Global gene expression analysis of the effects of Vinblastine on endothelial cells, when eluted from a thermo-responsive polymer

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Abstract: In-stent restenosis remains a significant problem associated with bare metal stents. This drawback has prompted research into improving stent design and the development of novel coatings, including drug-eluting stents. A number of drug-eluting stents are currently on the market; however, the success rate of these stents in complex situations has been found to be quite low. Thus, there remains potential for the development of more suitable drug-eluting stents. The aims of this study were to use a thermoresponsive polymer to develop a system to locally deliver vinblastine, an antimitotic agent currently used as an anticancer drug, and in addition, assess the effects of this drug at the gene expression level *in vitro*. An *N*-isopropylacrylamide/*N*-*tert*-butylacrylamide (NiPAAm/NtBAAm) copolymer solution in the ratio 65:35 was prepared and appropriate volumes of vinblastine were added to generate two final drug concentrations of 22 nanomoles/film or 0.022 nanomoles/film. Stainless steel discs (316) were coated with the copolymer solution or this solution containing drug. Human endothelial cells were cultured on collagen type 1 gels and then incubated with the coated discs for 24 h. Gene expression studies using oligonucleotide microarray analysis and quantitative RT-PCR were then performed. Microarray analysis revealed that vinblastine causes the differential expression of a range of genes involved in a variety of different functions, including cell cycle and apoptosis. The changes in expression of some of these genes culminate in cell cycle arrest and apoptotic pathways. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 246–253, 2006

Key words: HUVECs; NiPAAm/NtBAAm copolymer; real-time PCR; microarray analysis; collagen type 1

INTRODUCTION

Undesirable postimplantation reactions, such as thrombogenicity and restenosis, following stenting and the failure of systemic administration of drugs to inhibit these processes have encouraged the development of novel systems to improve the efficacy of the implantation procedure.[1] This research has brought about the development of two types of coatings, biocompatible coatings and drug-eluting stents. A drug-eluting stent is defined as ''a device that presents or releases one or more bioactive agents to tissue at and near implant.''[2]

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Despite the proven advantages of certain drug-eluting stents, this new development has encountered some opposition. It is being questioned whether it is necessary to expose patients who are unlikely to experience restenosis to these bioactive substances, thus delaying the reendothelialization process.[2] In addition, it has been argued that many studies carried out have concentrated on simple lesions and that the success rate may not be as high in more complex situations.[3]

Significant potential still remains for the development of more biologically effective drug-eluting stents. Current systems available are not ideal for diabetic patients and more complex targets such as small diameter vessels.[4] The optimization of the three components of drug-eluting stents (i.e. the stent, the drug carrier, and the bioactive agent) offers great scope in the development of the ''ideal'' drug-eluting stent. There is much potential for the development of novel local delivery vehicles that will release the drug in a more controlled way over a longer period of time, and in addition, the

development of new bioactive agents can lead to further improvements of drug-eluting stents currently available.[5]

Poly(*N*-isopropylacrylamide) (pNiPAAm) is a thermoresponsive polymer, which undergoes phase transition at 32°C. This temperature can be altered to the individual requirements of the intended application by various processes such as crosslinking and the incorporation of ionic monomers.[6] This feature makes pNiPAAm an ideal candidate for the local delivery of bioactive agents to an implantation site. If a drug is integrated into the polymer and used as a stent coating, the conformational changes the polymer undergoes at 37°C facilitate controlled local delivery of the drug.[7] The development of a subclass of these thermoresponsive polymers, the *N*-isopropylacrylamide/*N*-*tert*-butylacrylamide (NiPAAm/NtBAAm) copolymer system, offers even more flexibility, whereby the lower critical solution temperature (LCST) can be lowered by the incorporation of more NtBAAm into the copolymer system.[8]

Vinblastine is a naturally occurring vinca alkaloid derived from the periwinkle plant that has antiproliferative properties due to its ability to arrest mitotic cells in metaphase.[9] Vinblastine prevents the progression of mitosis by preventing spindle formation. The drug destabilizes polymerized tubulin by binding to the site of tubulin dimer attachment, and thus spindle formation is prevented and mitosis cannot progress.[10] Because of its antiproliferative properties, vinblastine is used extensively as an anticancer drug.[11] Few studies have explored the use of vinblastine in drug-eluting stents. Furthermore, the effects of vinblastine on cells at the gene expression level to determine which genes are being affected by the treatment have not been examined.

In this study NiPAAm/NtBAAm at a ratio of 65:35 was used to develop a novel delivery system to administer vinblastine to human endothelial cells *in vitro*. Oligonucleotide microarray-based global gene expression analysis was then carried out to investigate the effect of vinblastine on endothelial cells in culture at the gene expression level, and the feasibility for the use of vinblastine in drug-eluting stents was explored.

MATERIALS AND METHODS

Material preparation

A 5% solution of 65:35 *N*-isopropylacrylamide/*N*-*tert*-butylacrylamide (NiPAAm/NtBAAm) copolymer was prepared in methanol. Appropriate volumes of vinblastine were then added so that the final drug concentration of each film was 22 nanomoles or 0.022 nanomoles. Stainless steel discs (316-Goodfellow Cambridge, England) were then coated with the vinblastine–polymer film or with the polymer only to give a film thickness of 4 μm and were then dried in an ethanol atmosphere overnight. A previous study carried out to measure vinblastine elution from 65:35 NiPAAm/NtBAAm copolymer films found that after 24 h a film loaded with 22 nanomoles and 0.022 nanomoles of vinblastine released 19.25 nanomoles and 0.01925 nanomoles respectively (H. Gallagher, unpublished data). Prior to being placed in contact with the cells, the coated discs were placed in the laminar flow hood to ensure the removal of any traces of ethanol.

Surface analysis

Coupons were analyzed by atomic force microscopy (AFM) and scanning electron microscopy (SEM) to characterize the surfaces. Contact mode AFM was used and a scanning rate of 0.5 Hz was used for scanning sizes of 50 μm, and five scans were taken for each disc. An overall average roughness was then calculated.

Cell culture

Because of concerns about oxygen and nutrient starvation caused by the contact between the coupon and the cells, collagen type 1 gels were used as the tissue culture substrate. Collagen type 1 gels allowed the diffusion of oxygen and nutrients across the gel to be taken up by the cells.

Rat-tail collagen type 1 extracted following a previously published protocol[12] was received as a gift from Dr. Irina Selezneva (Institute of Theoretical and Experimental Biophysics, Puschino, Russia). Collagen type 1 gels (1 mg/mL) were prepared in 6-well plates, at a thickness of 1 mm. The collagen was diluted in supplemented endothelial growth media-2 (Cambrex, UK) and then neutralized by the addition of 2*N* NaOH, using the media as an indicator of pH. The gels were then cast in 6-well plates, ensuring that no air bubbles were present and that the gel was spread evenly around the wells. The gels were then incubated overnight at 37°C to gelate.

Human umbilical vein endothelial cells (HUVECs) at a concentration of 25,000 cells/cm^2 were seeded onto the collagen gels and were left to attach overnight. After 24 h, the coated stainless steel discs (with and without vinblastine) were placed in contact with the cells and incubated for 24 h at 37°C. Cells were subsequently used for cell metabolism assays (alamarBlue) or for gene expression analysis.

Cell metabolism assays

The alamarBlue (Biosource, CA, USA) assay was used as a measure of cell metabolic activity. After 24-h incubations with the discs, cell culture media were removed and wells were washed with Hank's Balanced Salt Solution (Sigma) and then incubated with alamarBlue for 2 h at 37°C, after which 100-μL aliquots of this incubation solu-

tion was placed in a 96-well plate and excited at 530 nm wavelength and read at 590 nm using an FLx-800 Microplate Fluorescence Reader (Biotek Instruments, Vermont, USA). Three independent experiments were carried out and results are presented as treatment *versus* arbitrary fluorescent units.

Gene expression analysis

RNA preparation

After 24 h, the discs were removed and the cells were harvested from the collagen by collagenase treatment. One milliliter of a 2.5 mg/mL solution of collagenase (Sigma) prepared in PBS/DEPC was added to each well and incubated for 10 min at 37°C. After 10 min, the solution from six wells of each treatment were pooled and centrifuged at 1500 rpm for 5 min. The supernatant was then removed and the cell pellets were washed thrice in PBS/DEPC to remove any traces of collagenase. RNA extractions were carried out using the QIAGEN RNeasy minikit with on-column DNase treatment (QIAGEN, Crawley, UK). This RNA was then run on a denaturing formaldehyde gel to assess the integrity of the RNA and quantified using an ND-1000 spectrophotometer (Nanodrop Technologies, USA).

Global gene expression analysis

Global gene expression analysis was carried out on RNA prepared as described in the section *RNA Preparation*. Microarray analysis was carried out by Ocimum Biosolutions (Germany). Amplification was carried out using the Nano Version, ExpressArt mRNA Amplification Kit (AmpTec GmbH, Hamburg, Germany). Single-channel experiments were carried out and therefore all the samples were labeled with Cy3. Each sample was then hybridized to a human 40K OciChip. Each chip was then scanned with a laser at three different photomultiplier gain settings, and images generated were used for analysis. Genowiz software and MAVI software (Ocimum Biosolutions) were then used to generate fold increases or decreases induced by the two different drug concentrations relative to the values generated for the polymer alone. Genes upregulated by more than 1.6 or downregulated by more than 0.66 were considered significant.

Microarray confirmation using quantitative real-time PCR

Ten genes were selected for confirmation of the microarray results (6 differentially expressed by films containing 22 nanomoles/film, 2 differentially expressed by films containing 0.22 nanomoles/film, and 1 by both drug concentrations).

One microgram of each RNA sample was used to make cDNA. The RNA samples were denatured at 70°C for 5 min. Reverse transcription was performed at 42°C for 60 min in a reaction volume of 20 μL containing the following: oligo dT primer, Moloney murine leukemia virus (M-MLV) reverse transcription buffer (50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol) (Promega, UK), PCR grade water (Sigma–Aldrich), deoxyribonucleotide triphosphates (Promega), and M-MLV reverse transcriptase (Promega). Reverse transcriptase was inactivated by heating samples at 65°C for 10 min. Control RNA samples, in which no reverse transcriptase was added, were included to confirm that no genomic DNA contamination was present.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as a house-keeping gene, and primers for the selected genes were designed to published mRNA sequences from GenBank using Primer Express software (Applied Biosystems, Foster City, CA) and sequence specificity was confirmed by performing a BLAST (NCBI) search. Primers were then synthesized by MWG Biotech (Ebersberg, Germany).

DNA quantification standards, containing a known number of cDNA copies of each gene, were prepared by purifying PCR products for each gene using the QIAGEN gel purification kit. These purified products were then quantified using the Nanodrop, and appropriate dilutions were carried out to generate standards containing a certain number of cDNA copies for each gene, that is 1×10^7 cDNA copies, 1×10^5 cDNA copies, 1×10^3 cDNA copies, and 1×10 cDNA copies for each gene.

A 1 in 50 dilution of these cDNA samples was then used in real-time PCR reactions to determine the expression levels of the selected genes. Amplification reactions were carried out in real-time, with 12.5 μL of 1× SYBR Green I PCR Master Mix (Applied Biosystems), 12.5 nanomoles of each primer, and 2.5 μL template in a final volume of 25 μL per well. Amplification reactions were performed in 96-well optical reaction plates on the ABI 7000 (Applied Biosystems). A dissociation curve was generated for each primer set at the end of each run, and PCR products were run on 2% agarose gels (Sigma–Aldrich) to confirm the size of the product and the specificity of the primers.

Statistical analysis of real-time results

Three independent experiments were carried out and results were then analyzed using a one-way ANOVA, followed by Scheffe's test using the statistical package SPSS for Windows version 12.0.1 (SPSS, Chicago, IL). A p value of <0.05 for the ANOVA was considered to be statistically significant.

RESULTS

Surface characterisation

The surface characteristics of the coated stainless steel discs were determined by SEM and AFM as illustrated in Figure 1. There was no statistical significance in the roughness difference between all of the coating types (data not shown).

Cell metabolism assay

Cell metabolic activity was found to be significantly lower in cells incubated with the coupons coated with

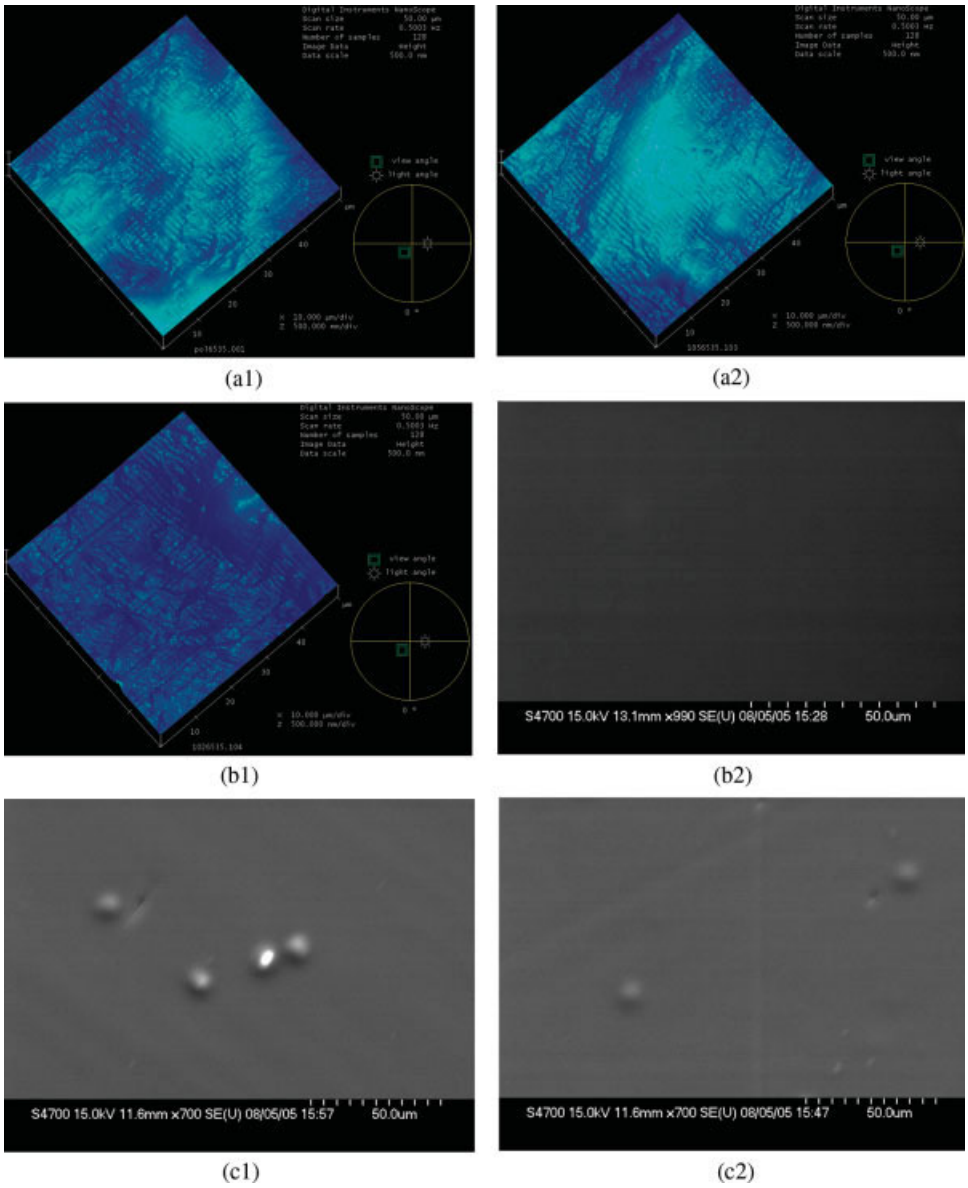

Figure 1. AFM and SEM images: (a) Disc coated with polymer only. (a1) AFM surface plot (a2) SEM image. (b) Disc coated with polymer containing 0.022 nanomoles/film (b1) AFM surface plot. (b2) SEM image. (c) Disc coated with polymer containing 22 nanomoles/film. (c1) AFM surface plot. (c2) SEM image. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

polymer films containing the higher concentration of drug than in cells incubated with the polymer only or in cells incubated with the films containing the lower drug concentration, as illustrated in Figure 2.

Global gene expression analysis

Microarray results

Differentially expressed genes were categorized according to function. Genes fell into seven broad categories: apoptosis, cell signaling, cytoskeleton, heat shock proteins, protein destruction, transcription factors, and miscellaneous. Genes and functions are summarized in Tables I–IV.

Microarray confirmation using quantitative real-time PCR

The results of the real-time experiments and the correlation of these results with the microarray data are summarized in Table V. In addition, calcyclin-binding protein was found to be significantly down-regulated by both drug concentrations, according to the real-time data.

DISCUSSION

The results of this study confirm that vinblastine can be successfully eluted from 65:35 NiPAAm/NtBAAm copolymer films coated on 316 stainless

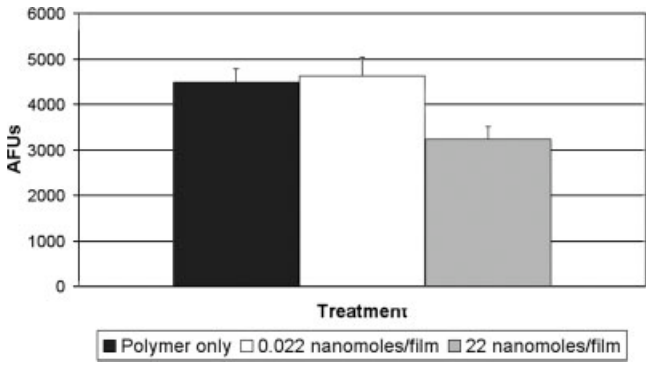

Figure 2. Alamar Blue Results: *indicates significance compared to control cells and + indicates significance compared to other drug concentration.

steel discs *in vitro*. The amount of vinblastine eluted is estimated to be a maximum of 19.25 nanomoles and 0.01925 nanomoles from each film concentration respectively after 24 h. The higher and lower vinblastine concentrations had a wide range of effects at the gene expression level on HUVECs after 24 h in culture. Considering the nature of this study, differentially expressed genes that fall into the apoptosis, cytoskeleton, cell cycle, and protein destruction categories are of particular interest.

The upregulation of proapoptotic genes and the downregulation of antiapoptotic markers are of particular significance. Proapoptotic markers galectins 1 and 7 and macrophage inhibitory cytokine (MIC-1) were found to be upregulated, and antiapoptotic genes such as tumor rejection antigen were found to be downregulated by the higher drug concentration. In a previous study, high doses of galectin 1 were found to have antiproliferative effects.[13] Another study found that treatment of cells with galectin 1 induced cell cycle arrest in G2.[14] Apoptosis was found to be induced when galectin 1 was added to activated T cells.[15] Galectin 7 has a similar function, with overexpression of the protein being found to cause apoptosis in keratinocytes.[16] MIC-1 is a member of the transforming growth factor β superfamily and has been found to have antitumorigenic and proapoptotic roles in human colorectal cells.[17] A downregulation of tumor rejection antigen (TRA-1) resulted from treatment by both drug concentra-

TABLE I
Genes Upregulated by Higher Drug Concentration

Accession No.	Gene	Function
L07769_1	Galectin 7	Apoptosis
BC001693	Galectin 1	Apoptosis
BC000529_1	Prostate differentiation factor/MIC-1	Apoptotis
AF065214	Cytosolic phospholipase a2 γ	Cell signaling
AF038955	G protein-γ5	Cell signaling
BC023533	Similar to ck2 interacting protein 1	Cytoskeleton
AJ271448	Protein phosphatase 4 regulatory subunit 2	Cytoskeleton
BC000573	hspc163 protein	Heat shock
AL512883	Elastase 2b	Miscellaneous
AF348700	Ubiquitin a-52 residue ribosomal protein fusion product 1	Protein destruction

TABLE II
Genes Down-Regulated by Higher Drug Concentration

Accession No.	Gene	Function
NM_003299	Tumor rejection antigen 1 (gp96)	Apoptosis
AF188611	bip protein; hspa5	Apoptosis
BC017069	HMG-box transcription factor 1	Cell cycle
AJ404873	NOA 36 protein	Cell cycle
AK000579	Bromodomain and WD repeat	Cell cycle
XM_037101	Nucleolar phosphoprotein b23	Cell cycle
AK001972	IMAP family member 4	Cell signaling
AF443871	ras-like gtp-binding protein	Cell signaling
L34657	pecam-1	Cytoskeleton
NM_033666	Integrin β1 isoform 1β	Cytoskeleton
NM_0022111	Integrin β1 isoform 1a precursor	Cytoskeleton
AJ320525	Fibronectin (fn)	Cytoskeleton
AK000889	Hspc055	Heat shock
BC034721	Hsp 40	Heat shock protein
BC028607	Kiaa1253 protein	Miscellaneous
AF032922	Syntaxin 4 binding protein unc-18c	Miscellaneous
AF054284	Spliceosomal protein sap 155	Miscellaneous
AF130062	Clathrin	Miscellaneous
AF160212	Vamp-associated 33-kDa protein	Miscellaneous
NM_134264	Socs box-containing wd protein swip-1 isoform 3; wsb1	Miscellaneous
AF072864	Peroxisomal membrane protein 24	Miscellaneous
XM_001732	Similar to calcyclin binding protein	Protein destruction
AF100762	Thyroid receptor trip15	Transcription factor
BC012043	Similar to steroid dehydrogenase	Transcription factor
D30655	Eukaryotic initiation factor 4aii;	Transcription factor
AF100620	Mortality factor 4 like 2	Transcription factor
AB037795	kiaa1374	Transcription factor
BC016295	Eukaryotic translation initiation factor 4A	Transcription factor
NM_016127	Transmembrane protein 66	Transcription factor

TABLE III
Genes Up-Regulated by Lower Drug Concentration

Accession No.	Gene	Function
NM_004636	Semaphorin	Apoptosis
BC007609	E2F transcription factor 4	Cell cycle
BC005098	E2F transcription factor 1	Cell cycle
NM_004423	Dishevelled 3	Cell signaling
AK024908	Ankyrin repeat and SOCS box-containing 8	Cell signaling
AF073710	Regulator of g-protein signaling 9	Cell signaling
AF521018	Socius protein isoform 2	Cytoskeleton
BC000573	hspc163 protein	Heat shock
BC000095	*n*-acetylgalactosaminidase	Miscellaneous
XP_173045	Hypothetical protein	Miscellaneous
L06175	p51	Miscellaneous
AF151351	Pyrroline 5-carboxylate reductase isoform	Miscellaneous
AK027315	Cyclophilin 10	Miscellaneous
AK024687	Dedicator of cytokinesis 5	Miscellaneous
BC029836	Similar to proteosome inhibitor subunit 1	Protein destruction
BC026027	Transcription factor binding to ighm enhancer 3	Transcription

tions. TRA-1 has an antiapoptotic role in cells, by preventing cell death through the maintenance of intracellular calcium concentrations in the cytosol, the endoplasmic reticulum, and the mitochondria.[18] Thus, the microarray data indicate that vinblastine causes the induction of proapoptotic pathways in endothelial cells. Confirmation studies found that galectin 1 was upregulated and TRA-1 was downregulated by the higher drug concentration, but not at a significant level. MIC-1 was found to be differentially expressed by both techniques, thus indicating that the cells are in an apoptotic state.

Calcyclin binding protein (CacyBP) is part of a ubiquitin-ligase complex concerned with the targeting of β-catenin for destruction by the proteasome via ubiquitination.[19] In this study, higher and lower concentrations of vinblastine resulted in a significant downregulation of CacyBP. It is possible that the reduction of CacyBP leads to increased β-catenin concentrations within the cell because of a reduction in proteasome-mediated degradation. A role for β-catenin in cell cycle control and apoptosis has been proposed, and one study found that decreased β-catenin destruction leads to a G2 cell cycle arrest and apoptosis in normal and transformed keratinocytes.[20] Thus CacyBP could contribute to the cell cycle arrest brought about by vinblastine and could contribute to the apoptotic response.

Microtubule interfering agents such as vinblastine are commonly used in the treatment of various cancers.[21] As a result many previous investigations have focused on this application of vinblastine and there are few reports assessing its potential as an antirestenotic agent. Previous studies have investigated the signal transduction pathways triggered by the microtubular disruption generated by vinblastine treatment of cancer cells and a role for the c-Jun NH_2-terminal protein kinase (JNK) signaling pathway has been elucidated.[21] The JNK is a subgroup of the mitogen activated protein kinases, which is activated as a result of various environmental stresses.[21] This study demonstrates that apoptosis signal-regulating kinase (ASK-1), RAS, and JNK are involved in postvinblastine treatment signal transduction pathways, and it was demonstrated that RAS and ASK-1 are required for the activation of JNK after treatment with vinblastine. JNK can then mediate the appropriate stress response through the activation of various transcription factors. It has been put forward that JNK may mediate apoptosis via both nontranscriptional and transcriptional methods.[22,23] One study revealed that JNK phosphorylates Bcl-2 in cancer cells treated with vinblastine, thus hampering its antiapoptotic properties, leading to cell death.[23] In this study JNK was found to phosphorylate the nuclear proteins c-Jun and AFT-2, reinforcing the possible role of JNK in mediating apoptosis by two measures. The transcription factor activator protein 1 (AP-1) is a major target of JNK.[24] The results of this study suggest that activation of the JNK signaling pathway causes an upregulation of TNF-α, Bak, and insulin-like growth factor binding protein 4 (IGFBP-4) and the downregulation of p21, which in turn may mediate the apoptotic pathway in response to microtubule disruption.[22] This study also suggests that these differentially expressed genes are targets of vinblastine-inducible AP-1. A further study found that vinblastine activated c-Jun/AP-1, whereas two other anticancer drugs (doxorubicin and etoposide) did not.[25] The results of this current study imply

TABLE IV
Genes Downregulated by Lower Drug Concentration

Accession No.	Gene	Function
NM_003299	Tumor rejection antigen	Apoptosis
BC007653	SH3KBP1 binding protein 1	Apoptosis
AB008109	rgs5	Cell signaling
AF161401	hspc283	Heat shock protein
AF072864	Peroxisomal membrane protein 24	Miscellaneous
NM_012295	Calcineurin binding protein	Signal transduction
U24578	Rp1	Transcription
BC002411	Similar to y box protein 1	Transcription factor

TABLE V
Summary of Real-Time Results

Microarray Data	Real-Time Data	
Differentially Expressed	Differentially Expressed	Statistical Significance
Calcyclin binding protein	Yes	Yes
CKIP-1	Yes	Yes
MIC-1	Yes	Yes
NOA36	Yes	Yes
Y box protein 1	Yes	Yes
Galectin 1	Yes	No
TRA 1	Yes	No
Integrin β1	No	
Socius protein isoform 2	No	
Ubiquitin	No	

that both concentrations of vinblastine induced apoptosis in HUVECs after 24 h, while none of the genes identified in the study discussed earlier (TNF-α, Bak, and IGFBP-4) were differentially expressed. However, the findings of these previous studies may give an insight into the possible mechanism of action in this study. It is possible that apoptosis was induced via the JNK signaling pathway and that the apoptotic mediators were differentially expressed via transcriptional regulation activated by JNK. The genes identified in this study may be putative vinblastine-inducible AP-1 target genes or alternatively may be downstream effectors of this signaling cascade.

According to the microarray data, vinblastine causes the differential expression of a number of genes associated with progression through the cell cycle. Since vinblastine is known to cause cell cycle arrest, these results are to be expected. Nucleolar autoantigen 36 (NOA 36) is a protein of unclear function that has putative roles in cell cycle control.[26] This protein has been found to be associated with centromeres during mitosis and at the midbody during cytokinesis. Although the exact mechanism of cytokinesis is yet to be elucidated, it is proposed that actin filaments, in association with a range of associated proteins, including NOA36, mediate the final cell division.[26,27] The findings of this current study may confirm the role of NOA36 in cell cycle progression. It is a possibility that spindle disassembly arose as a consequence of vinblastine treatment, and a downregulation of NOA36 resulted because these cells were not ready for cytokinesis.

CONCLUSIONS

Vinblastine causes the differential expression of a range of genes involved in a variety of different functions. These effects culminate in cell cycle arrest and apoptotic pathways. Thus, it must be considered whether this inhibition of endothelial cell growth, which is an inevitable result of treatment with an antiproliferative drug such as vinblastine, has sufficient benefits in treating restenotic pathways. It may be more appropriate to utilize this copolymer system for the release of a bioactive agent that would encourage EC growth rather than inhibit smooth muscle cell (SMC) proliferation.

The supposed role of NOA 36 in cell cycle regulation has been reinforced by the gene expression studies carried out. In addition, possible target genes of the AP-1 transcription factor complex or downstream effectors of the JNK signaling cascade may have been identified. This study also highlights that, although microarrays are a fast and efficient means of identifying genes involved in particular processes, there is still a need for confirmation experiments to be carried out to authenticate the data generated.

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