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Multidimensional Proteomics for the Identification of Endothelial Post Mortem Signals of Importance in Vascular Remodeling

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1. Introduction

1.1 Endothelial apoptosis and vascular remodeling

Atherosclerotic diseases (AD) and immune-mediated vasculopathy of the transplanted organ (referred to as transplant vasculopathy (TV)) are both characterized by vessel wall thickening and fibrotic changes that lead to progressive vascular obliteration (Al-Lamki et al., 2008; Cailhier et al., 2006; Cornell et al., 2008; Mitchell, 2009; Rahmani et al., 2006; Valantine, 2003). The endothelium, positioned at the interface of blood flow and the vessel wall, serves as a physiological barrier and sensor of environmental stress. The "response to injury hypothesis" proposed by Russell Ross in the 70's suggested that endothelial injury prompts vascular smooth muscle cell (VSMC) migration and proliferation, therefore initiating neointima formation (Ross et al., 1977; Ross and Glomset, 1976). Initially, vascular remodeling is beneficial but repeated cycles of injury, proliferation and repair lead to maladaptive remodeling and lumen narrowing. To date, *in vitro* and *in vivo* studies in animals and humans confirmed that endothelial apoptosis is a key determinant in the development of AD and TV (Rossig et al., 2001). Various immune and non-immune factors, such as cytotoxic T-cells, donor-specific antibodies, high cholesterol and hyperglycemia account for increased endothelial apoptosis (Cailhier et al., 2006). In turn, migration and accumulation of VSMC, surviving and accumulating within a hostile environment through acquisition of an anti-apoptotic phenotype, form the initial neointima. Histological and biochemical features characterizing AD and TV include 1) extracellular matrix (ECM) degradation that likely facilitate VSMC migration; 2) acquisition of a synthetic and anti-apoptotic phenotype by neointimal cells (VSMC), mesenchymal stem cells (MSC) and fibroblasts associated with Bcl-xl overexpression (Gennaro et al., 2004; Hirata et al., 2000; Pollman et al., 1998) and 3) differentiation of fibroblasts into myofibroblasts of importance in fibrogenic vascular changes (Tomasek et al., 2002) (Figure 1). The molecular interplay regulating intercellular communication between apoptotic endothelial cells (EC) and neointimal cells are only beginning to be unraveled.

1.2 Proteomics for studying Post Mortem Signals (PMS) exported by apoptotic EC

Apoptotic programmed cell death is classically considered a silent process. The first clues suggesting that apoptotic endothelial cells may not "go quietly" stems from pharmacological

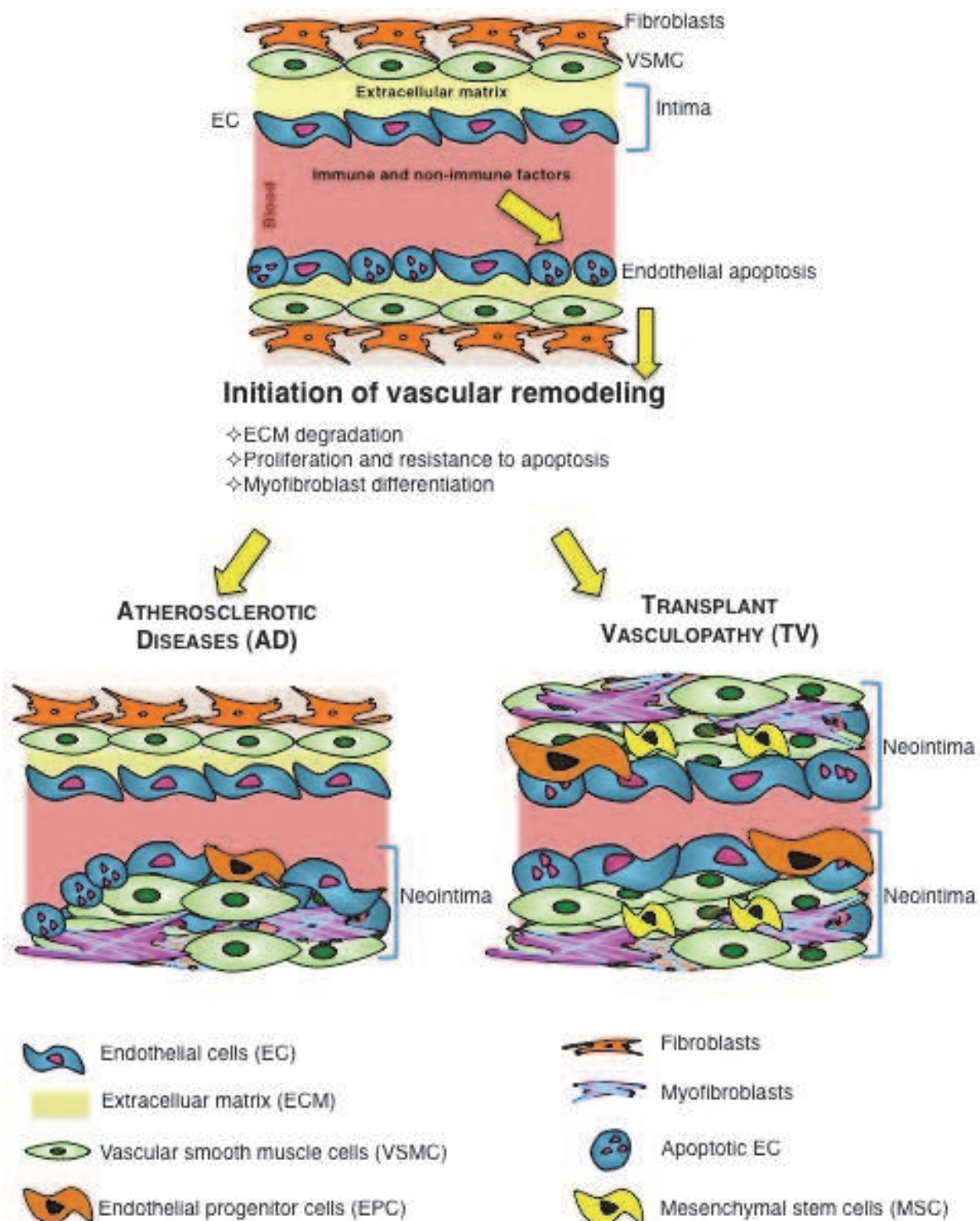


Fig. 1. Schematic diagram of the initiation of vascular remodeling characteristic of AD and TV. Immune and non-immune factors induce endothelial apoptosis. Endothelial apoptosis precedes neo-intima formation. The latter is accompanied by ECM degradation and proliferation and resistance to apoptosis of neo-intimal cells (VSMC, MSC, EPC, fibroblasts and myofibroblasts). Homing of MSC and EPC as well as myofibroblast differentiation contribute to fibrogenic changes observed with vascular remodeling.

or genetic approaches aimed at inhibiting endothelial apoptosis in models of AD or TV. Inhibition of endothelial apoptosis was shown to block the development of vascular

remodeling, suggesting a paracrine role for the apoptotic endothelium in triggering pathways of importance in neointima formation (Cailhier et al., 2006; Choy et al., 2004a; Choy et al., 2004b; Shimizu et al., 2000a; Shimizu et al., 2000b, 2002a, b). Cell biology approaches supported this contention and showed that medium conditioned by apoptotic EC regulates the survival and differentiation of major cellular constituents of the vessel wall (Cailhier et al., 2006; Laplante et al., 2005; Raymond et al., 2004; Soulez et al., 2010). Execution of the apoptotic program relies mainly on post-translational modifications, such as protein-protein interactions, protein translocation and proteolysis that will set in motion the molecular pathways regulating the various phases of apoptosis (Thiede and Rudel, 2004; Wang and Chen, 2011; Mahrus et al., 2008). The caspase family of cysteine proteases is central to the regulation of the various phases of apoptosis. Their activation in association with mitochondrial destabilization or extracellular death receptor activation leads to modifications in the architecture of intracellular organelles and fragmentation of the cytoskeleton, the ER and the nucleus (Taylor et al., 2008). Apoptosis triggers changes in the cell membrane including blebbing and extracellular exposure of PS of importance as a phagocyte recognition signal (Leroyer et al., 2008; Martinez et al., 2005; Pober and Sessa, 2007; Verhoven et al., 1995). In addition, mounting evidence suggests that the apoptotic program also regulates the extracellular export of a finely regulated set of signals of importance in leukocyte trafficking, phagocytosis and coagulation (Bournazou et al., 2009; Lauber et al., 2003; Truman et al., 2008).

The complete set of mediators released by a cell at a given time, defined as a secretome, can be decrypted through high-throughput methods based on mass-spectrometry. Use of technology focusing on post-transcriptional events bears special importance in dying cells where the various levels of molecular regulation depend on protein degradation, translocation and specific protein-protein interactions rather than gene transcription. Proteomics was instrumental in characterizing the complex mixture of several secretomes composed of both soluble and vesicular mediators including microparticles and exosomes (Mathivanan and Simpson, 2009). As illustrated by the following reports, large-scale mass-spectrometry also eased the identification of paracrine signals (lipids, proteins and microparticles) specifically enriched within the secretome of apoptotic cells. For example, apoptotic Burkitt lymphoma cells release lysophosphatidylcholine (LPC) through activated caspase-3 dependent mechanisms, which in turn favors recruitment of macrophages and clearance of apoptotic bodies (Lauber et al., 2003). Apoptotic MCF7 epithelial cells secrete lactoferrin as a means of promoting migration of mononuclear leukocytes while inhibiting migration of polymorphonuclear leukocytes (Bournazou et al., 2009). Apoptotic EC shed microparticles with potent immunogenic and pro-coagulant abilities (Smalley and Ley, 2008; Smalley et al., 2007). In sum, these proteomic-based reports suggested that a paracrine response embedded within the apoptotic program and herein referred to as post mortem signals (PMS), controls a finely orchestrated network of intercellular communication.

In the following sections, we will highlight the advantage of different proteomic strategies for characterization of PMS released by apoptotic cells. The systematic analysis of the secretome of apoptotic EC is central to gain insights into novel mechanisms of intercellular communication of importance in TV and AD. Also, the characterization of endothelial apoptotic secretome represents a unique opportunity to identify biomarkers of the initial stage of vascular remodeling.

2. Studying the secretome of apoptotic EC: Methodological aspects

2.1 *In vitro* experimental systems aimed at studying endothelial apoptosis

Two major pathways, the intrinsic and extrinsic pathways, regulate the initiation of apoptosis. The intrinsic pathway is activated by metabolic disturbances, such as nutrient deprivation and oxidative stress, leading to mitochondrial permeabilization, release of cytochrome C and activation of caspase-9. The extrinsic pathway is activated by death receptors that, upon ligand-mediated activation, recruit an initiator caspase (ex. caspase-8). The effector phase of apoptosis responsible for cleavage of key substrates that bring about the morphological changes of apoptosis is controlled by a common phase regulated by effector caspases (-3, -6, -7) (Taylor et al., 2008). Serum starvation (SS) is a classical inducer of the intrinsic apoptotic pathway in EC and offers several advantages for the characterization of an apoptotic secretome. First, four hours of SS in cultured EC induces sequentially mitochondrial permeabilization, activation of caspases -9 and -3, PARP cleavage and chromatin condensation characteristic of apoptotic cell death. The functional importance of caspase activation in SS-induced apoptosis was validated with caspase inhibitors (the pan-caspase inhibitor (ZVAD-FMK) and caspase-3 inhibitor (DEVD-FMK)) as well as small interfering RNA (siRNA) targeting caspase-3 (Sirois et al., 2011). Second, apoptosis induced by brief SS does not induce necrotic features and cell membrane permeabilization, as assessed by fluorescence microscopy with propidium iodide and evaluation of lactate dehydrogenase (LDH) activity in medium conditioned by serum-starved EC (Laplante et al., 2010; Sirois et al., 2011). The absence of necrosis in this system is an asset for studying secretory events in absence of uncontrolled leakage secondary to cell membrane damage. Finally, SS circumvents contamination of the secretome by residual components of culture medium (such as albumin) that could interfere with the identification of less abundant proteins specifically released by apoptotic EC downstream of caspase activation.

2.2 Identification of endothelial PMS by multidimensional proteomics

A comparative and multidimensional proteomic analysis was undertaken to characterize the secretome of apoptotic EC (Sirois et al., 2011) (Figure 2). Proteins specifically released by apoptotic EC were identified through comparison of the secretomes generated by equal numbers of serum-starved apoptotic EC (SSC-apo) and serum-starved EC in which apoptosis was blocked by the irreversible pan-caspase inhibitor ZVAD-fmk (SSC-no-apo). Cell media were cleared of cell debris and apoptotic blebs prior to proteomic analysis (Cailhier et al., 2008; Laplante et al., 2010; Sirois et al., 2011; Soulez et al., 2010). An equivalent amount of proteins were fractionated either by SDS-PAGE or by HPLC anion exchange chromatography followed by protein identification by MS/MS (Pshezhetsky et al., 2007). The two comparative strategies were complemented by a functional approach aimed at identifying proteins with an anti-apoptotic activity on VSMC, therefore recapitulating induction of the neointimal anti-apoptotic phenotype (Raymond et al., 2004). Proteins present in SSC-apo were fractionated by ultrafiltration followed by ion-exchange FPLC. Eluted fractions were individually tested *in vitro* for their ability to inhibit apoptosis of VSMC and the fraction displaying a significant anti-apoptotic activity was further fractionated by SDS-PAGE followed by protein identification by LC-MS/MS. Computational analysis of the peptides identified by mass-spectrometry generated three lists built by the functional and the two semi-quantitative comparative approaches.

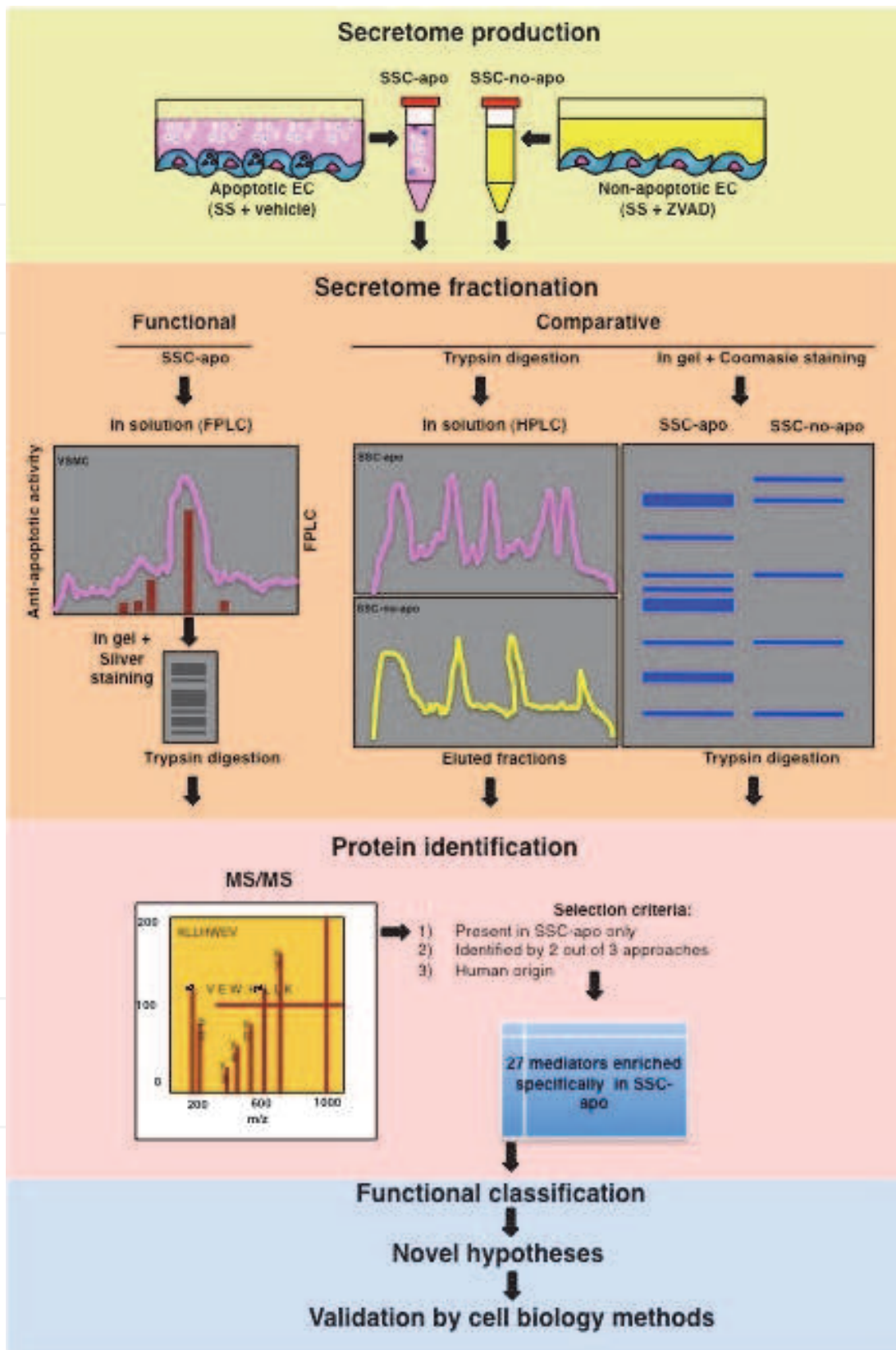


Fig. 2. Schematic representation of the experimental strategy for generating serum-free media (conditioned by equal EC numbers in equal volumes of serum-free media for 4 hours) by apoptotic (SSC-Apo) and non-apoptotic EC (SSC-No-Apo). Secretomes were collected

and depleted of cell debris and apoptotic blebs prior to fractionation. Multidimensional proteomics of the secretomes was performed using one functional and two comparative approaches. SSC-apo was fractionated by FPLC and each eluted fraction was tested for its anti-apoptotic activity in serum-starved VSMC. The fraction with the most significant activity was further separated by SDS-PAGE followed by silver staining and in-gel trypsin digestion. SSC-apo and SSC-no-apo proteins were also compared and fractionated by HPLC or SDS-PAGE prior to protein identification by mass-spectrometry analysis. Identification of specific components of the SSC-apo was achieved using stringent selection criteria. To be considered a specific component of the apoptotic secretome, the protein had to meet the following criteria: protein present in SSC-apo only; protein identified by 2 out of the 3 proteomic approaches; protein of human origin. 27 proteins were identified and classified according to their mode of secretion and the presence of signal peptide, generating novel hypotheses that were further validated by cell biology methods.

3. The caspase-specific endothelial secretome

A targeted screening strategy was developed to focus on the proteins with the highest likelihood of representing caspase-specific secretome components of importance in vascular remodeling. 1300 proteins were identified by LC- MS/MS analysis, 2385 were detected by SDS-PAGE-MS/MS and 28 proteins were identified by the functional approach. To be considered a specific component of the secretome of apoptotic EC, identified proteins had to meet concomitantly the following criteria: 1) they had to be identified by at least 2 out of the 3 different MS/MS approaches, 2) they had to be found exclusively in SSC-Apo, and 3) they had to be of human origin. According to these criteria, 27 proteins were classified as specific components of endothelial apoptotic secretome (Table 1) (Sirois et al., 2011). In the following section we will describe some of the observed changes and discuss the potential function of this apoptotic secretome.

3.1 Enrichment of proteins associated with non-classical modes of secretion

Most proteins that are directed to the cell surface or the extracellular space through a conventional secretory pathway contain a signal peptide (Nickel and Rabouille, 2009). Recent evidence suggests alternative modes of secretion for leaderless proteins, i.e. proteins without a signal peptide (Schotman et al., 2008) (Nickel and Rabouille, 2009). To define the contribution of classical and non-classical secretory pathways during apoptotic cell death, the 27 specific constituents of the endothelial apoptotic secretome were classified according to the presence of a signal peptide in their primary amino acid sequence, their mode of secretion, and their intracellular distribution (Table 1). This analysis showed that 25 out of the 27 proteins appeared to be associated with non-classical modes of secretion, based on recent literature and/or the absence of a secretion signal. 13 out of the 27 proteins were previously identified as a component of exosomal nanovesicles. Reevaluation of the comparative and functional proteomic results identified ten additional exosomal proteins in SSC-apo only, whereas only two exosomal proteins were identified in SSC-no-apo (Sirois et al., 2011). Finally, 4 proteins (Table 1 group 2) were annotated as potential components of exosome-like nanovesicles in other cell types. In total, 31 proteins associated with exosome-like nanovesicles were considered to be specific components of the secretome of apoptotic EC.

TYPE OF SECRETION (CLASSICAL OR NON-CLASSICAL)	GENE NAME	SIGNAL PEPTIDE	SECRETION MECHANISM	REF.	CELLULAR LOCALIZATION				
					Mem.	V.E.	C	N	Ext.
Group 1: Non-classical associated to the exosome pathway (13 proteins)	ALDH1A1	NO	Exosome	(Gonzales et al., 2009)			◆		◆
	EIF4G1	NO	Exosome	(Gonzalez-Begne et al., 2009)			◆		◆
	HSPA1L	NO	Exosome	(They, et al., 2001)			◆		◆
	TPT1	NO	Exosome	(Yu et al., 2006) (Lespagnol et al., 2008)		◆	◆		◆
	NAPA	NO	Exosome	(Pisitkun et al., 2004)	◆	◆			◆
	HLA-DQA1	YES	Exosome	(Wubbolts et al., 2003)	◆	◆			◆
	LRP2	YES	Exosome	(Pisitkun et al., 2004)	◆	◆			◆
	IGFR2	YES	Exosome	(Pisitkun et al., 2004)	◆	◆			◆
	PLA2G2D	YES	Exosome	(Subra et al., 2010) (Bette-Bobillo and Vidal, 1995)	◆	◆			◆
	TGFBR3	YES	Exosome**	(Pisitkun et al., 2004) (Lopez-Casillas et al., 1994)	◆				◆
	EGF	YES	Exosome**	(Pisitkun et al., 2004) (Le Gall et al., 2003)	◆	◆			◆
	L1CAM	YES	Exosome**	(Gutwein et al., 2005; Looze et al., 2009; Mechtersheimer et al., 2001)	◆	◆			◆
LRP1	YES	Exosome **	(Nguyen et al., 2004)	◆	◆	◆	◆	◆	
Group 2: Non-classical potentially associated to the exosome pathway (4 proteins)	BRCA2	NO	Exosome (potential)	N.D.		Sec. Gran		◆	N.D.
	ABCC2	NO	Exosome (potential)	N.D.	◆				N.D.
	ATPA4	NO	Tubulovesicles and apical membrane Exosome (potential)	(Wang et al., 2004)	◆				◆
	ITGA10	YES	Grasp-dépendant Exosome (potential)	(Schotman et al., 2008)	◆				◆
Group 3: Non-classical (8 proteins)	ANLN	NO	P2 particles prominin-1 ^{+ve}	(Dubreuil et al., 2007)			◆	◆	◆
	ASH1L	NO	ER et microsomes	(Aronov et al., 2007)					◆
	NOS2A	NO	N.D.	N.D.			◆	◆	N.D.
	GVIN1	NO	N.D.	N.D.			◆	◆	N.D.
	MTPN	NO	N.D.	N.D.			◆		N.D.
	COQ4	NO	N.D.	N.D.	N.D.	Mito	◆		N.D.
	SPIN2A	NO	N.D.	N.D.	N.D.		◆		N.D.
	GOLGA2	NO	N.D.	N.D.	N.D.	Golgi			N.D.
Group 4: Classical (2 proteins)	ADAMTS4	YES	Trans golgi	(Wang et al., 2004)					◆
	tPA	YES	WPBs	(Knipe et al., 2010)					◆

Abbreviations: Mem: Membrane; V.E.: endocytic pathway including endosomes, MVB and lysosomes; C: cytoplasmic; N: nuclear, Ext.: Identified in the extracellular milieu; N.D.: information non available; Mito: mitochondria; WPBs: Weibel Palade Bodies; **: shedding; Sec. Gran. : Secretory granules

Table 1. Specific components of the apoptotic secretome (SSC-apo) regrouping 27 mediators selected according to stringent criteria (see Figure 2 and the text). Proteins were listed according to their mode of secretion, the presence of a signal peptide and their intracellular localization. Classical type of secretion was defined as a protein containing a signal peptide with secretion mechanism described in the literature. Non-classical type of secretion was defined by the absence of a signal peptide or by reports describing their non-classical secretion.

Initially characterized by Rose Johnstone in the 80's, exosomes are now recognized as important intercellular carrier devices detected in most biological liquids including plasma and urine as well as in the media of cultured mammalian cells (Mathivanan et al., 2010; Pan and Johnstone, 1983; Pan et al., 1985). These nanovesicles with a diameter ranging for 50-100 nm are generated from inward budding of multivesicular bodies (MVB). Exosomes contain proteins of the MVB machinery including TSG101 and Alix, both considered classical exosome markers (Keller et al., 2006; They et al., 2002). Exosomes express MHC class I and II associated proteins and play important role in the innate immune system and in antigen presentation (They et al., 2009). They also contain different cargos including proteins, lipids, microRNAs and mRNA (Valadi et al., 2007). Their extracellular release stems from the fusion of MVB with the cell membrane but the molecular regulation of MVB exocytosis remains ill defined. A wide diversity of cell types have been shown to secrete exosomes but their protein composition appears to be cell specific and/or dependent on the metabolic state of the cell.

Guided by the proteomic results, we hypothesized that apoptotic cells release nanovesicle-associated mediators and that this process was triggered by caspase activation. This hypothesis was further validated by several biochemical techniques, cell biology approaches and electron microscopy (Sirois et al., 2011). Apoptotic nanovesicles were shown to express classical constituents of exosomes. Electron microscopy with morphometry analysis demonstrated that secreted nanovesicles are structurally and functionally distinct from apoptotic bodies and represent a novel entity of potential significance in vascular remodeling.

3.1.1 Nanovesicular PMS as novel anti-apoptotic factors exported by apoptotic EC

Translationally Controlled Tumour Protein (TCTP) was identified by both functional and comparative proteomics in SSC-apo (Table 1, group 1). TCTP is an evolutionarily conserved protein of crucial importance during development (Chen et al., 2007) and for intracellular inhibition of apoptosis (Telerman and Amson, 2009). TCTP does not contain a secretion peptide signal and its extracellular export depends on the exosomal pathway (Amzallag et al., 2004; Lespagnol et al., 2008). Using electron microscopy in association with immunogold labeling we showed that TCTP was present on the outer surface of endothelial apoptotic nanovesicles (Sirois et al., 2011). Caspase-activated apoptotic VSMC and fibroblasts also released TCTP-positive nanovesicles in association with apoptosis, suggesting that this pathway is active in various cellular components of the vessel wall. TCTP was found to play a central role in the activation of an anti-apoptotic phenotype in neointimal cells. VSMC exposed to TCTP(+) apoptotic nanovesicles mounted a robust anti-apoptotic response whereas VSMC exposed to nanovesicles generated by TCTP-silenced EC failed to develop an anti-apoptotic phenotype. Collectively these results suggest that TCTP released by apoptotic nanovesicles is a novel and central inducer of resistance to apoptosis in VSMC and a biomarker of apoptotic endothelial nanovesicles.

3.2 PMS characterized as biological mediators of vascular remodeling

We further addressed the relevance of the secretome released by apoptotic EC in vascular remodeling. Since development of AD and TV depends initially on ECM degradation and phenotypical changes within neointimal cells (i.e. anti-apoptotic and fibrogenic), the list of proteins generated by the multidimensional proteomic strategy was screened for the presence of mediators sharing these biological functions. Functional studies on EC, VSMC, MSC and fibroblasts highlighted a multifunctional and biochemically complex paracrine

activity of the endothelial apoptotic secretome (Cailhier et al., 2008; Laplante et al., 2006; Raymond et al., 2004; Raymond et al., 2002; Sirois et al., 2011; Soulez et al., 2010).

3.2.1 Anti-apoptotic PMS

The importance of ECM proteolysis in association with endothelial apoptosis was highlighted by the identification of the C-terminal perlecan fragment referred to as LG3 by MS/MS and validated by western blot analysis (Raymond et al., 2004). This fragment induces a significant anti-apoptotic activity on MSC through alpha-integrin-dependent activation of the ERK1-2 pathway leading to Bcl-xl overexpression (Soulez et al., 2010). LG3 also interacts with beta-integrins on fibroblasts to induce an anti-apoptotic response but the intermediate signaling component differs (Laplante et al., 2006). LG3-integrin interactions in fibroblasts leads to sequential activation of Src family kinases with downstream phosphatidylinositol 3-kinase (PI3K)-dependent induction of Bcl-xl (Laplante et al., 2006). In support of a functionally important role for LG3 in TV, increased LG3 urinary levels were reported in renal allograft recipients with chronic rejection (Goligorsky et al., 2007).

Comparative and functional proteomics of media conditioned by apoptotic and non-apoptotic EC also revealed the presence of proteases, including ADAM17, ADMTS4, SPUVE, tPA and cathepsin L of potential importance in ECM proteolysis (Cailhier et al., 2008). The extracellular export of cathepsin L, which was validated by WB analysis and functional studies, was found to occur through caspase-3 dependent pathways and to play a central role in cleavage of perlecan and generation of the bioactive LG3 anti-apoptotic fragment (Cailhier et al., 2008). Apoptotic EC export a complex array of soluble and vesicular transport-assisted mediators sharing a common anti-apoptotic activity. Interestingly, these mediators target differentially the cellular components of the vascular wall through non-redundant signaling mechanisms, adding specificity to the secreted signals.

3.2.2 Fibrogenic PMS

Vascular remodeling is associated with fibrogenic changes characterized by the accumulation of myofibroblasts within the vessel wall. Myofibroblasts represent a differentiated and activated subset of fibroblasts characterized by *de novo* expression of contractile stress fibers and alpha-smooth-muscle actin (α -SMA) and enhanced production of collagen I and II. The accumulation of myofibroblasts plays an important role in myointimal thickening and vascular stiffness characteristic of AD and TV. The fibrogenic mediator Connective Tissue Growth Factor (CTGF) was identified with an abundance ratio of 2.5 in medium conditioned by apoptotic EC as compared with medium conditioned by non-apoptotic EC (Laplante et al., 2010). Western blotting confirmed that caspase activation significantly increased the release of CTGF by EC during apoptosis. The central importance of CTGF in the fibrogenic response triggered by the endothelial secretome was highlighted by injecting mice sub-cutaneously with medium conditioned by apoptotic or non-apoptotic EC. A significant fibrogenic response with increased skin thickness and enhanced production of collagen I developed in mice injected with medium conditioned by apoptotic EC. Also, CTGF immunodepletion abrogated the fibrogenic activity of medium conditioned by apoptotic EC.

3.2.3 PMS with potential biological activity on vascular repair

Besides PMS characterized and described above, other components of the secretome released by apoptotic EC are potential regulators of vascular remodeling. PLA2G2D was

enriched in the secretome of apoptotic EC (Table 1, Group 1) and recent evidence suggests that it could participate in vascular remodeling. PLA2G2D belongs to a family of secreted phospholipases (sPLA₂), which catalyze hydrolysis of membrane glycerophospholipids to release fatty acids and lysophospholipids (Murakami et al., 2010). PLA2G2D secreted through the exosomal pathway favors intercellular transfer of inflammatory molecules, including prostaglandins (Subra et al., 2010). Tissue plasminogen activator (tPA) was also identified in the secretome of apoptotic EC (Table 1, Group 4) (Cailhier et al., 2008). Recent studies suggest that extracellular release of tPA fosters the development of fibrogenic changes (Edgton et al., 2004; Hu et al., 2008b; Zhang et al., 2007). Convincing evidence also suggests a predominant role for tPA in atherosclerotic diseases (Gramling and Church, 2010). In fibroblasts and myofibroblasts, tPA favors myofibroblast differentiation and induces anti-apoptotic phenotypes through phosphorylation of Bad and the inhibition of the intrinsic apoptotic pathway (Hu et al., 2008a).

4. Conclusion

Characterizing secretomes released by apoptotic cells implies inherent experimental challenges. Cell death is regulated by post-transcriptional events based on protein translocation and cleavage. Failure to take into consideration the importance of proteolysis, protein translocation and activation of non-classical secretion pathways during apoptosis will undermine the experimental strategy. The type of initiating apoptotic signal and the phase of apoptosis to be studied should also guide the design of the proteomic strategy. Creative data mining based on a combination of technical and functional criteria is necessary to gain novel insights into the modes of intercellular communication associated with cell death. The use of a multidimensional proteomics was instrumental in characterizing the importance of caspase activation as a novel regulator of non-classical modes of secretion. It allowed us to demonstrate that apoptotic cells release apoptotic nanovesicles, a novel type of membrane vesicle distinct from apoptotic bodies and reminiscent of exosomes. Mediators of importance in vascular remodeling and of potential use as biomarkers of endothelial injury, such as TCTP, LG3, CTGF, cathepsin L, EGF, PLA2GD2 and tPA were also identified. Further analysis of the complex secretome of apoptotic cells, including biochemical and functional characterization of apoptotic blebs and nanovesicles, should provide further insights into the mechanisms of intercellular communication between dying cells and the local microenvironment.

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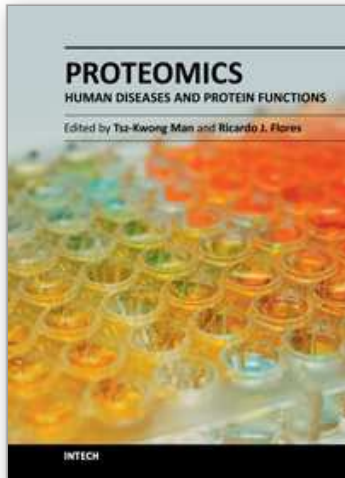
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