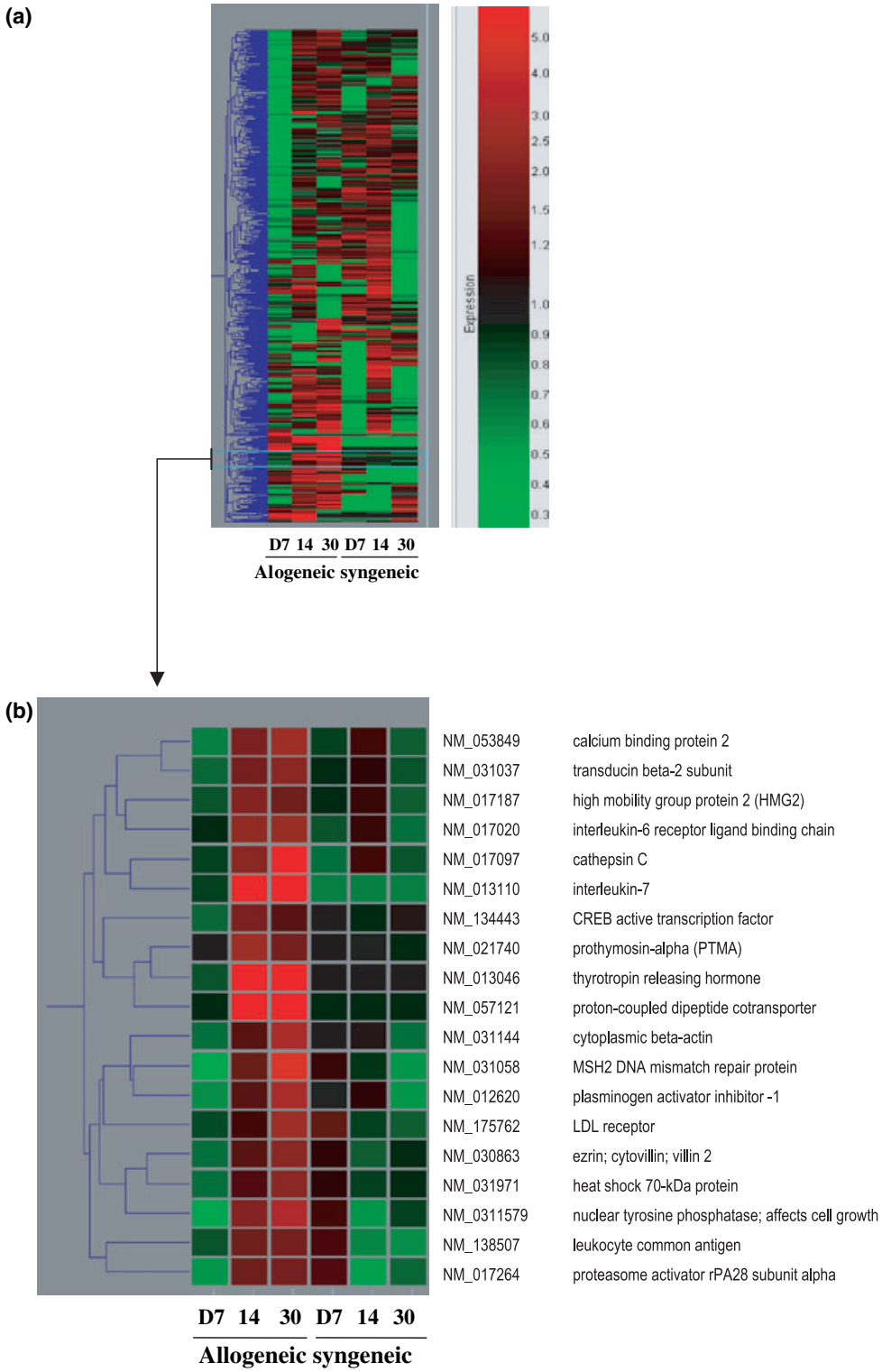

Figure 1 Cluster analysis of gene expression profiles. (a) Dendrogram tree of hierarchical clustering analysis of genes expressed in allogeneic (WF to DA) and syngeneic (DA to DA) transplanted aortas 7, 14 or 30 days after transplantation, relative to normal, nontreated aortas. Fold change of gene expression levels (log_2 scale) are indicated by red (increased) or green (decreased). (b) Cluster of 19 genes that show increased expression in allogeneic transplantations but not in syngeneic transplantations.

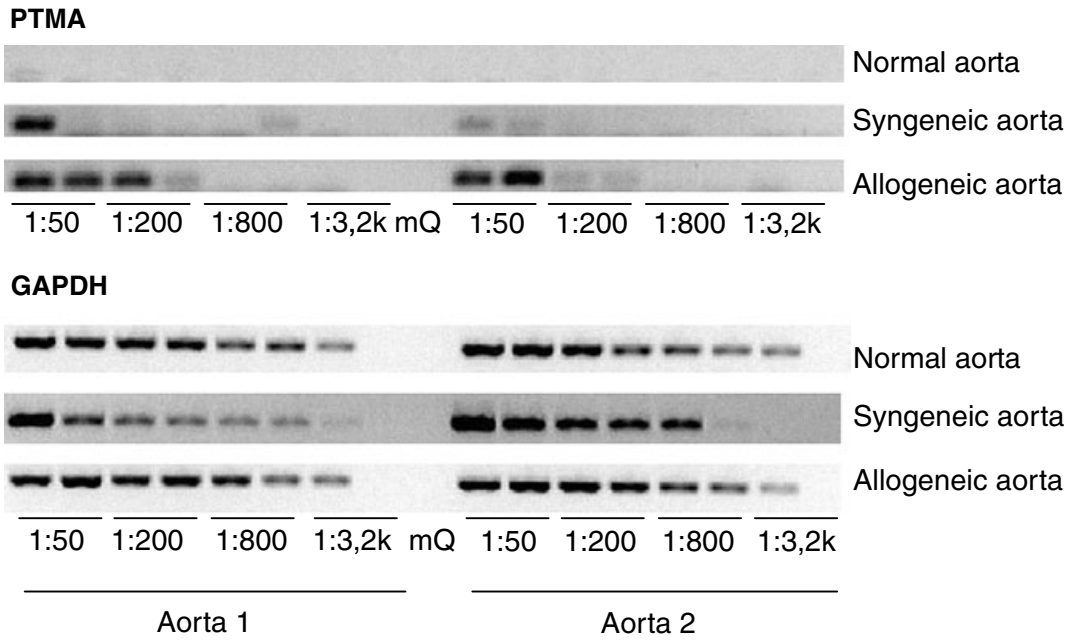

Figure 2 Expression of PTMA in aortic grafts. RT-PCR of normal, allogeneic or syngeneic aortic grafts for PTMA gene expression and GAPDH as house keeping gene on day 14 after Tx. PCR was performed in duplicate on two different aortas for all groups and all dilutions.

because of its interesting link to estrogen signaling and the importance of estrogens in the development of vascular lesions. In addition PTMA was not increased to the maximum of the current analysis and therefore thought to be a rate-limiting step.

The PTMA is almost threefold up regulated in allogeneic grafts compared with syngeneic grafts. The expression of PTMA is increased on day 14 post-Tx and decreases again at day 30. Increased expression levels of PTMA RNA in allogeneic compared with syngeneic aortas were confirmed by RT-PCR (Fig. 2).

The PTMA protein expression was quantified in paraffin-embedded aortas. Normal aortas showed almost no PTMA positive cells (Fig. 3a), whereas allogeneic aortic grafts at day 14 post-Tx showed increased numbers of PTMA positive cells (Fig. 3b). PTMA staining was observed in nuclei in the intima. Staining of ERβ was increased in allogeneic grafts at day 14 compared with normal aortas (Fig. 3c and d). ERα staining was not present in these grafts (data not shown). Cyclin D1 staining was also increased in the intima and media of allogeneic aortas at day 14 post-Tx (Fig. 3e and f).

Discussion

Genetic profiling of vascular changes in an experimental model for transplant arteriosclerosis resulted in the identification of PTMA as potential target. PTMA expression was high in allogeneic aortas on day 14 and 30 post-Tx, but remained stable in syngeneic grafts in time. The expression of PTMA protein in the intima was increased in allogeneic grafts compared with syngeneic or normal aortas. In addition the expression of ERβ was also increased in allografts only.

For genetic profiling of intimal changes using gene-arrays, isolated vascular grafts are preferable to complete organs to allow discrimination of the specific vascular processes from other changes in full-organ allografts. In this rat model, intimal proliferation is similar to the vascular changes observed in kidney allografts.

In the present study PTMA gene and protein expression were specifically up regulated in allogeneic grafts compared with syngeneic or normal aortas. PTMA, a 12.5 kDa nuclear protein, has been described to enhance the transcriptional activity of the ER, resulting in increased expression of downstream target genes such as cyclin D1 [7,8]. In the presence of estrogen, estrogen-ER complexes can induce the expression of PTMA [9] thereby resulting in increased transcriptional activity of the ER [7]. In the aortic allografts increased expression of ERβ is present (Fig. 3c and d), this might be the consequence of vascular endothelial damage [4]. Downstream of ER signaling, cyclin D1 as well as other cell cycle regulators including cyclin D3, p21 and p27 were upregulated in allogeneic aortas according to the gene array data (data not shown). Confirmation of the expression of both the cyclin D1 gene and protein showed an up regulation specifically in the allogeneic grafts. This suggests that

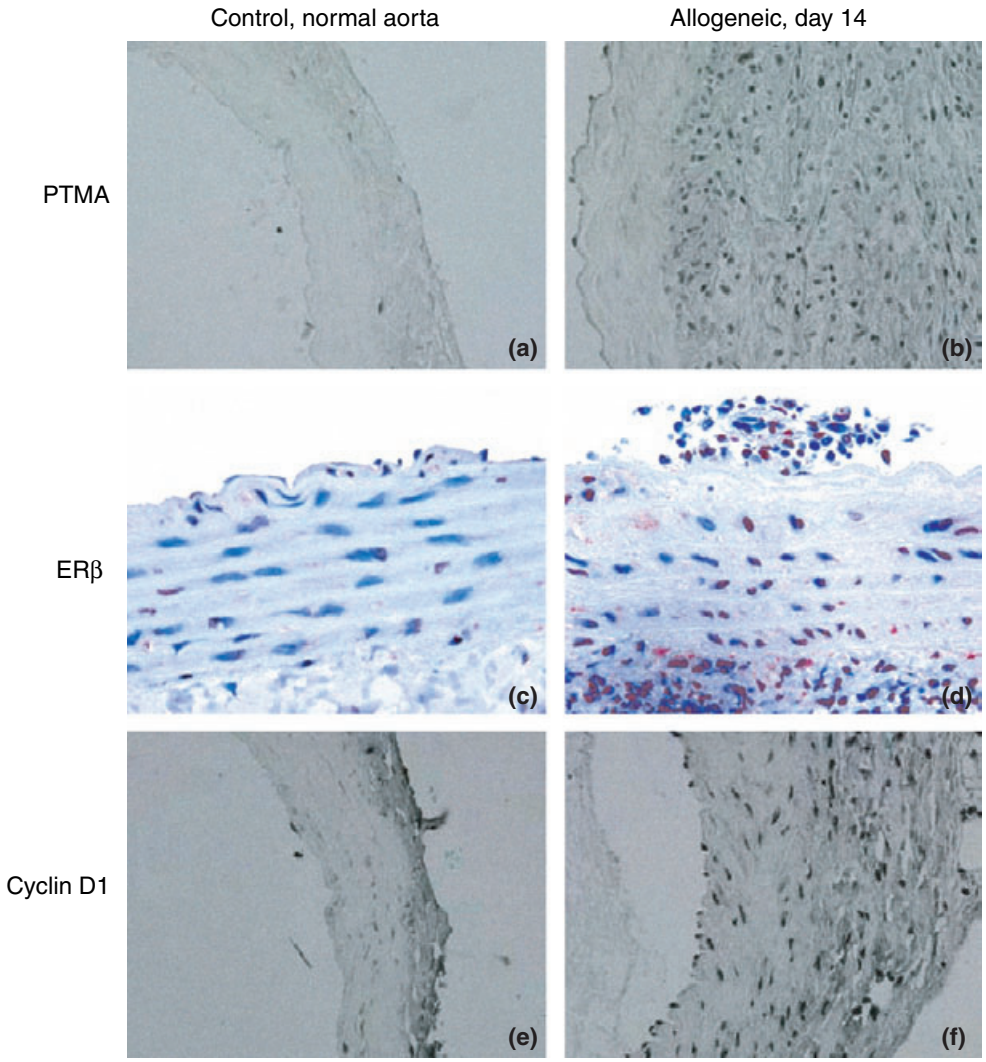

Figure 3 Immunohistochemistry for PTMA, ERβ and cyclin D1 in aortic allografts (original magnification ×400). (a) PTMA staining in control aorta. (b) PTMA staining in allogeneic aortic graft at day 14 post-Tx. (c) ERβ staining in control aorta. (d) ERβ staining in allogeneic aortic graft at day 30. (e) Cyclin D1 staining in control aorta. (f) Cyclin D1 staining in allogeneic aortic graft at day 30.

increased expression of PTMA has consequences for the expression of downstream cyclin D1, and thereby influences intima proliferation.

However, functional studies blocking PTMA gene expression or using PTMA knockout animals will have to be performed to demonstrate a rate-limiting role for PTMA in transplant arteriosclerosis. In general estrogens are vasculoprotective, however in this model the damaging effects, i.e. activation of cellular proliferation, appear superior to the beneficial effects.

The increased expression of PTMA was only found in allogeneic aortic grafts and not in syngeneic aorta grafts. This suggests that immune recognition is involved in induction of PTMA expression. One possible explanation is that the increased expression of PTMA is the result of increased PTMA expression in infiltrating leukocytes. This is further supported by the observation that the majority of mononuclear cells aligning the endothelium in allografts were PTMA positive. To obtain more detailed insights in the role of components of the immune system for expression of PTMA and transplant arteriosclerosis, aortic transplantations will have to be performed using either donor or recipient rats with immunodeficiencies, for example Nude rats, or mouse models.

The PTMA was not the only gene that was upregulated in allogeneic but not syngeneic aorta transplantations. Other potentially interesting genes that can be studied in the future are cathepsin C, interleukin-7 and proton-coupled dipeptide cotransporter. Of special interest is TRH as it clusters closely with PTMA. So far, TRH is only

known the have its effects in the central nervous system, but more specific analysis might reveal a role in maintenance of blood vessel architecture. Indirect evidence suggests a direct link between PTMA and thyrotropin, the hormone released by TRH [6].

In conclusion, we describe a gene array characterization of transplant arteriosclerosis using a rat aorta transplantation model. One of the genes identified in the present paper is PTMA, an enhancer of estrogen signaling, that might result in increased expression of cell cycle regulators and a potential target for future research.

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