Understanding endothelial cell apoptosis: what can the transcriptome, glycome and proteome reveal?

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Endothelial cell (EC) apoptosis may play an important role in blood vessel development, homeostasis and remodelling. In support of this concept, EC apoptosis has been detected within remodelling vessels in vivo, and inactivation of EC apoptosis regulators has caused dramatic vascular phenotypes. EC apoptosis has also been associated with cardiovascular pathologies. Therefore, understanding the regulation of EC apoptosis, with the goal of intervening in this process, has become a current research focus. The protein-based signalling and cleavage cascades that regulate EC apoptosis are well known. However, the possibility that programmed transcriptome and glycome changes contribute to EC apoptosis has only recently been explored. Traditional bioinformatic techniques have allowed simultaneous study of thousands of molecular signals during the process of EC apoptosis. However, to progress further, we now need to understand the complex cause and effect relationships among these signals. In this article, we will first review current knowledge about the function and regulation of EC apoptosis including the roles of the proteome, transcriptome and glycome. Then, we assess the potential for further bioinformatic analysis to advance our understanding of EC apoptosis, including the limitations of current technologies and the potential of emerging technologies such as gene regulatory networks.

Keywords: endothelial cell; apoptosis; transcriptome; gene network

1. INTRODUCTION

Apoptosis is the programmed suicide of a cell (Wyllie & Arends 2003). It serves to remove superfluous cells during development and tissue remodelling (Meier et al. 2000); maintain homeostasis in adult tissues; remove self-reactive or non-reactive immune cells (Adams et al. 1999) and delete damaged, infected or transformed cells. It is an energy-requiring process, throughout which membrane integrity is maintained to avoid the initiation of an inflammatory response. This contrasts with necrosis in which cells die relatively passively following overwhelming damage, leading to loss of membrane integrity and to inflammation (Kerr et al. 1972).

Like other organs, blood vessels and the heart appear to employ apoptosis for remodelling during development and to maintain homeostasis during adulthood. Within blood vessels, vascular endothelial cells (ECs) play an especially important role in regulating overall vessel structure. Most ECs in adult blood vessels are relatively quiescent and resistant to apoptosis. However, they are thought to retain the latent capacity for proliferation and apoptosis to mediate angiogenesis and regression, respectively. In this review, we will discuss what is known about the function of EC apoptosis in health and cardiovascular disease. We will describe how this process appears to be controlled at the level of proteins (the proteome). Then, we will discuss the role that regulated RNA transcript abundance profiles (the transcriptome) and cell surface polysaccharide profiles (the glycome) may play in the EC apoptotic programme. To progress our knowledge of the regulation of EC apoptosis further, we now need to understand the cause and effect
relationships between the molecular signals that control this process. In §10 of this review, we will assess whether a deeper analysis of the EC transcriptome using emerging technologies such as gene regulatory network analysis may help us understand the complex signals that control EC fate. Our conclusion is that these emerging technologies have great potential for EC research, especially when their results are integrated with those of cell biology studies, proteomics and in silico databases. However, it is critical that the cause and effect relationships predicted using technologies such as gene regulatory networks are thoroughly validated by laboratory experiments.

2. THE FUNCTION OF EC APOPTOSIS IN NORMAL PHYSIOLOGY

(a) EC apoptosis may contribute to blood vessel remodelling

Several lines of evidence support the view that EC apoptosis plays an important role in vascular biology and pathology. Firstly, apoptosis can be detected during developmental vessel regression in the aortic arches (Fisher et al. 2000), the abdominal aorta (Cho et al. 1995) and the ductus arteriosus (Kim et al. 2000). EC apoptosis may play an especially important role in the eye, in development of the tunica vasculosa lentis of the ocular lens (Mitchell et al. 1998) and the pupillary membrane of the anterior chamber of the eye (Lang et al. 1994). Macrophages may promote this EC apoptosis (Lang et al. 1994), possibly through Wnt signalling (Lobov et al. 2005).

Apoptosis in these regressing vessels is thought to promote their remodelling to meet the changing requirements of the tissues they supply (Carmeliet 2000). Vessel regression may involve a classical series of events. For example, in one scenario following the inhibition of VEGF-A signalling, blood flow ceases, followed by EC apoptosis and loss of pericytes, leaving empty basement membrane sheaves that can act as scaffolds for future capillary re-growth (Baffert et al. 2006).

EC apoptosis appears to be less frequent in adult animals than during development. For example, EC apoptosis appears to be much less common in adult rat kidney than in the developing rat kidney (Fierlbeck et al. 2003). Nevertheless, adult ECs retain a latent capacity for apoptosis, and EC apoptosis is detected within specific adult tissues where substantial vessel remodelling occurs, such as healing wounds (Desmouliere et al. 1995; Greenhalgh 1998), regressing alveoli in the mammary gland after weaning (Matsumoto et al. 1992; Djonov et al. 2001), the developing placenta (Tertemiz et al. 2005) and during the cyclical remodelling of female reproductive tissues (Modlich et al. 1996; Niswender et al. 2000; Dickson et al. 2001; Gaytan et al. 2002). Further support for a role of EC apoptosis in vessel remodelling comes from in vitro studies. For example, incubation with pro-survival growth factors (Satake et al. 1998) or retroviral-mediated anti-apoptotic Bcl-2 expression (Pollman et al. 1999) reduces the incidence of EC apoptosis and inhibits the regression of vessel-like structures.

It is important to note that two studies have been unable to detect significant EC apoptosis within regressing tissues where blood vessel remodelling would be expected to occur (Hughes & Chang-Ling 2000; Arfuso & Meyer 2003). In addition, apoptosis was not observed in one in vitro study where vessels were disrupted by angiogenic inhibitors (Fris et al. 2006). However, the majority of studies suggest that EC apoptosis is frequently seen in remodelling vessels, implying that EC apoptosis plays a role in the remodelling process.

(b) EC apoptosis may contribute to blood vessel morphogenesis and lumen formation

In addition to participating in vessel regression, EC apoptosis may also contribute to angiogenesis and lumen formation. Several EC types spontaneously form vessel-like structures in vitro (vascular morphogenesis) when cultured on an extracellular matrix or with matrix-producing cells. Incubation of EC with anti-apoptotic caspase inhibitors or overexpression of the anti-apoptotic protein Bcl-2 appears to inhibit vascular morphogenesis in vitro (Segura et al. 2002 and M. Harris & C. Print 2003, unpublished results). It initially seems counterintuitive that EC apoptosis may contribute to vascular morphogenesis. However, it is possible that EC apoptosis contributes to this process.
by deleting wrongly placed EC as nascent vessels enlarge. Furthermore, the negatively charged membrane surface of apoptotic EC may promote angiogenic sprouting of adjacent vessels by causing localized plasma membrane hyperpolarization (Weihua et al. 2005). Angiogenesis may also be promoted through the engulfment of apoptotic EC debris by viable neighbouring EC, which may lead to the release of pro-angiogenic factors including VEGF-A (Golpon et al. 2004). However, since in vitro angiogenesis models only approximately recapitulate in vivo angiogenesis, these in vitro experiments should be interpreted with caution until they are confirmed by in vivo studies.

As well as supporting vessel regression and possibly promoting angiogenesis, EC apoptosis may in addition play a role in lumen formation. In support of this hypothesis, ECs undergoing apoptosis are seen in the centre of developing vascular structures (Duval et al. 2003), and newly formed lumen appear to contain post-apoptotic endothelial fragments (Bishop et al. 1999). Blocking apoptosis in the capillaries of the developing rat kidney by inhibiting the pro-apoptotic factor TGF-β1 appears to retard lumen formation (Fierlbeck et al. 2003). In addition, apoptotic EC can be detected during lumen formation in placental vessels in vivo (Tertemiz et al. 2005).

(c) Mice in which regulators of EC apoptosis are disrupted have vascular phenotypes

Additional evidence that EC apoptosis plays a role in vascular biology is provided by the inactivation of genes encoding regulators of EC apoptosis in mice. For example, the growth factors vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1 (Ang-1) are important inhibitors of EC apoptosis. Inactivation of Ang-1 (Suri et al. 1996) causes severe vascular abnormalities early in development, and inactivation of VEGF-A (using either a genetic-based or protein inhibitor-based method) causes an increase in EC apoptosis and embryonic mortality (Gerber et al. 1999). An inherited mutation in the Tie2 receptor causes vascular malformations by inhibiting EC apoptosis through constitutive activation of p52 ShcA (Morris et al. 2006). Vascular endothelial (VE) cadherin is an EC–EC adhesion molecule that transduces anti-apoptotic signals into EC in conjunction with VEGF-A. Inactivation of VE-cadherin (Carmeliet et al. 1999) causes embryonic lethality due to failed vascular development. Disruption of gap junction formation by inactivation of connexin 43 alters coronary vessel development in association with altered apoptosis (Walker et al. 2005). In mice in which the anti-apoptotic gene Bcl-2 has been inactivated in the germ line retinal vascular development is retarded (Wang et al. 2005). Although these phenotypes imply an important role for EC apoptosis, since these signals also regulate EC proliferation, it is difficult to assess the proportion of each mutant phenotype that results specifically from the loss of EC apoptosis control.

Therefore, more specific studies to assess the role of EC apoptosis are underway in our laboratory and others. In these ongoing studies, EC apoptosis is being inhibited directly by the EC-specific expression of anti-apoptotic transgenes. We hope that the results of these studies will reveal the definitive functions of EC apoptosis in vivo.

### 3. EC APOPTOSIS IN CARDIOVASCULAR DISEASE

Altered EC apoptosis is associated with several cardiovascular pathologies, in particular with atherosclerosis and thrombosis. EC apoptosis can be detected in atherosclerotic plaques (Mehta et al. 2002; Norata et al. 2002) and may provide an important step in the transition from a stable atherosclerotic plaque to a plaque with overlying thrombosis. The tissue environment of an atherosclerotic plaque may promote EC apoptosis. For example, oxidized LDL (ox-LDL), which is present in most atherosclerotic plaques, appears to downregulate EC expression of the anti-apoptotic protein cFLIP and subsequently activate Fas-mediated EC death in vitro (Sata & Walsh 1998). Ox-LDL also appears to activate the release of
apoptosis-inducing factor (AIF) from coronary artery EC mitochondria. Suppression of AIF expression using antisense oligonucleotides suppresses ox-LDL-induced EC death (Zhang et al. 2004). In contrast, high-density lipoprotein (HDL) cholesterol, which is anti-atherogenic, appears to inhibit EC apoptosis in part by promoting the activity of nitric oxide (NO) synthase (Mineo et al. 2006). Oxidative stress has also been implicated in activating the apoptosis of EC in atherosclerosis (Valgimigli et al. 2003). The apoptosis of EC overlying atherosclerotic plaques may cause exposure of the underlying pro-thrombotic plaque tissues (Bombeli et al. 2002; Hall & Jevnikar 2003). This chain of events can be broken by infusion of anti-apoptotic caspase inhibitors (Durand et al. 2004).

In addition, apoptotic EC are thought to become pro-coagulant by a process that does not require the exposure of underlying plaque tissues (Bombeli et al. 1997). The coagulation cascade initiator tissue factor (TF, thromboplastin) may provide an especially important link between EC apoptosis and thrombosis. TF activity is increased by phosphatidyserine (PS) exposure on the surface of EC during apoptosis (Tedgui & Mallat 2003). Women with systemic lupus erythematosus (SLE) are at increased risk of atherosclerosis and superimposed thrombosis. This may be due in part to an increased incidence of EC apoptosis and increased TF activity, since elevated numbers of PS-expressing post-apoptotic EC are detected in the blood of SLE patients.

In atherosclerotic plaques, vascular smooth muscle cells (VSMC) become activated, migrate into the tunica intima, survive and proliferate. This proliferation may be promoted in part by factors derived from apoptotic EC overlying the atherosclerotic plaque. Raymond et al. (2004) suggest that proteolytic activity initiated by caspases from apoptotic EC causes degradation of proteoglycans, which release unidentified factors that activate VSMC ERK phosphorylation and Bcl-\(x_L\) expression. Other cardiovascular pathologies with which EC apoptosis has been associated include vasculitis (Woywodt et al. 2003) and transplant rejection (Krupnick et al. 2002; Hall & Jevnikar 2003). EC apoptosis is also associated with inflammatory disorders (Winn & Harlan 2005) and non-cardiovascular pathologies such as neoplasia, reviewed by Carmeliet & Jain (2000), which are beyond the scope of this review.

4. THE CONTROL OF EC APOPTOSIS BY PROTEIN-BASED SIGNALLING AND CLEAVAGE CASCADES

Whether an EC lives or whether it dies by apoptosis is determined by the interplay of numerous signals. It is the integration of all these signals, rather than any
individual signal, that ultimately determines an EC’s fate. The signals that regulate EC apoptosis are closely integrated with the signals that regulate other activities such as cell division, stress responses, activation and migration (Blagosklonny 2003). The most widely studied mechanisms that regulate EC apoptosis are in the realm of proteins (the proteome). These include regulated protein translation, phosphorylation, re-localization, cleavage and oligomerization. We have reviewed these mechanisms in detail previously (Duval et al. 2003), and they can be broadly divided into those that regulate (i) the mitochondrial and (ii) the death receptor apoptosis pathways. When triggered, these two pathways converge to activate the ‘executioner’ caspases 3, 6 and 7 and nuclease enzymes that, along with other enzymes such as cathepsins (Madge et al. 2003) and calpains (Porn-Ares et al. 2003), cleave structural proteins, metabolic enzymes and nucleic acids. This results in a series of biochemical and morphological changes including (in approximate order of appearance) loss of cell–cell and cell–matrix contact, cytoplasmic retraction, re-localization of cytochrome c from mitochondria to cytoplasm, activation of caspases, phosphatidyl serine exposure on the plasma membrane, membrane blebbing and chromatin condensation (Granville et al. 1999). This process can be seen in the time-lapse sequence in figure 1 (Johnson et al. 2004). The end result is post-apoptotic EC corpses that retain their membrane integrity and are either dispersed in the bloodstream or occasionally phagocytosed by neighbouring cells. Interestingly, components of EC corpses that are dispersed in the bloodstream may be taken up by endothelial progenitors and promote their proliferation and differentiation (Hristov et al. 2004).

The mitochondrial apoptosis pathway is initiated by intracellular damage or by the loss of extracellular survival signals, for example, by growth factor deprivation when vessels become occluded during the process of regression (Lang & Bishop 1993; Meeson et al. 1999). This activates caspase 9 and releases pro-apoptotic molecules such as AIF from the mitochondria, thus initiating the apoptotic programme described above.

A plethora of extracellular survival signals normally suppress the mitochondrial apoptosis pathway. These include integrin-mediated cell adhesion to basement membrane (Meredith et al. 1993; Re et al. 1994; Frisch et al. 1996), adhesion to adjacent cells mediated by VE-cadherin (Carmeliet et al. 1999) and platelet EC adhesion molecule-1 (PECAM-1; Gao et al. 2003; Limaye et al. 2005), haemodynamic shear forces from blood flow (Meeson et al. 1996), vascular EC-specific phosphotyrosine phosphatase (VE-PTP; Baumer et al. 2006), VEGF-A (Lobov et al. 2002), interleukin (IL)-8 (Li et al. 2003), basic fibroblast growth factor (FGF; Fuks et al. 1994) and survival signals derived from pericytes or VSMC (Benjamin et al. 1998). These extracellular survival signals are transduced into the cytoplasm where they activate intracellular signalling intermediates, including pro- and anti-apoptotic members of the Bcl-2 family. Overexpression of anti-apoptotic Bcl-2 or Bcl-xL proteins inhibits EC apoptosis (Schechner et al. 2000; Zheng et al. 2000), while antisense oligonucleotide-mediated inhibition of the pro-survival Bcl-xL protein induces EC apoptosis in vitro (Ackermann et al. 1999). The radio-protective drug amifostine may act in part by elevating the expression of anti-apoptotic members of the Bcl-2 family (Khodarev et al. 1999). The radio-protective drug amifostine may act in part by elevating the expression of anti-apoptotic members of the Bcl-2 family (Khodarev et al. 1999).
development, possibly by reducing the expression of thrombospondin (TSP)-1 and -2 (Chen et al. 2005).

The death receptor apoptosis pathway is activated when tumour necrosis factor (TNF)-family proteins (Slowik et al. 1997) such as TNFz, Fas ligand and vascular endothelial growth inhibitor (VEGI; Yu et al. 2001) bind to TNF-family ‘death receptors’ on the EC surface. Death receptors activate cytoplasmic adapter proteins, which in turn activate caspase 8 and the p38 mitogen-activated protein kinase (MAPK) pathway (Cardier & Erickson-Miller 2002; Pru et al. 2003). Although ECs express death receptors on their surface (Richardson et al. 1994; Sata et al. 2002), TNFz and Fas ligand only activate apoptosis in a subset of EC (Biancone et al. 1997; Sata et al. 2002; Filippatos et al. 2004). The apparent resistance of many ECs to death receptor-induced apoptosis may be in part due to their expression of the caspase 8-antagonist cFLIP (Aoudjit & Vuori 2001; Bannerman et al. 2001). It may also be due to a second action of the TNF-receptor family—activation of the transcription factor NFkB (Yasumoto et al. 1992; Rath & Aggarwal 1999) which upregulates the expression of RNAs encoding apoptosis inhibitors (Bach et al. 1997) including A1 (Duriez et al. 2000), A20 and xIAP (Steihlik et al. 1998). There are additional ‘death ligands’ for ECs that are not members of the TNF superfamiliy. For example, adult capillary and venous EC, but not arterial EC, are sensitive to bone morphogenetic protein 4 (BMP4)-induced apoptosis. The relatively higher resistance of arterial EC to apoptosis may be due in part to their expression of inhibitory (I) Smads. After the knockdown of I-Smad expression using short interfering RNA, the resistant arterial EC become sensitive to BMP4-induced death. In contrast, ectopic expression of I-Smads in BMP4-sensitive cells suppressed BMP4-induced death. Furthermore, intravenous administration of BMP4 into mice caused haemorrhage of capillary EC in brain and lung (Kiyono & Shibuya 2006).

5. COULD CHANGES TO THE TRANSCRIPTOME AND GLYCOME POTENTIATE EC APOPTOSIS?

In addition to the extensively studied protein-based events described above, EC apoptosis may also be regulated by altered RNA transcript abundance profiles (an altered transcriptome), altered cell surface polysaccharide profiles (an altered glycome) or by altered concentrations of intracellular small molecules. The potential interplay between the intracellular events that form the apoptotic programme is shown schematically in figure 2.

To map the transcriptome and glycome changes that accompany EC apoptosis, our laboratory recently modelled the apoptosis of EC that may occur during vessel regression by culturing primary human umbilical vein EC (HUVEC) in conditions of partial survival factor deprivation (SFD). Time-lapse microscopy of the last events in this process is shown in figure 1. Within 1 h of the onset of SFD, HUVECs showed signs of stress. After 28 h, progression through the cell cycle had ceased and only approximately 60% of original cell numbers remained. At this time, approximately 85–90% of the cultures consisted of stressed cells that, without intervention, were destined to die over the next 3 days and approximately 10–15% of the cultures consisted of cells actively undergoing apoptosis. Since almost all post-apoptotic cells appeared to detach and float into the medium within 3 h of death, the remaining adherent cultures, which were subsequently analysed, contained very few post-apoptotic corpses. To determine whether SFD-induced EC apoptosis was associated with transcriptome changes, we used Affymetrix human U95A gene arrays to compare an abundance of 12 600 transcripts in HUVEC cultured in their optimal medium with the corresponding abundance in HUVEC after 28 and 48 h of SFD. Each experiment was replicated five times, each time using primary HUVEC derived from a different individual. We found that most of the 12 600 transcripts were not significantly affected by SFD. However, Bayesian Student’s t-tests identified 171 transcripts that were consistently upregulated and 495 downregulated in all five experiments (p<0.01). Independent component analysis (ICA; a machine learning method for the analysis of noisy data that makes very few statistical assumptions) (Saidi et al. 2004) confirmed the selection of regulated transcripts made by the Bayesian Student’s t-tests (Johnson et al. 2004).

Interestingly, very few of the apoptosis-associated transcriptome changes indicated a protective stress response. Instead, most changes appeared to be either directly pro-apoptotic or to indirectly prime cells for future apoptosis. They are summarized in figure 3, some of which are of special interest. For example, transcripts encoding the death receptor LARD (DR3) and the death receptor ligands TRAIL and TWEAK were upregulated. RNAs encoding survival signals and their receptors, such as VEGF-C, IL-8, flow-induced G-protein-coupled receptor, GP130, IL1 receptor component-L1 and Flt-1, were downregulated. Transcripts encoding intracellular inhibitors of apoptosis were also downregulated including cIAP1, TRAF-2, STAT2 and the integrin-associated kinase ICAP-1a. Bax, a pro-apoptotic member of the Bcl-2 family, was upregulated. At the final stage of the apoptotic programme is engulfment of apoptotic corpses by phagocytes. Transcript abundance changes may assist this process, since RNAs encoding the chemokine monocyte chemoattractant protein-1 (MCP-1) and clusterin (apolipoprotein J, thought to promote the uptake of apoptotic bodies by non-professional phagocytes) were also upregulated.

We also observed changes in the abundance of RNAs encoding enzymes that determine the structure of heparin-like cell surface glycosaminoglycans (HSGAG). Highly sulphated HSGAG are required to present extracellular matrix-binding survival factors, such as FGF and VEGF-A, to their receptors on the EC surface (Park et al. 1993; Venkataraman et al. 1999). After 28 h apoptosis, the RNA encoding N-deacetylase N-sulphotransferase (which promotes HSGAG sulphation) was downregulated, the RNA encoding iduronate-2-sulphatase (which removes sulphate groups from HSGAG) was upregulated and serglycin (a proteoglycan core protein secreted by EC) was downregulated. In line with these transcript abundance changes, when we
analysed HSGAG profiles, we found that during EC apoptosis there was a relative loss of tri-sulphated disaccharides and a relative increase in mono-sulphated disaccharides (figure 4). This apoptosis-associated reduction in HSGAG sulphation may further reduce the ability of stressed or apoptotic EC to respond to survival factors such as VEGF-A. In agreement with this hypothesis, we found that the ability of EC to mount a response to VEGF-A (as measured by the degree of VEGF-induced transcriptome change) was dramatically reduced following SFD (Johnson et al. 2004).

Based on these results, we suggest that the cell biology of stress/deprivation-induced EC apoptosis is underpinned by synergy between previously described protein-based changes and the changes to the transcriptome and glycome identified in this study. This provides a new paradigm for the regulation of EC apoptosis, and the possibility that a small number of the apoptosis-associated transcriptome and glycome changes identified by this study may provide future drug targets for the regulation of EC apoptosis.

6. WHAT IS THE TIME COURSE OF TRANSCRIPTOME CHANGE DURING EC APOPTOSIS?

To understand the kinetics of transcriptome change during EC apoptosis, we have performed a time course experiment. HUVEC (a pooled culture from 10 donors) were exposed to SFD conditions identical to those used in the study described above. RNA was prepared from the cultures before the induction of apoptosis (time 0) and after 0.5, 1.5, 3, 6, 9, 12 and 24 h, hybridized to CodeLink Human Uniset 20K gene arrays. This experiment was repeated three times independently. The regulation of transcripts during EC apoptosis can be visualized in the scatterplots shown in figure 5a–d. We then selected a subset of 276 transcripts that were consistently regulated over the time course of EC apoptosis for further analysis. To do this, we excluded all transcripts from our analysis that were not regulated by $Z \geq 1.5a$ at 3 or more adjacent time points in all three experimental replicates (see Appendix for methods). These data are available in file 1 in the electronic supplementary material. From these 276 transcripts, $k$-means clustering was used to select eight groups of transcripts (in addition to an unclassified group) with similar time-course profiles (figure 6). From these profiles and from the scatterplots shown in figure 5a–d, it can be seen that a number of transcripts start to be regulated form 1.5 h and that, in general, the rate of change appears to low after 12 h. When we plotted the individual transcripts identified in the previous study, we noted that, in general, the transcripts encoding growth factors (such as Ang-2 and IL-8) were among the earliest transcripts to be regulated, while transcripts associated with the cell cycle (such as cyclins A2, H, and E, CDC6, CDC28 and kinesin-like spindle protein) were regulated later (table 1). The regulation of many of these transcripts following 28 h SFD has been validated by our previous Affymetrix study. These data suggest that some functional classes follow common patterns of regulation during the SFD-induced apoptosis of EC cultures. More sophisticated analysis (see below) suggests common upstream regulators of these transcripts.

7. PROGRAMMED CHANGES TO THE TRANSCRIPTOME MAY POTENTIATE APOPTOSIS IN OTHER CELL TYPES

Contribution of the transcriptome and glycome to the apoptotic programme is not unique to EC. It has been known for some time that regulation of RNA transcript abundance may turn the apoptotic programme on or off during development. For example, ecdysone-dependent expression of RNAs encoding the caspases DRONC (Dorstyn et al. 1999) and DRICE (Kilpatrick et al. 2005) appears to prime Drosophila tissues for histolysis at specific developmental stages. Interestingly, the strategy of regulated transcript abundance activating the apoptotic programme during development may also be employed in plants. In the tissues of barley undergoing developmental cell death, RNAs encoding plant homologues of the endonucleases that cleave DNA during mammalian apoptosis are upregulated (Zaina et al. 2003).

Further support for the hypothesis that transcriptome change may play a role in apoptotic programmes comes from experiments in which apoptosis of some cell types can be blocked by inhibiting RNA synthesis (Galli et al. 1995; Schulz et al. 1996). In addition, several studies have identified programmed changes in RNA transcript abundance as cultured cells undergo apoptosis. For example, infection with group A Streptococcus pyogenes induces apoptosis in some human epithelial cells. Microarray analysis suggests that this apoptosis is associated with altered gene expression including induction of RNA transcripts encoding caspases and pro-apoptotic members of the Bcl-2 family (Nakagawa et al. 2004). Ionizing radiation-induced apoptosis of lymphoblastoid cells is accompanied by the induction of RNAs encoding pro-apoptotic proteins such as Fas and Bak (Akerman et al. 2005). 5-Fluorouracil-induced apoptosis of colon carcinoma cells is accompanied by a programme of altered gene expression including the induction of RNAs encoding p53, Fas and TNF receptor-2 (Zhang et al. 2003). Apoptosis of human lung cancer cells is associated with the upregulation of a number of genes including pro-apoptotic members of the Bcl-2 family (Robinson et al. 2003). The dexamethasone-induced apoptosis of lymphoid cells is also accompanied by alterations in gene expression including the induction of BimEL, a pro-apoptotic member of the Bcl-2 family (Medhi et al. 2003). In neurons, a set of RNA transcripts appears to be upregulated during apoptosis including those encoding caspase 3, the death receptor DR6 (Chiang et al. 2001), Id2 (Gleichmann et al. 2002) and the pro-apoptotic Bcl-2 family member Bax (Wullner et al. 1998).

As would be expected, different cell lineages appear to regulate different cohorts of RNA transcripts during their apoptotic programmes. However, some apoptosis-associated RNA changes are conserved across different cell lineages (including EC), such as the induction of transcripts encoding pro-apoptotic members of the Bcl-2 family. Taken together, these data suggest that in many cell types the altered abundance, during or immediately before apoptosis, of transcripts encoding apoptotic regulators or...
apoptotic machinery may play a ‘feed-forward’ role to assist the apoptotic programme.

8. WHAT ARE THE LIMITATIONS OF TRADITIONAL GENE ARRAY BIOINFORMATIC TECHNIQUES FOR THE STUDY OF EC APOPTOSIS?

As discussed above, traditional bioinformatic techniques have revealed interesting new paradigms for the control of EC apoptosis. However, they do have real limitations. While these limitations should not deter prospective EC researchers from using transcriptome profiling studies, they require consideration.

(i) Owing to the false discovery introduced by noise or individual differences (Schoenfeld et al. 2004), in addition to any annotation or contamination errors inherent in the gene array platform itself, a proportion of the results of every gene array experiment will be incorrect. Researchers and clinicians using microarray techniques must accept this low rate of error. However, accepting a low rate of error is not as difficult as it sounds. After all, deciding on an acceptable probability of error forms the basis of most statistical analyses of scientific experiments.

(ii) The results obtained when the same RNA is analysed using different gene array platforms are not always concordant (Jordan 2004). This has shaken the confidence of some researchers in gene array technology (Tan et al. 2003). This problem is illustrated by the microarray data we describe in this paper. Our previous study of the effect of 28 h SFD on HUVEC used Affymetrix U95A genechips and highlighted 79 regulated transcripts (listed in file 3 in the electronic supplementary material of Johnson et al. 2004). The CodeLink genechip time-course study presented in this paper used the same cell type and SFD conditions and contained a 24 h time point. We compared the transcripts apparently regulated by 28 h SFD in the Affymetrix study with the transcripts apparently regulated by 24 h SFD in the CodeLink study. Of the 79 transcripts highlighted by the Affymetrix study, 48 could be identified by reference to Unigene IDs in the CodeLink gene arrays, but only 30 of these appeared to be regulated by 1.5-fold or more in a congruent direction to the Affymetrix study. Although the lack of full concordance is concerning, some of the differences between the results of our two studies may be due to experimental differences. Other differences may be due to occasional misannotation of one or both gene array platforms, since it was recently

Figure 5. A time course of transcriptome change during EC apoptosis. Scatterplots show each transcript represented by a dot, with abundance at time 0 shown on each x-axis and abundance at other time points shown on the y-axes: (a) 1.5 h, (b) 6 h, (c) 9 h and (d) 24 h SFD. Those transcripts that are not regulated remain approximately on the diagonal. Transcripts that appeared to be upregulated when cultures were exposed to SFD conditions for 24 h compared with healthy cultures at time 0 are highlighted in white. The gradual upregulation of these transcripts over time can be seen.
shown that matching of gene array probes
between platforms by actual sequences rather
than by Unigene IDs dramatically improved the
apparent concordance (Mecham et al. 2004).
This microarray cross-platform discordance
problem urgently requires further study. The
safest approach when possible may be to focus on
results that have been confirmed by two inde-
dendent gene array platforms or by one platform
and quantitative polymerase chain reactions.

(iii) Finally, there is an important but hidden
limitation of traditional bioinformatic tech-
niques. Although techniques such as clustering
(Eisen et al. 1998), ICA (Saidi et al. 2004) and
self organizing maps (Tamayo et al. 1999) have
been extremely valuable in revealing hidden
patterns of transcript co-regulation, in general
they are not good at revealing the causal
relationships among molecular signals within
cells. However, it is the combination of the cause

Table 1. The kinetics of SFD-dependent regulation of transcript abundance. Values represent median fold change (relative to
0 h) of normalized transcript abundance of the triplicate time-course data. Negative values represent downregulation. Positive
values represent upregulation.

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Figure 6. $k$-means clustering revealed eight groups of transcripts (from a shortlist of 685 highly regulated transcripts) that
followed distinct temporal patterns of regulation.

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and effect relationships between all the signals operating in a cell, rather than the isolated actions of individual signals, that drive and regulate processes such as apoptosis.

9. THE CONTRIBUTION AND LIMITATIONS OF THE PROTEOME

An obvious limitation of gene array data is that it does not give direct information about proteins or post-transcriptional regulation. Although the use of gene array technology has helped to enrich our knowledge of the key regulatory genes involved in processes such as EC apoptosis, understanding regulation at the level of the proteome will be of more direct value. Large amounts of useful information have already emerged from proteomic studies of EC (Bruneel et al. 2003; Kamino et al. 2003; Nylund & Leszczynski 2004; Sprenger et al. 2004; Yu et al. 2004). Notable among these is a subtractive proteomics study that identified tumour-associated EC surface proteins, which may be targets for anti-tumour therapy. In fact, radio-immunotherapy directed against one of the EC surface proteins identified, annexin A1, is effective in vivo (Oh et al. 2004). Another notable study from the same research group illustrated the differences between in vitro and in vivo lung microvascular ECs, and highlighted the need for replicated in vivo confirmation of in vitro results (Durr et al. 2004). A study of the proteomic changes that occur during etoposide-induced EC apoptosis has suggested that multiple protein-based signalling cascades, including the mitochondrial and the ER stress pathways, may play a role (Bruneel et al. 2005).

The information derived from these proteomics studies could not have been derived from transcriptome studies, since it is very difficult to correlate gene expression levels with protein abundance (Gygi et al. 1999; Lian et al. 2002). Although technologies such as protein microarrays are rapidly developing and now enable relatively specific and sensitive analysis of proteins (Letarte et al. 2005), we believe that at present transcriptome studies still remain extremely viable. The best approach in the future may be to combine the results of proteomics with that of transcriptome analysis using systems biology techniques (see below).

10. INFERENCE OF CAUSAL RELATIONSHIPS FROM TRANSCRIPTOME DATA

Methods to infer cause and effect relationships based on transcriptome data are emerging. Of these methods, some researchers believe the technique of gene regulatory network analysis may hold particular promise (Brazhnik et al. 2002; Schlitt & Brazma 2005). Practitioners of gene regulatory network transcriptome analysis claim to produce ‘circuit diagrams’ that show the hierarchical networks of cause and effect transcription control relationships that underlie a biological process like EC apoptosis or interventions like drug treatment. These gene regulatory networks are specific to the cell type under study. Unlike the traditional post-translational signalling network charts that most biologists are used to seeing, the nodes on gene regulatory network graphs represent transcripts rather than proteins. The links between the nodes (called edges) represent putative cause and effect relationships between transcripts, where the abundance of one transcript, through one or more intervening protein-based steps, can regulate the abundance of a second transcript. Therefore, although proteins are not explicitly shown in gene regulatory network graphs, they provide the hidden engine that underlies the network structure.

Several mathematical methods have been used to infer gene regulatory networks such as differential equation models (Chen et al. 1999; de Hoon et al. 2003), state space models (Rangel et al. 2004), Boolean network models (Akutsu et al. 1998; Shmulevich et al. 2002) and Bayesian network models (Friedman et al. 2000; Imoto et al. 2002). In some situations, a combination of these techniques is helpful. For example, Boolean and Bayesian network algorithms can be combined (Imoto et al. 2003b) to infer gene networks for response to a drug—the Boolean network approach is used initially to identify the set of transcripts regulated in abundance by the drug, and a Bayesian network approach is then used to map the upstream regulators of the drug-affected genes, which provide potential ‘druggable’ targets. This combination of approaches has been used successfully in yeast (Aburatani et al. 2003) to determine that the protein regulated by the oral antifungal drug griseofulvin is CIK1 (Savoie et al. 2003).

Some features of gene regulatory network inference techniques appear especially important when inferring networks from microarray data. For example, transcripts are not always regulated in a linear manner; therefore, capturing nonlinear relationships between transcripts can greatly increase the power of network analysis. This can be achieved by the use of non-parametric additive regression models (Imoto et al. 2002). In addition, most gene expression data contain many outlying data points and show heteroscedasticity (the dispersion of distributions differs between transcripts). Therefore, modelling which takes the effects of outliers and heteroscedasticity into account is especially well suited to inferring networks from microarray data (Imoto et al. 2003a). Like all bioinformatics techniques, both the quality and quantity of the available data determine the statistical power of gene network techniques.

To illustrate the principles of applying gene regulatory networks to endothelial biology, we have used the dynamic Bayesian network inference method (Kim et al. 2003) to generate a gene network based on the median of the HUVEC apoptosis time-course gene array data which was described above. A graph representing this network is shown in figure 7a. In this network, the regulation of abundance of most parents and their multiple children over the apoptosis time course appears to be correlated, with the up- or downregulation of the putative children often lagging behind that of their parents (figure 7b and file 2 in the electronic supplementary material). In this gene network, the parent node with the most children is GABA receptor-associated protein (GABARAP). According to this network, GABARAP is potentially acting as a
novel transcriptional ‘hub’ during the process of EC apoptosis. Interestingly, GABARAP has recently been shown in other laboratories to induce apoptosis by interacting with DEAD box polypeptide 47 (Lee et al. 2005) and appears to be encoded by a tumour suppressor gene mutated in breast cancer (Klebig et al. 2005). To illustrate the potential function of GABARAP in the regulation of EC apoptosis, we knocked down the expression of GABARAP and luciferase (a negative control) in HUVEC using siRNA. RT-PCR analysis indicated that 24 h after anti-GABARAP siRNA treatment, the abundance of GABARAP mRNA was reduced to less than 20% of its former levels (data not shown). siRNA-mediated reduction of GABARAP expression appeared to inhibit HUVEC apoptosis. The median incidence of apoptosis induced in HUVEC by 24 h of SFD (performed as in Johnson et al. 2004) was 11.7% in cells treated with anti-luciferase siRNA (control) but only 5.8% in cells treated by anti-GABARAP siRNA (figure 7c). Analysis of this data using the Mann–Whitney two-tailed test, a non-parametric statistical method, gave a statistically significant result of \( U = 426.5 \) and \( p \leq 0.001 \). In addition, the morphological changes induced in HUVEC by 24 h of SFD appeared to be reduced by the inhibition of GABARAP expression (figure 7d). This suggests that GABARAP may indeed be a regulator of EC apoptosis, as is suggested by its prominent position in the gene network. Another node that appears to act as transcription hub during apoptosis is interferon regulatory factor-7 (IRF7), a transcription factor known to be regulated by the TNF and NFκB families. Interestingly, the TNF family member TRAIL and the NFκB family member RELB lie upstream of IRF7 in our gene network. The complete parent–child relationships for this gene network are given in file 2 in the electronic supplementary material. Web-based tools such as Cell Illustrator (http://www.cellillustrator.com) and Cytoscape (http://www.cytoscape.org) can be used by the reader to explore this putative network and the gene-to-gene relationships it symbolizes.

As a further illustration of gene network analysis techniques, we also generated a second gene network from the same apoptosis time-course gene array data. This network differs from the one shown in figure 7a, from which it was derived by the incorporation of new gene array data from eight siRNA disruptant experiments. This new gene array data was used as a Bayesian prior and is provided as file 3 in the electronic supplementary material. In these eight disruptant experiments, specific RNAs related to cell cycle control (CDC45L, CCNE1, CENPA, CENPF, CDC2, CDC25C, MCM6 and CDC6) were reduced in abundance by more than 65% in HUVEC using siRNA pools. Gene array analysis showed that these siRNA treatments caused large numbers of transcripts to be regulated (presumably as a consequence of the targeted RNA downregulation, or possibly in some cases also as a consequence of unexpected off-target siRNA effects). An example of the effects of siRNA treatment on the HUVEC transcriptome is shown in figure 5a,b. The putative time-course gene network modified by the incorporation of this disruptant information as a Bayesian prior is shown in figure 8c and its complete parent–child relationships are listed in file 4 in the electronic supplementary material. Of the 488 edges contained in the modified gene network shown in figure 8c, 338 are shared with the unmodified gene network shown in figure 7a. Of the 150 edges found in the modified but not the unmodified networks, 94 have as parents one of the eight RNAs that were targeted in the siRNA disruptant experiments. These particular edges appear to have inherited cause and effect information from the disruptant gene array data, since they all correctly predict the effects on children of disrupting parents by siRNA treatment (data not shown). Therefore, the inclusion of disruptant data as a Bayesian prior appears to enhance the ability of dynamic time-course gene networks to predict specific directional relationships between transcripts. It seems possible that the addition of data from biological database annotations and proteomics experiments could further enhance the predictive power of this type of gene network. We and other researchers are generating larger-scale gene regulatory networks using gene array data from hundreds of disruptant experiments, which will be combined with time-course data using Bayesian techniques, as illustrated above (Imoto et al. 2006). We believe these large networks will provide valuable hypotheses about functional interactions within ECs, which can then be tested experimentally.

Therefore, gene regulatory network technologies, along with other techniques for inferring cause and effect relationships in cells from transcriptome data, promise a great deal for vascular biology research and for accelerating the rational design of new cardiovascular medications. They will be especially valuable if their results are used to complement those of traditional cell biology studies and EC proteomic studies currently underway. Cautious integration of the cause and effect relationships that are predicted by gene networks into systems biology databases (Aggarwal & Lee 2003) may be especially useful. A study showing the usefulness of this approach modelled a yeast galactose utilization pathway by analysing experimental perturbations using information from microarrays, proteomics and computer databases (Idecker et al. 2001). Another example is a study that defined pathways that control endoderm and mesoderm specification during sea urchin development. In this study, the results of perturbation analyses were combined with information from computer databases, cis-regulatory analysis and molecular embryology (Davidson et al. 2002). In Professor Eugene Butcher’s laboratory, systems biology approaches using small amounts of data from cultured ECs have already revealed important new information about the role of NFκB and Ras EC signalling pathways (Plavec et al. 2004). The addition of data from gene regulatory networks may dramatically increase the power of this type of systems biology analysis.

However, a critical challenge for technologies such as gene regulatory networks is that the predictions they make must be extensively tested using traditional ‘wet’ laboratory experiments. This will require clear communication and extensive cooperation between bioinformaticians and biologists/clinicians. Hopefully, the emerging generation of vascular biologists fluent in

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Figure 7. An example of a dynamic time-course gene regulatory network. (a) A graph representing a dynamic Bayesian gene network generated from the median of triplicated apoptosis time-course data. Dots represent transcripts ('nodes') and lines between the dots represent potential cause and effect interactions ('edges'). (b) A graph showing apoptosis time-course gene array transcript abundance data for a parent RNA transcript in the network (CDKN1C, black) and its putative children (C1QTNF5, green; AKR1C3, blue; MLF1, orange; SUV39H1, red). (c) Reduction of GABARAP mRNA abundance inhibits HUVEC apoptosis. Incidence of apoptosis induced by 24 h of SFD in anti-GABARAP siRNA-transfected HUVEC (GAB) and anti-luciferase siRNA-transfected HUVEC (LUC, control) were assessed by immunocytochemical detection of caspase 3 cleavage. Horizontal bars indicate median values. (d) Images of HUVEC cultures deprived of serum for 24 h after treatment with anti-luciferase siRNA (control) and anti-GABARAP siRNA. Fields are 225 × 300 μM in size.

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both bioinformatics and wet laboratory experimentation will be able to meet this challenge.

11. CONCLUSIONS
EC apoptosis appears to play an essential role in blood vessel development and remodelling and has been associated with cardiovascular pathologies. Therefore, understanding the regulation of EC apoptosis with the goal of intervening in this process has become a current research focus. Much is known about the protein-based signalling and cleavage cascades that regulate EC apoptosis. However, the potential role of pro-apoptotic transcriptome and glycome changes in the orchestration of EC death has only recently been recognized. For a complete understanding of EC apoptosis, we now need to define the complex relationships between the molecular signals that operate in these cells. New technologies such as gene regulatory networks that promise to infer cause and effect relationships from EC transcriptome data provide powerful tools, especially when integrated with information from proteomic and cell biology studies and with computer databases. However, the predictions made by all of these techniques must be extensively validated by laboratory experiments. This will require close cooperation between bioinformaticians and experimental biologists and is likely to be driven by a new generation of cross-disciplinary scientists fluent in the languages of both fields.

APPENDIX: MATERIAL AND METHODS
HUVEC were isolated from umbilical cords by collagenase digestion and cultured at 37°C/5% CO₂ in basal culture medium supplemented with a proprietary mixture of heparin, hydrocortisone, epidermal growth factor, FGF and 2% foetal calf serum (FCS; EGM-2, Cambrex, Workingham, UK). HUVEC from 10 individuals were pooled, plated at 4.8 x 10⁵ cells in 25 cm² flasks and allowed to recover for 24 h, at which time they were approximately 60% confluent. Supplemented medium was then removed and replaced with basal culture medium containing only 2% charcoal stripped FCS (Invitrogen, Paisley, UK). At time points

Figure 8. An example of a dynamic time-course gene regulatory network modified by the inclusion of prior information from siRNA disruptant experiments. (a) A scatterplot comparing transcript abundance in untransfected HUVEC (x-axis) with transcript abundance in mock HUVEC transfected with control siRNAs directed against luciferase (y-axis)—little regulation of transcript abundance appears to have occurred. (b) A scatterplot comparing transcript abundance in HUVEC transfected with siRNAs directed against luciferase (x-axis) with transcript abundance in HUVEC transfected with siRNAs directed against NFκB p105 (y-axis)—NFκB p105 (circled) was downregulated more than fourfold and a large number of additional transcripts were also regulated in abundance. (c) A graph representing an apoptosis time-course gene network generated that had been modified by incorporating, as a Bayesian prior, gene array data from eight siRNA disruptant experiments, similar to those shown in (a,b). Edges in common with the network shown in figure 7a are green, and edges present in this network but not in the network shown in figure 7a are pink.
0, 0.5, 1.5, 3, 6, 9, 12 and 24 h, total RNA was prepared using Trizol reagent (Invitrogen) and assessed using an Agilent 2100 bioanalyser. Biotin-labelled complex cRNAs were prepared and hybridized to CodeLink UniSet Human 20K gene chips according to the manufacturer’s protocols (GE Healthcare, Amersham, UK). To generate triplicate data (of biological replicates), the protocol was repeated two further times using HUVEC from 10 different individuals for each pool.

The quality of the expression data from all gene chips was confirmed using CodeLink expression analysis Software (v. 4.1). To ensure that expression levels were comparable between the arrays, the data was normalized using intensity-dependent scaling as described in Schoenfeld et al. (2004). To knock down, the abundance of RNA transcripts in HUVEC siRNA ‘smartpools’ from Dharmacon Inc. (Lafayette, Colorado, USA) were transfected into HUVEC using the siFectamine transfection reagent (ICVEC, London, UK) used according to the manufacturers instructions. The incidence of HUVEC apoptosis was assessed by cytospinning HUVEC onto glass slides followed by immunohistochemical staining using an anti-active caspase 3 antibody (rabbit polyclonal, Promega, Southampton, UK, 1:250 dilution as described in Johnson et al. (2004)). For each experimental condition, the staining of more than 1000 cells was counted. Data were log_{2}-transformed and log ratios between each time point and the 0 h control calculated. In addition, the ratio between each time point and 24 h was also calculated to capture regulation at both ends of the time course. For each transcript at each time point, Z-scores were calculated. Log_{2} ratios of individual transcripts were subtracted from the mean of log_{2} ratios for that time point and then divided by the standard deviation of log_{2} ratios for that time point. For each time-course replicate, transcripts that had ‘good’- or ‘low’-quality flags (based on CodeLink expression analysis software results) and a Z-score of −2 ≤ Z ≤ +2 at three or more adjacent time points were selected for further study. Transcripts that contained ‘good flags only’ and had a Z-score of −1.5 ≤ Z ≤ +1.5 at three or more adjacent time points were also selected. Those regulated transcripts concordant between the triplicate time-course data were selected for the network generation. In addition, transcripts that were known to be involved in apoptosis and were significantly regulated in a previous Affymetrix apoptosis study (Johnson et al. 2004), that were also regulated by greater than 1.5-fold in the Affymetrix apoptosis study (Johnson et al. 2004), that were also regulated by greater than 1.5-fold in the triplicate CodeLink data were included. This gave a list of 276 transcripts for apoptosis network building. Dynamic Bayesian apoptosis time-course networks were generated from the list of 276 apoptosis regulated transcripts, as described by Kim et al. (2003). Data generated from eight siRNA knock down arrays were used to make an array prior to the apoptosis time-course network. The eight knockdown arrays were selected from a list of 270 different knockdown arrays, based on the following criteria: (i) the transcript knocked down is in the list of 276 transcripts regulated in the apoptosis time-course triplicate data (as explained above) and (ii) a large number of the regulated genes resulting from the knock down were also contained in the 276 list of apoptosis-regulated transcripts. Gene networks, both with and without this prior information, were inferred from the apoptosis data as described (Kim et al. 2003). Comparisons of the gene networks generated with and without knockdown prior information, and their visualization and data mining, were carried out using the software iNet (now known as Cell Illustrator) which can be downloaded from http://www.cellillustrator.com

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