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Identification by differential display of eight known genes induced during in vivo intimal hyperplasia

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Abstract To achieve a better understanding of the mechanism of intimal thickening, we used a rabbit model in which aorta was denuded mechanically by a balloon catheter. Total RNA was prepared from each aorta 1, 2, 7, 14, 23, or 30 days after denudation, and from intact aorta of non-denuded control rabbits. Subsequently, using the differential display method, we identified eight genes that were expressed differently during the time course after injury. One of them, *RESP18* (encoding regulated endocrine secretory protein 18), was suppressed during the acute reaction. The other seven showed increases in expression during the acute phase: the genes for hTAFII68 (human TATA-binding protein associated factor), NPAT (nuclear protein mapped to the AT locus), OSF2 (osteoblast-specific factor 2), Pyst1, casein kinase 1α, integrin α1, and XP-C complementing protein. Although hTAFII68, NPAT, OSF2, and Pyst1 are thought to be related to transcription, not all four are positive regulators. Considering that none of these genes had previously been reported as being implicated in intimal hyperplasia, we conclude that many known or unknown genes play roles in this process. We believe that differential display is an effective method for screening genes whose variations in expression can provide clues toward understanding the molecular mechanism of intimal hyperplasia.

Key words Intimal hyperplasia · Differential display method · Endothelial denudation · RT-PCR · Masson-Trichrome staining

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Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a widely used therapeutic procedure for human coronary heart diseases, including angina pectoris and acute myocardial infarction. Re-stenosis after PTCA due to cellular hyperplasia within the neointima, which occurs in about 30%–50% of patients, is a serious problem. Cell–cell interactions, cell–matrix interactions, redox state, ligand–receptor interactions, tyrosine kinases, and transcription factors are all involved in endothelial dysfunction and neointimal formation (Gibbons and Dzau, 1996). However, a limited number of genes have so far been reported in relation to this highly complex phenomenon.

Differential RNA display is a powerful technique for identifying genes that are expressed differently among various tissues or cell lines (Liang and Pardee 1992). This approach is designed to screen a defined subpopulation of transcripts through the reverse transcriptase polymerase chain reaction (RT-PCR), using arbitrarily selected primers, and to display the results as bands on a gel by electrophoretic separation of the amplified cDNAs. Unlike subtractive hybridization techniques for comparing two subjects, differential display allows multiple samples to be compared simultaneously, so one can easily identify fragments corresponding to differentially expressed transcripts. In addition, it provides access to transcripts of low abundance that are difficult to detect without PCR. Our previous study reported that a clone whose transcript represented as little as 0.0002% in a preparation of total mRNA could be identified by means of this procedure (Ozaki et al. 1996).

As a model for better understanding the mechanism of intimal hyperplasia, we denuded rabbit aorta by catheterization and performed differential display to identify genes whose expression patterns changed during the time course following injury. Here, we report eight such genes, which therefore may be assumed to play roles in intimal hyperplasia.

Materials and methods

Endothelial denudation

Aortas of 12 three-month-old male JW rabbits were denuded by Fogarty catheter introduced through the femoral artery. Two of them were killed after 1, 2, 7, 14, 23, or 30 days, respectively, and excised aortas were stored at −80°C until use. Intact rabbit aorta served as a control. Half of each specimen was stained with Masson-Trichrome for confirmation of endothelial denudation and subsequent proliferation.

Differential display method

Total RNA was prepared using TRIZOL (Life Technologies, Houston, TX, USA) according to the supplier's instructions. After digestion of contaminating DNA by RNase-free DNase (Boehringer, Mannheim, Germany) and reverse transcription by SuperscriptII (BRL, Life Technologies), samples were subjected to the differential display procedure (Ozaki et al. 1996). In brief, PCR amplification was performed using 5′-primer (10-mer deoxyoligonucleotide primer with arbitrary sequence) and 3′-anchored $GT_{15}MN$ primer (where M indicates either G, A, or C; and N indicates any deoxynucleotide) under the following conditions: one cycle of 3 min at 95°C, 5 min at 40°C, and 5 min at 72°C, then 40 cycles of 30 s at 95°C, 2 min at 40°C, and 1 min at 72°C, followed by 5 min at 72°C. Samples were applied to a 6% acrylamide/7.5 M urea sequencing gel. Differentially amplified bands were excised from the gel, reamplified under the same conditions, and again applied to a sequencing gel. Reamplified products of the appropriate sizes were recovered and cloned into the pCRII vector (Invitrogen, San Diego, CA, USA). Sequencing of the clones was performed using a dye terminator cycle-sequencing kit (Perkin-Elmer, Foster City, CA, USA) according to the supplier's instructions. Electrophoresis was carried out using a 377 DNA sequencer (Perkin-Elmer).

Reverse transcriptase polymerase chain reaction

For confirmation of differential expression patterns, RT-PCR was carried out with synthetic internal primers of each clone. Expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was examined as an internal control. The protocol for RT-PCR was as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, followed by 72°C for 2 min. Quantitative analyses of the transcripts were carried out using the FluorImager SI (Molecular Dynamics, Sunnyvale, CA, USA).

5′ RACE (Rapid amplification of cDNA ends)

To isolate coding regions of the genes, 5′-RACE was performed using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Atto, CA, USA).

Homology search

Using the FASTA program (Pearson and Lipman 1988), we compared nucleotide and amino acid sequences to the nucleotide sequence database (nonredundant combination of GenBank, EMBL, and DDBJ databases), and the amino acid sequence database (nonredundant combination of Swiss-Prot, PIR, and PRF databases plus translations of DNA sequences in the GenBank database), respectively. Clones with amino acid sequences having greater than 90% identity with known genes of other species were considered to be rabbit homologues.

Results and discussion

Figure 1 shows the results of Masson-Trichrome staining which revealed intimal thickening in aortas after denudation by a balloon catheter. We isolated RNAs from the aorta of each rabbit and then performed differential display using a total of 29 combinations of arbitrary primers and reverse primers. We chose for cloning 67 PCR fragments that presumably corresponded to parts of the cDNAs that were differentially expressed during intimal hyperplasia. Figure 2 shows a representative autoradiogram of a differential display. Nucleotide sequences of five subclones from each PCR fragment (a total of 335 subclones) were deter-

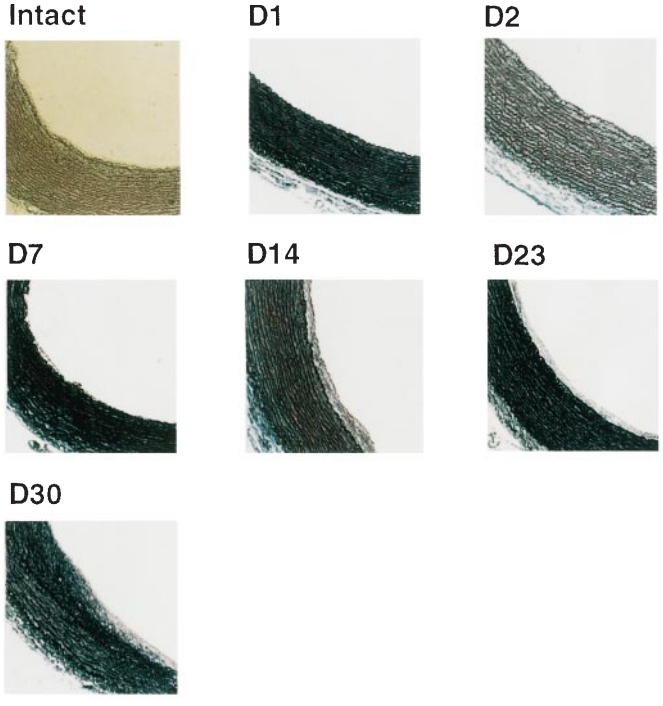

Fig. 1 Intimal hyperplasia after denudation of rabbit aorta, shown by Masson-Trichrome staining. Intact aorta retains endothelial cells on the internal elastic plate; on days *D1* and *D2*, they were scraped off in vivo by mechanical injury. Seven days after injury (*D7*), some endothelial cells had recovered. Thickening of intima occurred over time (*D14, D23,* and *D30*)

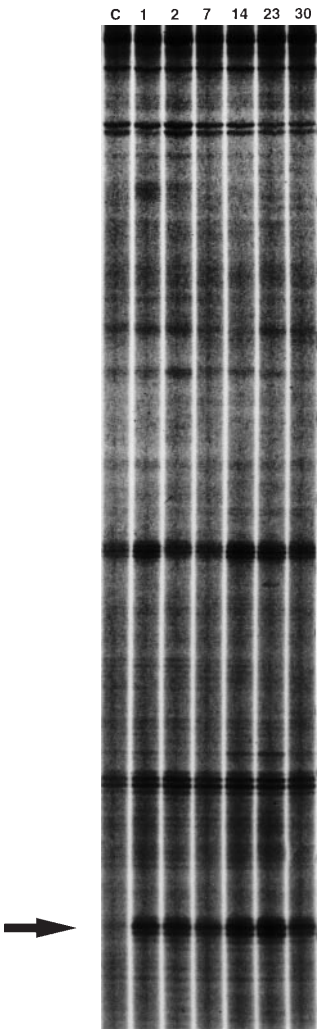

Fig. 2 Representative autoradiogram of a differential display. Primers in this experiment were 5′-GT_{15}MC-3′ and 5′-GATCATAGCC-3′. *Arrow* indicates a differentially expressed cDNA fragment, which turned out to represent the rabbit homologue of OSF2 (see also Fig. 3). *Numerals* indicate days after injury and *C* denotes intact aorta

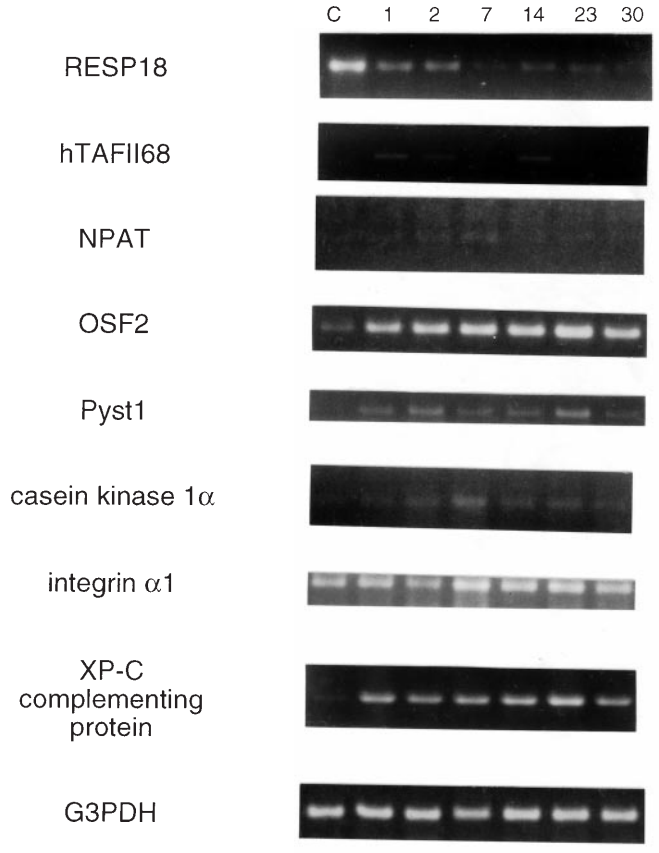

Fig. 3 Expression patterns of eight genes after balloon injury. The expression levels were examined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). *Numerals* indicate days after injury and *C* denotes intact aorta. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) was used as an internal control

mined. They were found to represent 145 different clones, of which 117 contained, at both ends, their respective arbitrary primer and/or reverse primer and included inserts whose sizes were identical to the corresponding original PCR fragments. When these 117 DNA sequences were analyzed by the FASTA computer program, 27 clones turned out to be repetitive sequences, ribosomal RNA, or mitochondrial DNA. The expression patterns of the remaining 90 sequences were examined by RT-PCR. Among the 90 sequences tested, eight were confirmed to show differential expression patterns after endothelial denudation of rabbit aorta. All eight represented known genes (Fig. 3); quantitative analyses by densitometry are summarized in Table 1. Interestingly, most of these genes were not previously known to be expressed in the aorta. Combined with our evidence that some of them were expressed at barely detectable levels in the intact aorta, our results strongly suggest that these genes function specifically in the process of intimal hyperplasia.

The patterns of expression after denudation were divergent, however. Although seven of the eight genes showed increased expression in the acute phase, expression of *RESP18* decreased significantly. RESP18 is a putative endocrine secretory protein regulated by dopaminergic agents

Table 1 Quantitative analysis of differential expression after balloon injury

	C	D1	D2	D7	D14	D23	D30
RESP18	100.0	19.3	17.2	4.4	6.9	6.2	3.9
hTAFII68	4.5	100.0	62.7	5.8	88.5	10.1	6.4
NPAT	10.6	22.3	64.5	100.0	30.8	45.7	9.4
OSF2	6.1	52.8	75.4	81.8	71.8	100.0	56.0
Pyst1	4.7	39.4	82.8	39.8	43.3	100.0	19.7
Casein kinase 1α	7.8	25.6	52.7	100.0	35.8	34.1	20.5
Integrin α1	31.6	41.3	28.6	100.0	80.5	83.9	52.7
XP-C complementing protein	4.4	67.1	45.3	59.1	78.6	100.0	32.9

Points where expression of each gene is highest are set to 100 and relative values are shown.

expressed in the pituitary and in the paraventricular and supraoptic nuclei of the hypothalamus (Bloomquist et al. 1994). It is thought to be a product of cells of somatomammotrope lineage, but its function is not yet clear.

Among the eight genes we found to be related to intimal hyperplasia, *hTAFII68* (Bertolotti et al. 1996), *NPAT* (Imai et al. 1996), *OSF2* (Takeshita et al. 1993), and *Pyst1* (Groom et al. 1996) are thought to be modulators of transcription. hTAFII68 contains a consensus RNA-binding domain which also binds single-stranded DNA; this gene product is associated with TFIID, the main sequence-specific DNA binding component of the RNA polymerase II transcriptional machinery, and it may play specific roles during initiation of transcription at distinct promoters. It may be able to enter the preinitiation complex together with RNA polymerase II. During intimal hyperplasia in our experiments, *hTAFII68* showed a nonlinear expression pattern; on day 7, its expression level seemed to be reduced (Fig. 2). This bipolar expression pattern could reflect genetic variation among the rabbits examined, or differences in how the procedure of denudation was accomplished and/or subsequent proliferative differences from one rabbit to another. However, the genetic backgrounds of our test animals were thought to be homogeneous, and the histological analysis indicated that our denudation procedures were consistent. We think the bipolar expression pattern of the *hTAFII68* gene reflects a complex phenomenon; i.e., the gene product might be necessary not only in the acute phase but also in the progression of intimal hyperplasia.

NPAT (nuclear protein mapped to the AT locus) is a recently discovered gene whose chromosomal location is only 0.5 kb from *ATM* (the gene responsible for ataxia telangiectasia) on chromosome 11q23, but it is transcribed in the opposite direction (Imai et al. 1996). It shares weak homology with yeast *cdc24*, and contains nuclear localization signals as well as target sites for phosphorylation by cyclin-dependent protein kinases associated with E2F. Although its function is not clear yet, the presence of phosphorylation sites suggests that NPAT may be involved in cell-cycle regulation mechanisms.

Osteoblast specific factor 2 (OSF2), a member of the family of PEBP2 alpha/AML-1 transcription factors, binds to OSE2, an osteoblast-specific cis-acting element (Geoffroy et al. 1995). OSF2 is known to be expressed in bone and to a lesser extent in lung (Takeshita et al. 1993); in our experiments, it was also expressed in aorta. Previous information indicated that OSF2 is down-regulated in embryonal-rhabdomyosarcoma cell lines in comparison with primary myoblasts (Genini et al. 1996). Combined with our results, we suggest that OSF2 is not osteoblast-specific, but may positively regulate transcription of genes required for differentiation of normal muscle cells.

Pyst1 encodes a cytosolic dual-specificity phosphatase which exhibits substrate selectivity for MAP kinases (Groom et al. 1996). Groom et al. recently reported that its expression in human fibroblasts was not altered by various stress treatments such as heat shock, hydrogen peroxide, or UVC radiation. As our data indicate that this gene is involved in the response to endothelial denudation, it may trigger a signal transduction pathway other than the ones affected by the aforementioned stresses; alternatively, endothelium may respond differently from fibroblasts. It is worth noting that *Pyst1* may contribute to the negative modulation of transcription, even though it showed increased expression in response to balloon injury. The balance between positive and negative modulators of transcription may be critical even in the proliferative state.

Of the remaining three genes indicated by differential display, one codes for XP-C complementing protein, which is able to complement the xeroderma pigmentosum-C defect in a cell-free repair system containing UV-damaged SV40 minichromosomes; it may be involved in repair of nucleotide excision (Masutani et al. 1994). The second gene product, integrin α1, is a cell-adhesion molecule that serves as a specific cell-surface receptor for extracellular matrices and contributes to the attachment, spreading, proliferation, and differentiation of vascular cells. It has been reported to be absent on endothelial cells from large vessels such as the aorta (Defilippi et al. 1991), information that is contrary to our results. It may be natural for its expression to increase in the proliferative state. The last of the eight genes listed here encodes a product, casein kinase 1α, related to enzymes that phosphorylate acidic substrates. To date, little is known about this protein (Zhai et al. 1992) and its function remains unclear.

On the basis of the work reported here, we believe the differential display method will allow us to identify many genes, known or unknown, that can yield new insights into the mechanism of intimal hyperplasia.

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